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Analysis of the structural heterogeneity of laminarin by electrospray-ionisation–mass spectrometry

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Abstract

Electrospray-ionisation–mass spectrometry (ESIMS) was used in conjunction with chemical derivatisation and degradation procedures to analyse the size heterogeneity and branching structure of laminarin from the brown alga, *Laminaria digitata*. Laminarin is a β-(1 → 3)-linked D-glucan with occasional β-(1 → 6)-linked branches. Electrospray-ionisation–mass spectrometry of permethylated laminarin distinguished two homologous series of molecules, a minor G-series containing 22–28 glucosyl residues, and a more abundant M-series containing 20–30 glucosyl residues linked to a mannitol residue. The relative abundance of all these molecular species could be determined simultaneously from a single mass spectrum, with a mean mass error of 0.6 atomic mass units and a mean mass accuracy of 0.011%. Both series had a mean degree of polymerisation of 25 glucosyl residues, and an approximately 3:1 molar ratio of M-series to G-series molecules was maintained across the range of molecular sizes. Treatment of laminarin with periodate, followed by reduction with borohydride, degraded terminal glucosyl residues on both the main chain and the branches, and allowed the detection of isomers differing solely in their degree of branching. M-series molecules were thus shown to contain 0, 1, 2, 3 or 4 branches, with an average of 1.3 branches per molecule; branched G-series molecules were also detected. Subsequent treatment with acid (Smith degradation) showed that 75% of the branches were single glucosyl residues. This study thus shows how the speed, resolution and mass accuracy of electrospray-ionisation–mass spectrometry can be used in the detailed structural analysis of a polydisperse polysaccharide.

Keywords: Laminarin; β-(1 → 3)-D-Glucan; Electrospray-ionisation; Mass spectrometry; Smith degradation

Abbreviations: amu, atomic mass units; dp, degree of polymerisation; ESIMS, electrospray-ionisation–mass spectrometry; GLC–MS, gas chromatography–mass spectrometry

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1. Introduction

Polysaccharides isolated from natural sources generally exhibit a range of molecular sizes, and often a range of isomeric branching structures. Traditional chemical analysis can accurately determine the average chemical structures of these molecules, but does not always give information on their polydisperse nature. Mass spectrometry has long been a favoured technique for the accurate determination of molecular masses of small molecules, and the mass-spectrometric analysis of polysaccharide mixtures has the potential to determine the exact sizes of all the molecules present and to detect small chemical modifications. Until recently, however, this approach has not been feasible in practice.

A variety of methods have been used to analyse oligosaccharides and polysaccharides by mass spectrometry. Liquid secondary-ion or fast-atom bombardment mass spectrometry have generally been the methods of choice [1,2], but are restricted to molecules of less than 4000 atomic mass units (amu) unless instruments with an extended mass range are used. Direct chemical ionisation [3] has been used to analyse polysaccharides of a degree of polymerisation (dp) of up to 40 glycosyl residues, but information on mass distribution is lost due to the severity of the method used to introduce the sample to the mass spectrometer. Matrix-assisted laser-desorption time-of-flight mass spectrometry is a promising technique for carbohydrate analysis [4–6], and has been used to analyse polysaccharides up to dp 40 [7]. However, it lacks molecular weight resolution at high mass, which causes problems in polysaccharide mixtures where information on both molecular weight distribution and small chemical differences is required. Electrospray-ionisation–mass spectrometry (ESIMS) solves many of the problems associated with the nondestructive desorption and ionisation of biological macromolecules [8–10], and thus is being increasingly used to determine the profiles of carbohydrate side chains associated with proteoglycans and glycoproteins, often in association with preanalysis liquid chromatography, chemical degradation or chemical derivatisation [11–15]. Here, we show how the resolution, accuracy and simplicity of ESIMS can be used to determine both the molecular heterogeneity in backbone length and the branching structure of laminarin, a small storage polysaccharide found in brown algae [16,17].

Laminarin is a β-(1 → 3)-D-glucan containing approximately 25 glucosyl residues [18,19], and its small size and simplicity of structure led to laminarin being a model polysaccharide for early work on structure determination [18–25]. However, in common with many other polysaccharides, laminarin is polydisperse, displaying a degree of structural heterogeneity (reviewed in refs. [16,17]). Firstly, the length of the 3-linked glucosyl backbone varies, that is, not all molecules are of identical dp. Secondly, a small but variable proportion of laminarin molecules terminate with a reducing 3-linked glucose residue and are designated G-chains, whereas the majority terminate with a nonreducing 1-linked D-mannitol residue and are designated M-chains [21,22,25,26]. Lastly, laminarins from several species of *Laminaria* are water-insoluble and contain only linear β-(1 → 3)-linked residues, whereas preparations of laminarin from *Laminaria digitata* are water-soluble and contain small but significant levels of β-(1 → 6)-linked branches [21,23–25,27,28].

Our results demonstrate that a much more detailed analysis of this heterogeneity is

obtained by ESIMS than is possible by chemical analysis. We show here the distribution of sizes of both M-chain and G-chain molecules of laminarin from *L. digitata*, and the heterogeneity in branch number in laminarin molecules of each size.

2. Experimental

Materials.—Laminarin from *Laminaria digitata* was obtained from Sigma Chemical Co. (lot 110H3841).

Reduced laminarin was prepared by treating laminarin (1 g) with aqueous $NaBH_4$ (400 mL, 0.5 M in 2 M NH_4OH) for 1 h at 60 °C, followed by decomposition of excess $NaBH_4$ with acetic acid (100 mL, 25% aq). The sample was rotary-evaporated to a syrup, then evaporated 5 times with 10% (v/v) acetic acid in methanol (200 mL) to remove boric acid, redissolved in water (200 mL), precipitated with 4 vol of ethanol (2 h, −20 °C), and freeze-dried. Yield: 970 mg (97%).

Oxidised-reduced laminarin was prepared by treating laminarin (100 mg) with aq $NaIO_4$ (5 mL, 0.25 M in 50 mM CH_3CO_2Na, pH 5.0) for 1 h at 20 °C, followed by addition of ethylene glycol (300 μL) and incubation for a further 1 h. The sample was gel-filtered into water on Sephadex G-25, then reduced with $NaBH_4$ and processed as above. Yield: 54 mg (56%).

Oxidised-reduced laminarin (2 mg aliquots) was subjected to Smith degradation [29] by hydrolysis in aq trifluoroacetic acid (1 mL, 1 M, 20 °C) for various times up to 108 h; the samples were then evaporated under a stream of nitrogen, and freeze-dried.

Analytical techniques.—Linkage analysis was performed using the NaOH method of Ciucanu and Kerek [30] modified as follows. Samples (200 μg) were dissolved in dimethyl sulfoxide (100 μL) and permethylated by addition of a slurry of solid NaOH in dimethyl sulfoxide (100 μL, 120 mg mL^{-1}, 5 min), followed by three sequential additions of methyl iodide (20, 20 and 40 μL) at 10 min intervals. After a further 10 min the reaction was stopped by addition of sodium thiosulfate (2 mL, 10% aq), permethylated products were extracted into chloroform (1 mL), and the organic phase was washed 4 times with water and evaporated.

The permethylated samples were then hydrolysed in aq trifluoroacetic acid (250 μL, 2 M, 4 h, 102 °C), then dried under a stream of nitrogen, reduced with aq $NaBD_4$ (200 μL, 0.5 M in 2 M NH_4OH, 2.5 h, 20 °C), and the reaction was stopped by three sequential additions of acetic acid (10 μL). Samples were then evaporated 3–5 times with 5% (v/v) acetic acid in methanol (500 μL), followed by two evaporations with methanol (500 μL), then acetylated with acetic anhydride (500 μL, 2.5 h, 100 °C). Water (4 mL) was added, and the partially methylated alditol acetates were extracted twice into dichloromethane (1 mL), washed twice with water (2 mL), and analysed by gas chromatography–mass spectroscopy (GLC–MS) on a 1020B Finnigan MAT using both a high-polarity column (BPX-70, SGE-Australia) [31] and a low-polarity column (CP-Sil-5, Chrompack International, The Netherlands) [32]. Inositol hexa-acetate was used as standard.

Reducing power was measured colorimetrically using the Cu^{2+}/2,2′-bicinchoninic acid (BCA) assay (Pierce Chemical Co., USA) [33].

Electrospray-ionisation–mass spectrometry.—ESI–mass spectra were obtained on a Finnigan MAT 95 reverse-geometry sector mass spectrometer, equipped with a Finnigan electrospray and a DEC 3100 data system. Spectra were acquired in the positive-ion mode, using a 5 kV accelerating potential and a nominal resolution of 800 (full-width-at-half-maximum definition). The instrument was calibrated by creating a magnet calibration curve using CsI in liquid secondary-ion mode; in ESI mode, this calibration curve was locked in place by setting the accelerating voltage to give a value of 1293.6 amu for the $[M + Na]^+$ sodium adduct of permethylated maltohexaose.

Samples (125 μg) for ESI analysis were permethylated by the NaOH method [30] as described above, then dissolved in a solution of 0.005% CH_3CO_2Na in 50% aq methanol (1 mL) and infused at 5 μL min^{-1} into the ESI needle with a syringe pump (Harvard). A sheath liquid of methanol was used at a flow rate of 4 μL min^{-1}. ESI was performed with the capillary at 220 °C, tube lens at 120 V, capillary at 50 V and needle at 3.4 kV. The magnet was scanned from mass/charge 700 to 3200 at 15 s $decade^{-1}$, resulting in a scan time of 7 s.

Typically, 10–15 scans were acquired and averaged. Spectrum deconvolution was performed with the standard Finnigan transformation function [34], to a mass range of 4000–7000 amu, taking sodium as the adduct ion; a minimum step of 0.8 amu was used during deconvolution, which set an upper limit on the resultant mass accuracy. Values shown on deconvoluted spectra and quoted in the text are the peak of ^{13}C abundance spectra. Expected masses were calculated using H = 1.007976, C = 12.011137 and

Table 1
Glucosyl linkage analysis of laminarin fractions [a]

	Laminarin [b]	Reduced laminarin [b]	Oxidised-reduced laminarin	Oxidised-reduced laminarin, acid-treated [c]
Mol%				
t-Glc	8.1	9.6	0.0	5.2
3-Glc	87.1	85.0	94.9	93.5
3,6-Glc	4.8	5.3	5.1	1.2
Glucosyl residues/mol [d]				
t-Glc	2.0	2.4	0.0	1.2
3-Glc	21.8	21.2	21.8	21.5
3,6-Glc	1.2	1.3	1.2	0.3
total	25.0	25.0	23.0	23.0

[a] t-Glc denotes 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, 3-Glc denotes 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol, and 3,6-Glc denotes 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylglucitol.

[b] A small amount (0.7–1.0 mol%) of a derivative corresponding to l-mannitol was found in laminarin and reduced laminarin, and a trace (0.1–0.2 mol%) of a derivative corresponding to 3-glucitol was found in reduced laminarin.

[c] Treated with 1 M trifluoroacetic acid for 36 h at 20 °C.

[d] Calculated assuming a mean of 25 glucosyl residues/mol for laminarin and reduced laminarin, and 23 glucosyl residues/mol for oxidised–reduced laminarin and acid-treated oxidised–reduced laminarin (assuming oxidative degradation of a terminal nonreducing glucosyl residue on the main chain, and a terminal nonreducing glucosyl residue from one branch per molecule).

O = 15.999304, which gives the weighted mean of all possible ^{13}C isotope values for each species, and were then rounded to the nearest 0.1 amu.

3. Results and discussion

Laminarin.—The laminarin sample from *Laminaria digitata* was completely soluble in water at 20 °C. Linkage analysis by methylation, analysed by GLC–MS, showed the

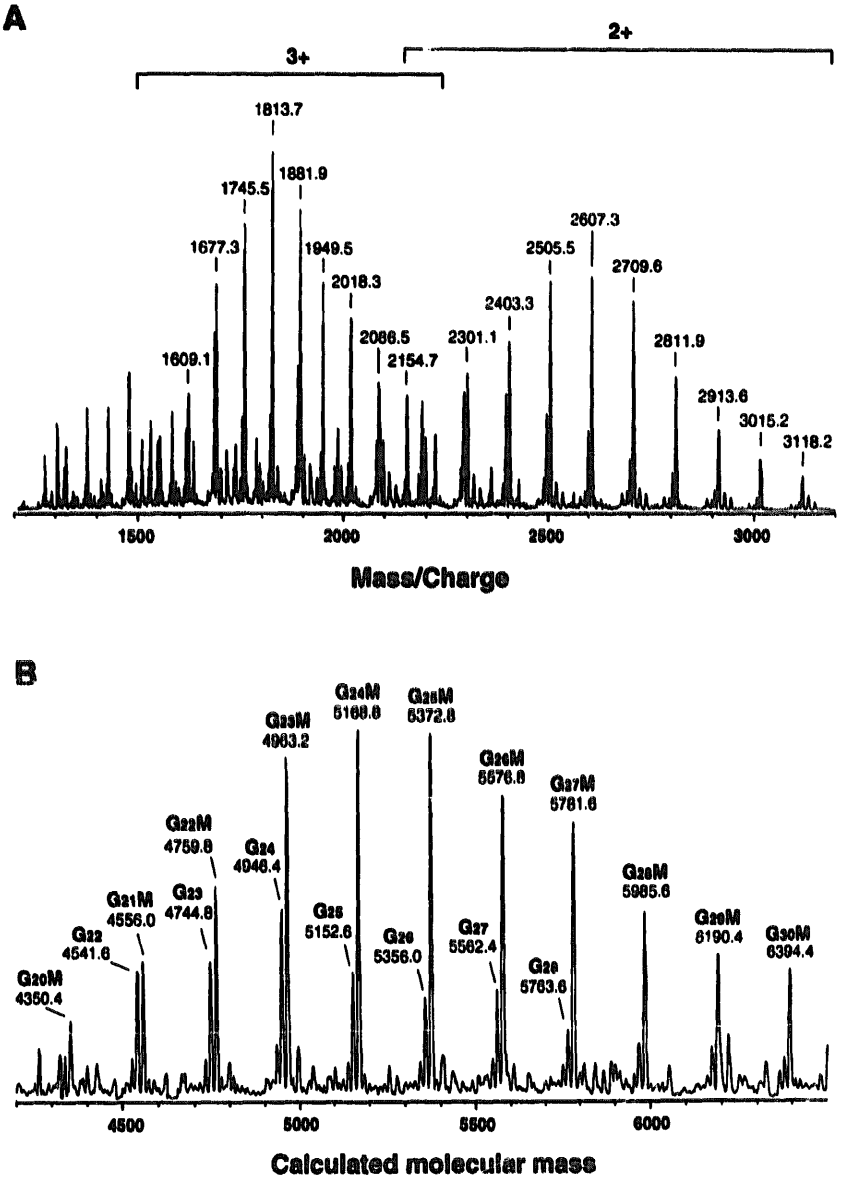

Fig. 1. ESIMS of permethylated laminarin. (A) Native spectrum. A 2+ indicates doubly charged sodium adducts $[M+2Na]^{2+}$, and 3+ indicates triply charged sodium adducts $[M+3Na]^{3+}$. (B) Deconvoluted spectrum. G25M, for example, denotes a permethylated oligosaccharide with 25 glucosyl and 1 mannitol residue, and G25 denotes a permethylated oligosaccharide with 25 glucosyl residues.

presence of a 3-linked glucan backbone with about 5% of the residues branched through O-6 (Table 1). The excess of terminal glucosyl residues over 3,6-linked glucosyl residues corresponded to an approximate dp of 25–30 residues and an average of just over one branch point per molecule.

Measurement of reducing power with Cu^{2+}/bicinchoninic acid showed that the same reducing power was present in 1 mg laminarin as in 7.3 μg of glucose. 1 mg of a glucan of dp 25 (the dp deduced for laminarin, see below) is calculated to have the same reducing power as 44.2 μg of glucose, which indicates that only 17% of the laminarin molecules had reducing termini. These reducing molecules were allocated to the G-series of molecules terminating with a 3-linked glucose residue [21,22,25,26]. Close examination of the GLC–MS data revealed a small peak of a 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methylhexitol (diagnostic MS fragment ions at 101, 117, 145 and 161; $R_{inositol}$ values of 0.211 on BPX-70, 0.418 on CP-Sil-5) eluting before the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol derived from terminal glucosyl residues ($R_{inositol}$ values of 0.342 on BPX-70, 0.541 on CP-Sil-5). The detection of this pentamethylated hexitol acetate is consistent with a proportion of the glucosyl chains of laminarin terminating with a

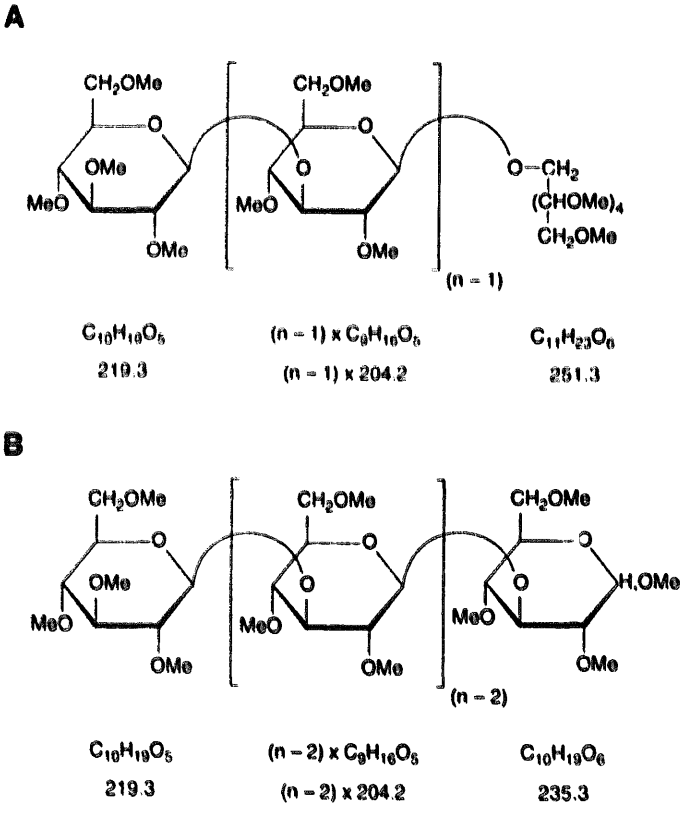

Fig. 2. Structures and expected masses of permethylated laminarin M-series and G-series molecules. (A) Unbranched member of M-series, G25M, for example, has $(n-1)=24$ and an expected mass of 219.259434 + (24 × 204.224369) + 251.301779 = 5371.946069, rounded to 5371.9 amu. (B) Unbranched member of G-series, G25, for example, has $(n-2)=23$ and an expected mass of 219.259434 + (23 × 204.224369) + 235.258738 = 5151.678659, rounded to 5151.7 amu. Me denotes a methyl group added during permethylation before ESIMS.

nonreducing, 1-linked mannitol residue; these chains are members of the M-series [21,22,25,26]. Mannitol residues linked through O-1 and O-6 are symmetrical and give the same derivative on linkage analysis, and similar fragment ions derived from "prereduced 6-linked hexosyl residues" were observed by Waeghe et al. [35]. The deduced 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methylmannitol was present at 0.7–1.0 mol% of the total recovered residues, a relative yield of approximately 20% (calculated assuming that 83% of the laminarin molecules were nonreducing). Such substoichiometric yields are expected for pentamethylated hexitols, and have been attributed variously to their relatively high volatility and water solubility [35,36] or to oxidation [37].

The ESI spectrum of permethylated laminarin contained doubly and triply charged sodium adducts $[M + 2Na]^{2+}$ and $[M + 3Na]^{3+}$, forming a complex set of peaks (Fig. 1A). After deconvolution, two distinct series of molecules were clearly resolved, with masses between 4300 and 6500 amu (Fig. 1B). Adjacent members of the more abundant series differed by a mean of 204.3 ± 0.4 amu (all ± values are the standard errors of the mean), which represents a difference of a single methylated hexosyl residue. The masses of this series equalled those predicted for a permethylated glucan linked to a mannitol residue (Fig. 2A), with a mean difference from the expected of 0.8 amu, and it was thus

Table 2
Molecular species of laminarin determined by ESIMS [a]

Species	Masses (amu) [b]				Abundance (%)		Mean number of branches [c]
	Expected	Observed	Error	Accuracy (%)	G-series	M-series	
G20M	4350.8	4350.4	−0.4	−0.010		2	
G21	4334.8	4335.2	0.4	0.009	1		
G21M	4555.1	4556.0	0.9	0.021		4	0.7
G22	4539.0	4541.6	2.6	0.057	4		
G22M	4759.3	4759.8	0.5	0.011		6	0.9
G23	4743.2	4744.8	1.6	0.033	4		
G23M	4963.5	4963.2	−0.3	−0.006		10	1.0
G24	4947.5	4946.4	−1.1	−0.021	5		
G24M	5167.7	5168.8	1.1	0.021		11	1.1
G25	5151.7	5152.6	0.9	0.018	4		
G25M	5371.9	5372.8	0.9	0.016		11	1.2
G26	5355.9	5356.0	0.1	0.002	3		
G26M	5576.2	5576.8	0.6	0.011		9	1.4
G27	5560.1	5562.4	2.3	0.041	3		
G27M	5780.4	5781.6	1.2	0.021		8	1.4
G28	5764.4	5763.6	−0.8	−0.013	2		
G28M	5984.6	5985.6	1.0	0.016		5	1.8
G29	5968.6	5969.6	1.0	0.017	1		
G29M	6188.8	6190.4	1.6	0.025		4	2.0
G30	6172.8	6175.2	2.4	0.039	1		
G30M	6393.1	6394.4	1.3	0.021		4	2.0
subtotal					27	73	
total						100	

[a] From data in Fig. 1 (masses and abundances) and Fig. 4 (branch numbers).
[b] Mean error is 0.9 amu, giving a mean mass accuracy for this spectrum of 0.016%.
[c] M-series only; mean branch number was not measured for G-series molecules.

attributed to the M-series of laminarin molecules: G25M at 5372.8, for example, represents $Glc_{25}(1 \rightarrow 1)$mannitol, a glucan with a dp of 25 glycosidically linked to a mannitol residue (expected mass: 5371.9). Adjacent members of the less abundant series differed by a mean of 204.1 ± 0.4 amu, also representing a single methylated hexosyl residue. The masses of this series equalled those predicted for a permethylated glucan (Fig. 2B), with a mean difference from the expected of 0.9 amu, and it was thus attributed to the G-series of laminarin molecules: G25 at 5152.6, for example, represents Glc_{25}, a glucan with a dp of 25 (expected mass: 5151.7). The two homologous series differed from each other by a mean of 16.0 ± 0.3 amu, as required by the proposed structures (Fig. 2); undermethylation would result in a mass difference of 14.0 amu.

The full allocation of components from the typical laminarin spectrum shown in Fig. 1 is shown in Table 2. The data from this particular spectrum had a mean mass error of 0.9 amu, and a mean mass accuracy of 0.016%; a major source of error was the minimum step of 0.8 amu used in the deconvolution program. A mean mass error of 0.6 amu, and a mean mass accuracy of 0.011%, was calculated over all the mass spectra collected during this work.

The heterogeneity in backbone length of the population of laminarin molecules was immediately apparent from ESIMS analysis (Fig. 1). A symmetrical distribution of M-series molecules from 20 to 30 glucosyl residues was observed, with G23M to G27M predominating. ESI ion intensities and the consequent peak heights from the deconvoluted mass spectrum were considered to represent the true abundance of components within the homologous series, with different terminal units in the M-series and G-series (a permethylated hexitol and a permethylated hexose, respectively) not affecting the ionisation efficiency. Deconvoluted peak heights were thus used to calculate the abundances of all the molecular species present. This showed that G23M to G27M together contributed 49% of the laminarin molecules present (Table 2). A mean dp of 25.0 glucosyl residues was calculated for the M-series molecules, and a mean dp of 24.9 glucosyl residues was calculated for the G-series molecules. The M-series comprised 73% of the total laminarin molecules present, and the G-series 27% (Table 2). This value differs slightly from the value of 17% reducing molecules determined by the Cu^{2+}/bicinchoninic acid assay, probably because of discrepancies in the colorimetric assay. The M-series:G-series ratio of 73:27 was maintained approximately constant across the range of molecular sizes in the sample: for example, the G21M:G21 ratio was 78:22, and the G30M:G30 ratio was 74:26. Combined with the similar mean dp of M-series and G-series molecules, this implies that addition of a 1-linked mannitol residue at the reducing terminus occurs on a random 73% of all laminarin molecules, with no size preference for this modification.

Reduced laminarin.—The laminarin sample was reduced with sodium borohydride, giving a preparation in which less than 0.1% of the molecules had reducing termini as judged by the Cu^{2+}/bicinchoninic acid assay. Linkage analysis showed, as expected, that the reduced laminarin was very similar to the original laminarin sample (Table 1). A substoichiometric amount of a derivative from 1-mannitol was again found from the M-chain molecules, but reduced laminarin also gave rise to a trace of a derivative deduced to be 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylglucitol from the reduced G-chain molecules.

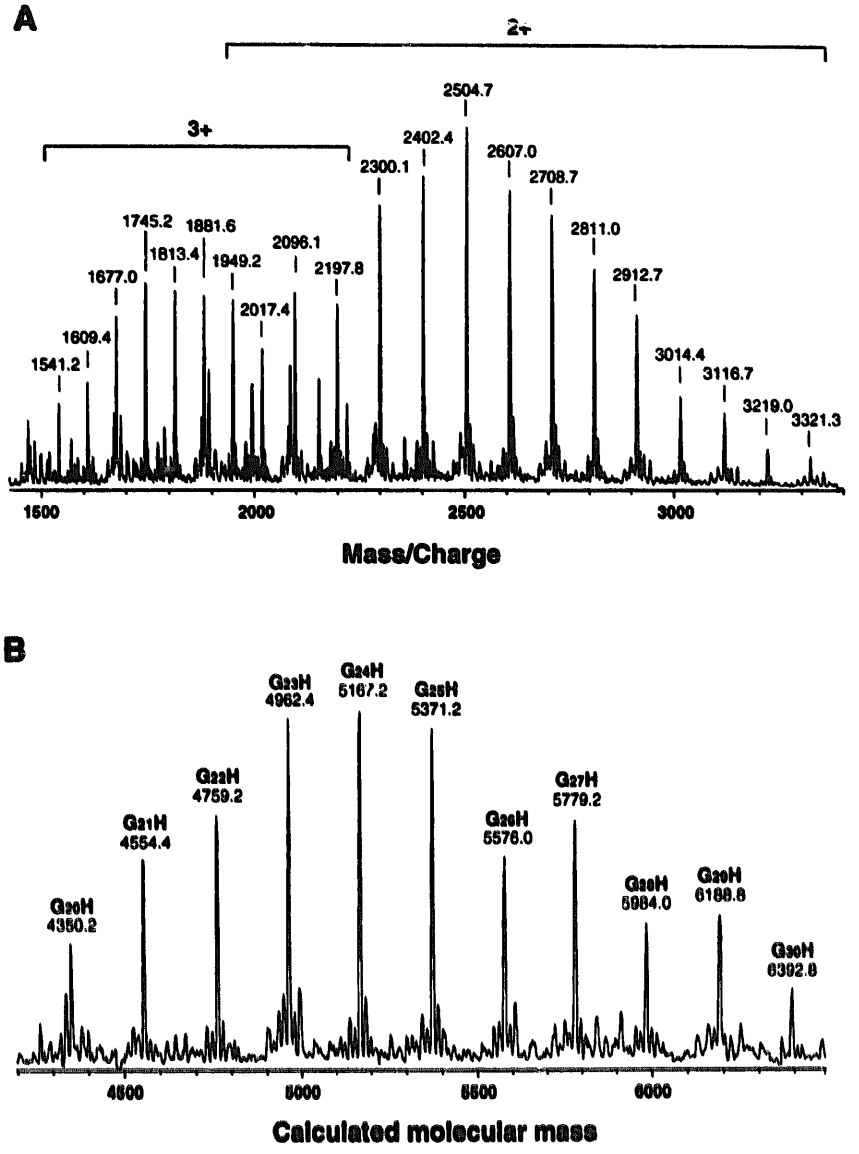

Fig. 3. ESIMS of permethylated reduced laminarin. (A) Native spectrum. A 2+ indicates doubly charged sodium adducts $[M + 2Na]^{2+}$, and 3+ indicates triply charged sodium adducts $[M + 3Na]^{3+}$. (B) Deconvoluted spectrum. G25H, for example, denotes a permethylated oligosaccharide with 25 glucosyl and 1 hexitol (mannitol or glucitol) residue.

Analysis by ESIMS of permethylated reduced laminarin again gave predominantly the doubly and triply charged sodium adducts $[M + 2Na]^{2+}$ and $[M + 3Na]^{3+}$ (Fig. 3A), but the spectrum was significantly simpler than that of permethylated laminarin and did not contain overlapping sets of peaks. Deconvolution showed that only a single homologous series of molecules was present (Fig. 3B), and adjacent members of the series differed by a mean of 204.3 ± 0.2 amu, representing a single methylated hexosyl residue. The masses of this series equalled those expected for a permethylated glucan linked to a hexitol (mannitol or glucitol) residue, with a mean difference from the

expected of 0.2 amu. This series was thus deduced to contain both M-series molecules and a smaller amount of reduced G-series molecules at the same masses; as expected from the chemical data, the reducing G-series molecules detected in laminarin were not detected in reduced laminarin. G25H at 5371.2 thus represents $Glc_{25}(1 \rightarrow 1)$mannitol (G25M, expected mass: 5372.0) plus $Glc_{25}(1 \rightarrow 3)$glucitol (expected mass: 5372.0) produced by reduction of Glc_{26} (G26).

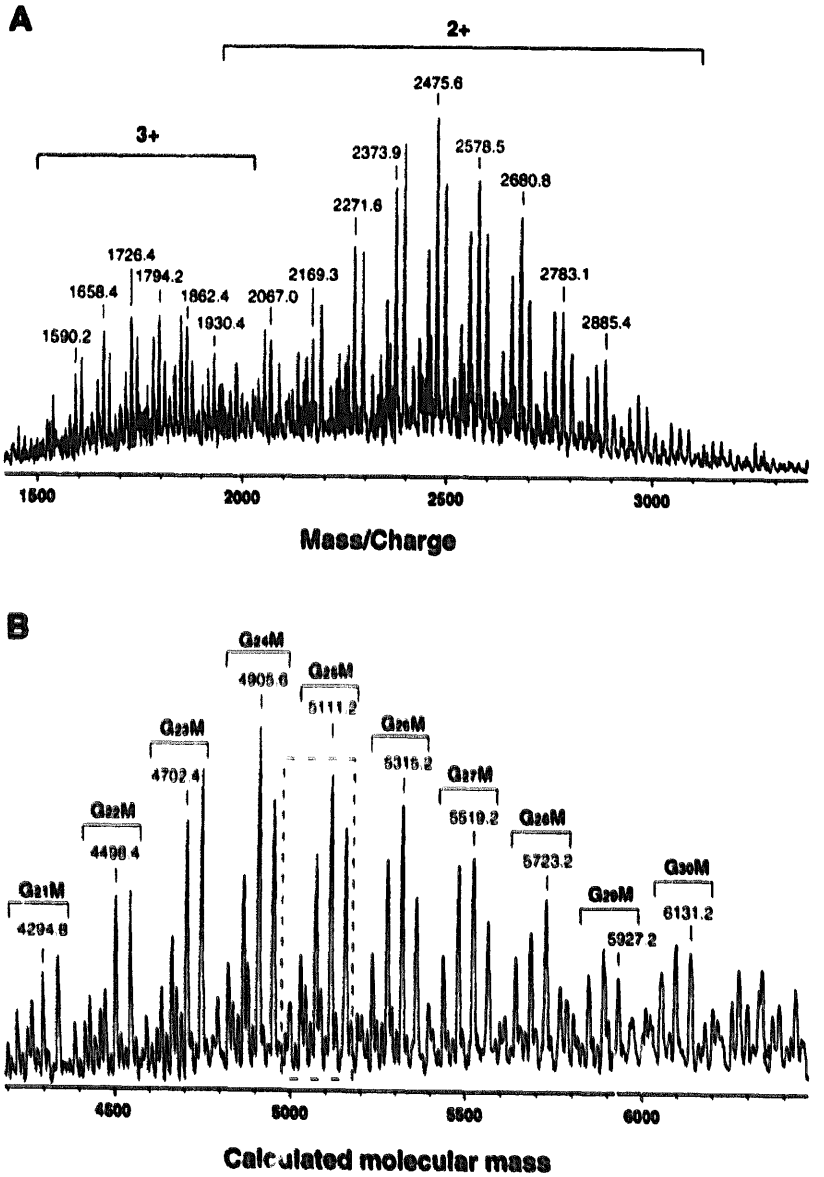

Fig. 4. ESIMS of permethylated oxidised-reduced laminarin. (A) Native spectrum. A 2+ indicates doubly charged sodium adducts $[M+2Na]^{2+}$, and 3+ indicates triply charged sodium adducts $[M+3Na]^{3+}$. Mass:charge ratios are shown only for ions corresponding to species with 1 branch point. (B) Deconvoluted spectrum. G25M, for example, denotes the differently branching species derived from a permethylated oligosaccharide containing 25 glucosyl and 1 mannitol residue; G-series branching species are not indicated. Molecular masses are shown only for species with 1 branch point. The box outlines the region of the spectrum that is expanded in Fig. 6.

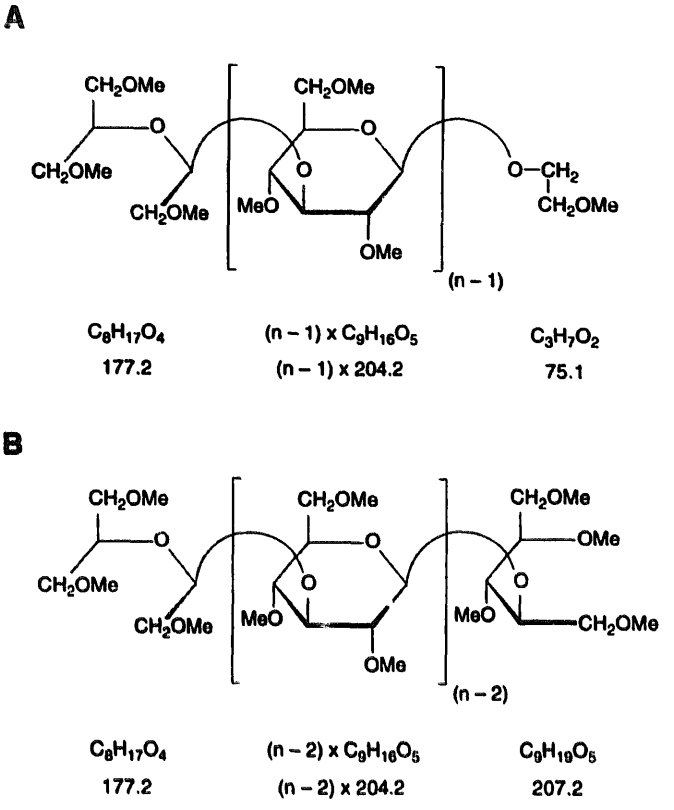

Fig. 5. Structures and expected masses of permethylated oxidised-reduced laminarin M-series and G-series molecules. (A) Unbranched member of M-series. Unbranched oxidised-reduced G25M, for example, has $(n-1)=24$ and an expected mass of $177.221902+(24\times204.224369)+75.087851=5153.694611$, rounded to 5153.7 amu. (B) Unbranched member of G-series. Unbranched oxidised-reduced G25, for example, has $(n-2)=23$ and an expected mass of $177.221902+(23\times204.224369)+207.248297=5081.630688$, rounded to 5081.6 amu. Each branch reduces the expected mass of oxidised-reduced M-series and G-series molecules by 42.0 amu. Me denotes a methyl group added during permethylation before ESIMS.

Oxidised-reduced laminarin.—Further structural information on the laminarin molecules was obtained by oxidation with periodate, followed by reduction with sodium borohydride [14,29,38]. Linkage analysis showed complete loss of terminal nonreducing glucosyl residues (Table 1). No derivative from 1-mannitol was detected, implying that this residue in M-series molecules had also been degraded.

The ESIMS spectrum of permethylated oxidised-reduced laminarin showed a complex spectrum of doubly and triply charged sodium adducts $[M+2Na]^{2+}$ and $[M+3Na]^{3+}$ (Fig. 4A). Deconvolution revealed a series of triplets and quadruplets of peaks (Fig. 4B). The masses of the major peaks equalled those expected for permethylated M-series laminarin molecules degraded at terminal residues at both ends of the molecules, as well as at terminal glucosyl residues on a variable number of branches (Fig. 5A). Periodate does not react with the 3-linked glucosyl residues of the laminarin backbone, but oxidises the 1-linked mannitol residues of M-series molecules, giving ethylene glycol moieties after reduction [21], and causing a loss of 176.2 amu after permethylation (Fig. 5A). Periodate also oxidises terminal nonreducing glucosyl residues on both

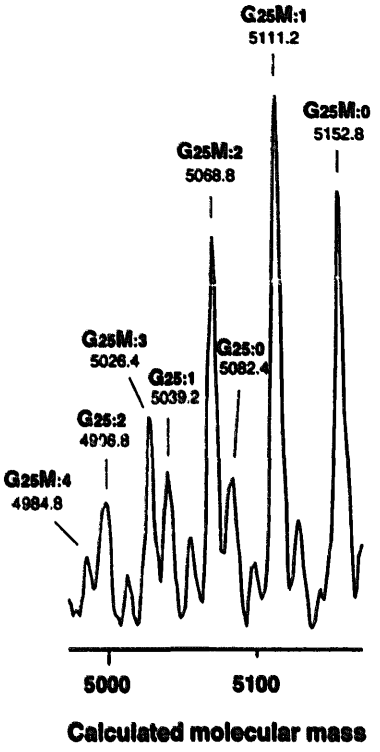

Fig. 6. Expansion of part of deconvoluted ESIMS spectrum of permethylated oxidised-reduced laminarin from Fig. 4. G25M:0, G25M:1, G25M:2 and G25M:3 denote species derived from G25M molecules with 0, 1, 2 and 3 branches, respectively, and G25:0, G25:1 and G25:2 denote species derived from G25 molecules with 0, 1 and 2 branches, respectively.

the main chain and the branches, giving after reduction mixed acetals of glycolaldehyde with O-2 of glycerol and the O-3 or O-6 of a laminarin glucose residue [21,29,38], and causing a loss of 42.0 amu from each terminal nonreducing glucosyl residue after permethylation (Fig. 5A) [14].

The observed triplets and quadruplets thus arise from splitting each member of the laminarin M-series into three or four molecular species in accordance with the number of terminal nonreducing glucosyl residues present, which is related to the number of branches per molecule. The quadruplet labelled G25M, for example, consists of species at 5152.8, 5111.2, 5068.8 and 5026.4 amu (expected masses: 5153.7, 5111.6, 5069.6 and 5027.5), derived from oxidation of G25M molecules in the laminarin M-series containing respectively 1, 2, 3 or 4 terminal glucosyl residues, and thus 0, 1, 2 or 3 branches (Fig. 6); G25M:0 denotes the molecule derived from a G25M isomer with 0 branches. The average difference between adjacent peaks within a triplet or quadruplet was 42.1 ± 0.1 amu, as required for isomers with different branch numbers, and the average difference between triplets or quadruplets was 204.3 ± 0.1 amu, as required for a difference of a single methylated hexosyl residue. A mean mass difference from the expected of 0.7 amu was calculated for all the species observed.

Each member of the laminarin M-series thus contained a number of branching isomers. Peak heights were used to calculate relative amounts of each isomer, and mean

numbers of branches (Table 2). Unbranched molecules were the most abundant isomers in molecules up to G23M, while G24M to G28M most commonly had one branch, and in G29M and above the 2-branched species was the most abundant with a significant proportion of molecules having three or four branches (data not shown). The mean branch number varied from 0.7 for G21M, through 1.2 for G25M, to 2.0 for G30M (Table 2); the mean branch number for the complete M-series was calculated as 1.3, which agrees well with the results of linkage analysis (Table 1).

Many of the minor peaks in the mass spectrum of oxidised-reduced laminarin were deduced to derive from oxidation of G-series molecules. Normal (Malapradian) oxidation of the open-chain form of the reducing 3-linked glucosyl residue, followed by "over-oxidation" (non-Malapradian oxidation) of the resultant $-CH(CHO)_2$ group (rapid in 0.25 M $NaIO_4$, 20 °C), would completely degrade the residue [39] and leave a shorter G-series molecule again susceptible to periodate attack, leading to general loss of G-series derivatives from the mass spectrum. Oxidation of the closed-chain form of the reducing 3-linked glucosyl residue would cleave the residue between C-1 and C-2, giving a formyl ester on O-5 that would be relatively stable under the conditions used (pH 5.0, 20 °C) [39]. However, this formyl ester would hydrolyse during the reduction in 2 M NH_4OH, giving a 2-linked L-arabinose residue [28] which would be immediately reduced to a 2-linked L-arabinitol residue, resulting in a loss of 28.0 amu after permethylation (Fig. 5B). Oxidation also occurs at the terminal nonreducing glucosyl residues on the main chain and branches of G-series molecules (Fig. 5B), which separates G-series branching isomers in the same way as for M-series branching isomers.

The portion of the oxidised-reduced laminarin mass spectrum boxed in Fig. 4 is expanded in Fig. 6, to show the species derived in this way from branching isomers of G25. G25:0, for example, denotes the molecule derived from a G25 isomer with 0 branches. These peaks were too small to give accurate quantitative information on branch distribution, but there was no indication that branch distribution within the G-series differed from that within the M-series.

Smith degradation [29] was employed to give information on the length of the branches. This technique has been previously used to demonstrate that branches in the soluble fraction of laminarin from *L. hyperborea* consisted of single glucosyl residues [28]. Treatment of oxidised-reduced laminarin with 1 M trifluoroacetic acid at 20 °C for various times [40,41] removed the oxidised fragments and regenerated a set of smaller laminarin molecules. The reaction was monitored by linkage analysis, which showed a progressive reappearance of the terminal glucosyl residue of the main chain, and a progressive loss of 3,6-linked glucosyl residues corresponding to degraded branches that had consisted of only a single glucosyl unit. Hydrolysis was complete by 36 h (data not shown). The resultant molecules retained 1.2 mol% 3,6-linked glucosyl residues, corresponding to branches that had consisted of more than one glucosyl unit (Table 1). Undegraded laminarin contained an average of 1.2 branch points per molecule as determined by linkage analysis, and after one round of Smith degradation this had dropped to an average of 0.3 branch points per molecule. Approximately 75% of the branches therefore consisted of a single glucosyl residue, with the remaining branches consisting of two or more residues.

4. Conclusions

Chemical analysis and degradation procedures had previously provided an average view of the laminarin molecule. ESIMS of permethylated laminarin, as reported here, resolved the individual members of the M-series and G-series of laminarin molecules, and provided quantitative data on backbone length distribution and reducing end heterogeneity. A mean molecular size of 25 glucosyl residues and an approximately 3:1 molar ratio of M-series and G-series molecules agreed with data from linkage analysis and the measurement of reducing content. The distribution of molecular sizes in the M-series and G-series suggested that the extent of modification with a 1-linked mannitol residue was independent of molecular size.

Combining ESIMS with periodate oxidation allowed measurement of heterogeneity in branch number. Both M-series and G-series molecules were branched, and the number of branches increased with increasing dp: the smallest laminarin molecules contained species with 0, 1 or 2 branches, whereas the largest laminarin molecules contained species with 0, 1, 2, 3 or 4 branches. Acid hydrolysis of periodate-treated laminarin (Smith degradation) showed that the majority (approximately 75%) of these branches consisted of single glucosyl residues.

The advantage of ESIMS is that it gives structural information across the range of molecular sizes present in the polydisperse population of laminarin molecules. Permethylation to give sample volatility is the only pretreatment required, and analysis is rapid and accurate; the mean difference between observed and expected masses of 0.6 amu across all the samples analysed gave a mean mass accuracy of 0.011%. The underivatised laminarin molecules had masses of up to 5000 amu, and thus these are some of the largest carbohydrates from which biologically useful information has been acquired by mass spectrometry.

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