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Carbohydrate Research 310 (1998) 203–210

CARBOHYDRATE
RESEARCH

Structural analysis of laminarans by MALDI and FAB mass spectrometry

Alexander O. Chizhov[a,c,*,1], Anne Dell[a], Howard R. Morris[a],
Andrew J. Reason[b], Stuart M. Haslam[a], Roy A. McDowell[a],
Oleg S. Chizhov[c], Anatolii I. Usov[c,2]

[a] *Biochemisty Department, Wolfson Laboratories, Imperial College, Exhibition Road, London SW7 2AZ, UK*

[b] *M-Scan Ltd., Silwood Park, Sunninghill, Ascot, Berkshire SL5 7PZ, UK*

[c] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 47 Leninsky Prosp. 117913 Moscow, Russian Federation*

Received 2 April 1998; accepted 29 June 1998

Abstract

MALDI and FAB mass spectrometry were applied to eight samples of laminarans from different algal species. The existence of both M-chains (mannitol-containing) and G-chains (mannitol-free) was confirmed for six of them, as well as the absence of M-chains for laminarans from two *Cystoseira* sp. It was found that *Cystoseira barbata* and *C. crinita* glucans contain a small percentage of N-acetylhexosamine-terminated chains. This is the first observation of nitrogen-containing sugars in laminarans. The presence of cyclic structures in some laminaran samples is suggested by the MALDI and FAB data. This study demonstrates the power of modern mass spectrometry for defining d.p. profiles in complex mixtures of polysaccharides and for rapidly and sensitively revealing the presence of novel components. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: Laminaran; β-Glucan; N-Acetylhexosamine; MALDI-MS; FAB-MS; Brown algae

1. Introduction

Laminarans (laminarins) are a class of low-molecular-weight storage β-glucans of brown algae consisting of (1→3)-β-D-glucopyranose residues in which some 6-O-branching in the main chain and some β-(1→6)-intrachain links are present [1–3]. The majority of laminarans contain polymeric chains of two types: one type is built only of Glc*p* residues (G-chains), whereas the other type is terminated with 1-O-substituted D-mannitol residues (M-chains) [1–3]. Laminarans from different algal species may vary in structural features, for example, in the M:G ratio (in some cases M-chains are absent), in the degree of branching and d.p. (up to 50 carbohydrate residues, usually ca. 25), and in

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Abbreviations: DHB, dihydroxybenzoic acid; DMSO, dimethyl sulphoxide; d.p., degree of polymerisation; FAB, fast atom bombardment; MALDI, matrix-assisted laser desorption/ionisation; MS, mass spectrometry; TFA, trifluoroacetic acid; TOF, time-of-flight.

* Corresponding author. E-mail: chizhov@ioc.ac.ru

[1] On leave from N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow.

[2] Second corresponding author.

PII S0008-6215(98)00177-3

the ratio of (1→3)- and (1→6)-glycosidic bonds. Several laminarans have been shown to have structures quite distinct from that originally suggested for the commercially available preparations from *Laminaria* sp. [2–4].

Laminarans are of increasing current interest because some of their derivatives have been shown to have interesting biological activities. For example, a fluorescent-tagged heptasaccharide prepared from *Eisenia bicyclis* laminaran has been demonstrated to have elicitor activity [5], and a glucan with immunostimulating activity was obtained by transglycosylation of laminaran from *Laminaria cichorioides* [6].

Laminarans were one of the subjects for the development of classical chemical methods of polysaccharide structural analysis [1]. More recently, progress in mass spectrometry, in particular, the invention of effective methods of ionisation from condensed phases makes direct MS analysis of laminarans as mixtures of oligosaccharides a powerful means of characterisation. Read et al. [7] used electrospray-ionisation mass spectrometry to elucidate the size heterogeneity and branching structure of permethylated derivatives of a commercial preparation of laminaran from *Laminaria digitata*; native laminaran of the same origin was recently studied using MALDI-MS [8]. In addition, FAB-MS has been widely used in the analysis of many polysaccharides whose size and complexity are comparable to the laminarans [9].

Here we report the application of FAB and MALDI mass spectrometry for the structural analysis of laminarans from several algal species. We show that MALDI-MS of native laminarins is a rapid and sensitive means of defining d.p. profiles whilst analysis of permethyl derivatives by either technique is particularly useful for investigating minor and/or novel components.

2. Experimental

Materials.—Laminaran from *Laminaria digitata* was obtained from Sigma Chemical Company (lot 50H3839). Laminaran from *L. hyperborea* was purchased from Koch-Light Labs. (lot 3423H46917). Laminarans from *Alaria angusta*, *Chorda filum*, *Cystoseira barbata*, *C. crinita*, *Laminaria cichorioides*, and *Sphaerotrichia divaricata* were isolated previously [4]. The following reagents and solvents were used: DMSO (Romil), methyl iodide, sodium borohydride and sodium borodeuteride (Aldrich Chemical Co.), sodium hydroxide (BDH), acetonitrile and chloroform (HPLC grade, Rathburn).

Analytical techniques.—Methylation of laminaran samples prior to FAB-MS was performed using a modification of the NaOH slurry method previously described [10]. Samples (0.1–1.2 mg) were solubilised in DMSO at 40 °C and a slurry of NaOH in DMSO was added to the solution followed by methyl iodide (0.2 mL). The mixture was vortexed for 20 min and then the addition of NaOH and MeI was repeated. The mixture was vortexed periodically for 1 to 1.5 h then cooled on ice before the reaction was terminated by the addition of 1 mL of water. Permethylated laminaran was extracted with chloroform (2 mL), and the organic layer was washed with water (3×2 mL) before drying under a stream of dry nitrogen.

Methylated laminarans were purified on Sep-Pak® C18 cartridges (Waters Ltd.) as previously described [10]. Eluates of 35, 50, 75, and 100% acetonitrile were collected and freeze-dried.

FAB MS.—Positive-ion mode FAB mass spectra of methylated laminarans were obtained with a ZAB-2SE-FPD mass spectrometer fitted with a cesium ion gun operated at 30 kV. Monothioglycerol (Sigma) was used as the matrix. Samples were dissolved in methanol proir to aliquoting into the matrix.

MALDI MS.—MALDI-TOF mass spectrometry was performed using a Voyager Elite PerSeptive Biosystems Biospectrometry Research Station laser-desorption mass spectrometer coupled with delayed extraction. Samples of native laminarans (0.2 to 1 mg) were dissolved in a minimum amount of UHQ water (100 to 300 μL) and ×10 and ×100 dilutions in UHQ water were made. Aliquots (1 μL) of each sample were analysed. The short methylation procedure described in [10] was used for preparation of methylated samples for MALDI-MS. Methylated laminarans were dissolved in 80:20 v/v methanol:water prior to addition to the matrix. 2,5-Dihydroxybenzoic acid (DHB) was used as the matrix. Insulin B chain was used as an external calibrant.

3. Results

Laminaran samples.—The following samples were examined (i) mixtures of M- and G-chains from *Alaria angusta*, *Chorda filum*, *Laminaria cichorioides*, *L. hyperborea*, *L. digitata* and *S. phaerotrichia divaricata*; (ii) G-chains from *Cystoseira barbata* and *C. crinita* [4,11].

MS of mannitol-containing laminarans.—Native laminarans were examined by MALDI-MS to establish d.p. profiles. In these experiments, M-chains and G-chains, which differ by only 2 mass units cannot be distinguished at high d.p. values. Therefore, in a second set of experiments, samples were converted to their permethylated derivatives thus increasing the mass interval between G and M oligomers to 16 mass units, allowing unambiguous discrimination by MALDI-TOF at all d.p. values. Native laminarans from *A. angusta*, *L. cichorioides*, *L. hyperborea*, *L. digitata*, and *S. divaricata* gave MALDI spectra of similar appearance which closely resemble the data from Novotny et al. [8] on *L. digitata*. Typical data are shown in Fig. 1 for *L. hyperborea* and *L. cichorioides*. Both samples contain oligomers up to at least d.p. 40 with abundance peaking at d.p. 26 (*m/z* 4259). This maximum falls between 5000 Da (determined by gel chromatography) and 3200 Da (d.p. ca 20, linkage analysis) reported for *L. cichorioides* in ref. [6]. The other laminarans studied differed slightly in the d.p. of the largest oligomer observed (range $n=31$ to $n=40$) and in the position of the maximum ion abundance (from $n=23$ to $n=26$). In contrast to these laminarans, the majority of *C. filum* chains are considerably smaller with abundance peaking at d.p.12, although minor oligomers up to d.p. 38 are present (Fig. 2). Interestingly, *C. filum* oligomers up to about d.p. 23 show significant satellite signals 18 mass units below each molecular ion, consistent with loss of water. Corresponding signals

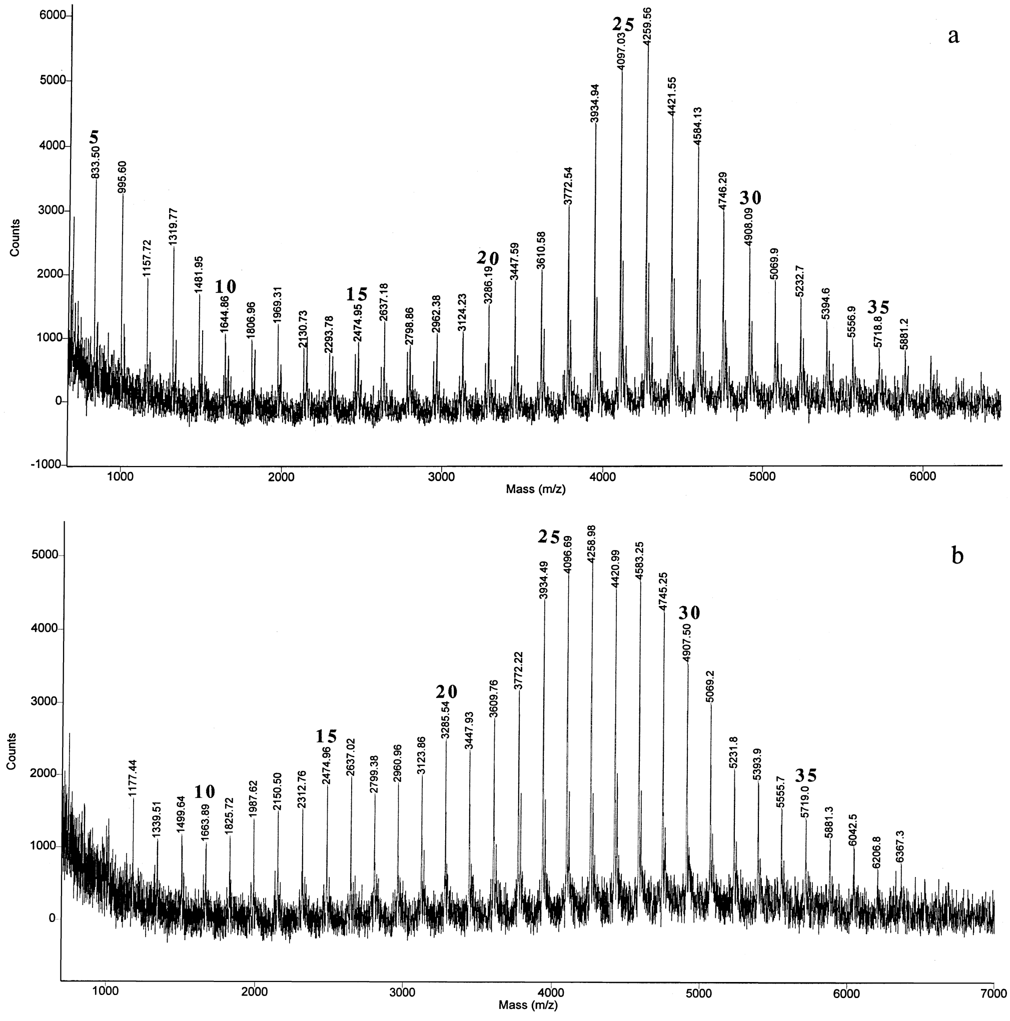

Fig. 1. MALDI-TOF mass spectra of native laminarans from (a) *L. hyperborea* and (b) *L. cichorioides*. The most abundant signals are $[M+Na]^+$; the minor satellites to higher mass are $[M+K]^+$ ions.

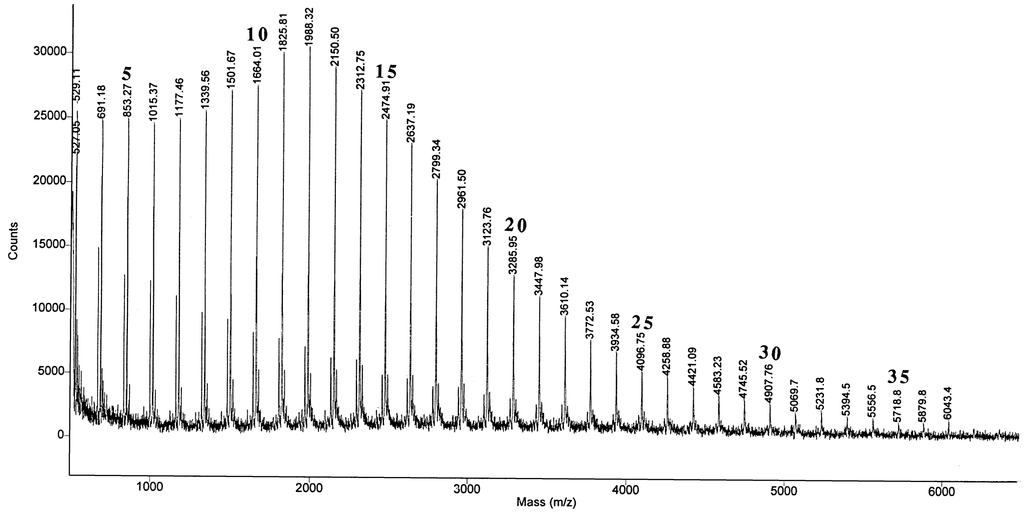

Fig. 2. MALDI-TOF mass spectrum of native laminaran from *C. filum*. Each major $[M+Na]^+$ molecular ion is accompanied by a minor $[M+K]^+$ ion to higher mass.

are absent in spectra of the other M- and G-chain-containing laminarans indicating that water loss does not normally occur during MALDI analysis of laminarans. Thus the "minus 18" signals in the *C. filum* laminaran spectrum are probably molecular ions of minor constituents of the sample which, prior to MALDI-MS, have lost water by cyclisation or elimination. Cyclisation was suggested by MALDI and FAB data of permethylated derivatives. Partial MALDI data for permethylated *L. hyperborea* and *C. filum* laminarans are shown in Fig. 3(a) and (b), respectively. The major molecular ions in Fig. 3(a) occur as pairs corresponding to G-chains and M-chains. In contrast, the spectrum of laminaran from *C. filum* is more complex with each abundant molecular ion cluster containing a prominent signal 46 mass units below the ion corresponding to the G-chain (reducing-end constituent) [Fig. 3(b), see legend for assignments]. Corroborative data were obtained in the FAB experiments (data not shown). The 46 mass units intervals are consistent with cyclisation. Further, the absence of analogous satellites for M-chains suggests that cyclisation involves the reducing end residue. Whether the cyclic components are macrocycles or have an anhydro sugar at the reducing end remains to be established.

MS of mannitol-free laminarans.—Laminarans from *C. crinita* and *C. barbata* are believed to be mannitol-free [4a]. This was confirmed by MS experiments on native and methylated samples. However, peaks distinct from $[G_n+Na]^+$ molecular ions were found for both laminarans suggesting the existence of novel minor constituents. Thus MALDI analysis of *C. crinita* laminaran showed molecular ions consistent with G-oligomers up to d.p. 31 with abundance peaking at d.p. 23 [Fig. 4; (a) native and (b) methylated]. However, close examination of the methylated data revealed two regions of the spectrum where clusters of ions indicated additional components, namely d.p. 5 to d.p. 10 and d.p. 16 to d.p. 17 [Fig. 4(b)]. The latter show abundant signals 46 mass units lower than the corresponding G_n value consistent with cyclic components (see discussion of *C. filum* data above). This was corroborated by FAB-MS of methylated, deuteromethylated and reduced/methylated derivatives (data not shown). The former are more interesting. They exhibit satellite signals 41 mass units higher than the sodiated ion for G_n. This mass interval is consistent with the presence of a Hex*N*Ac residue. These "plus 41" components were clearly observed in the FAB-MS experiment where purification on Sep-Pak C18 (see Experimental) resulted in their enrichment in the 50% acetonitrile fraction [Fig. 4(c)]. Notably these constituents gave both $[M+H]^+$ and $[M+Na]^+$ ions, in contrast to the G_n components which exhibited sodiated molecular ions only. This is in accord with the well established FAB-MS behaviour of HexNAc containing oligosaccharides [9]. Importantly, an A-type fragment ion at *m/z* 260 [Fig. 4(c), inset], which shifts to *m/z* 272 in the spectrum of the deuteromethylated derivative (data not shown) establishes the non-reducing terminal location of this putative HexNAc [9].

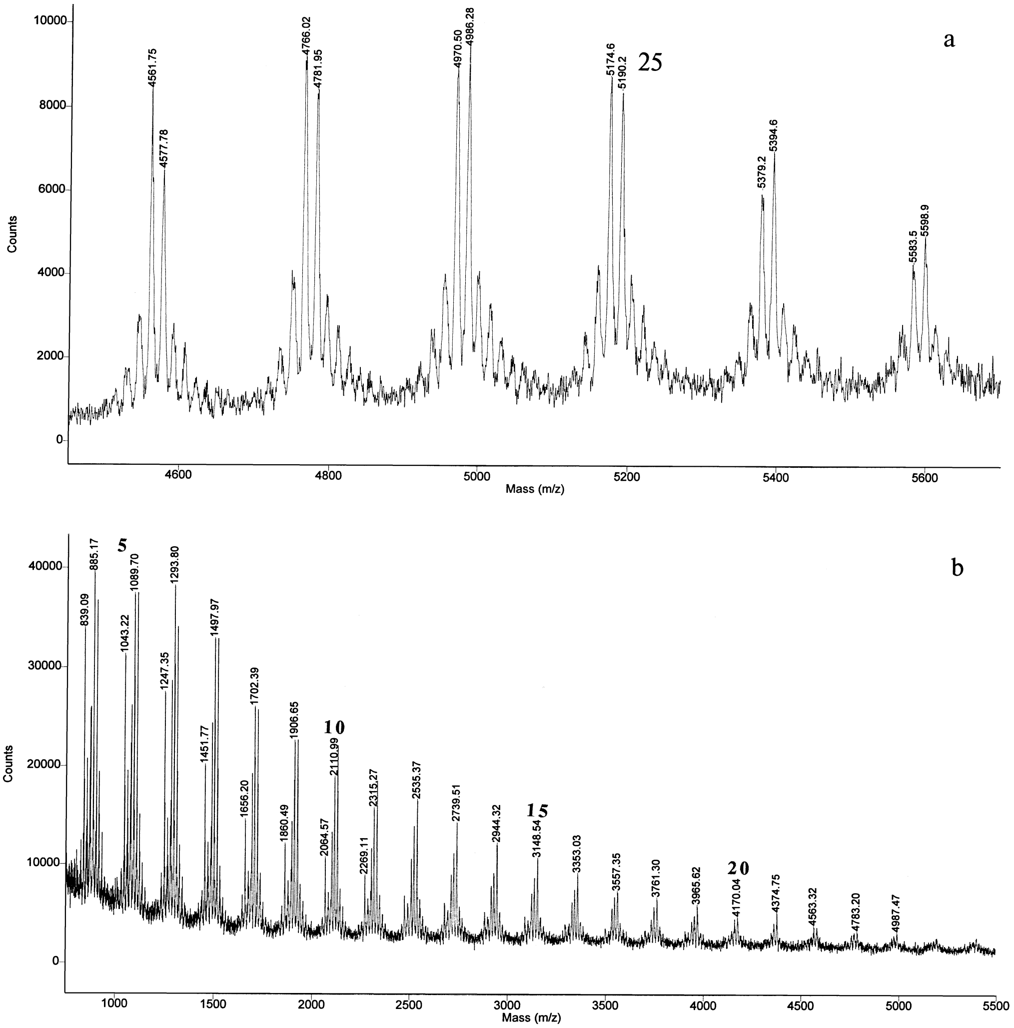

Fig. 3. Partial MALDI-TOF mass spectra of methylated laminarans from (a) *L. hyperborea* and (b) *C. filum*. In addition to the signals assigned in the text the C. filum spectrum shows a major signal in each cluster consistent with undermethylation at two sites in each of the M-chains. This is probably due to the mild methylation procedure which was employed in order to minimise peeling reactions (see Experimental).

These observations are very surprising because no amino sugars have been found in polysaccharides of brown algae except the recent finding of an N-acetylglucosamine-containing minor fraction of commercial fucoidan from *Fucus vesiculosus* [12]. Experiments are under way to define the amino sugar found in *C. crinita* laminaran. No NAc signals were observed in the ^{13}C NMR spectrum of this sample [4a] but this is not surprising in view of the low abundance of HexNAc which is present as only a single residue in oligomers of d.p. 5–10 which themselves constitute less than 5% of the total oligomer population.

The MALDI spectrum for native *C. barbata* laminaran contains major $[G_n + Na]^+$ signals for components up to d.p. 32. Mono-HexNAc-containing ions are observed for the higher oligomers, d.p. = 22–31 (Fig. 5). FAB analysis of the permethylated derivative confirmed the presence of terminal HexNAc (data not shown). Thus both *Cystoseira* species appear to be capable of incorporating HexNAc into their laminarans although they differ with respect to the d.p. values of the HexNAc-containing components.

4. Discussion

Eight laminarans from different species of brown algae were studied by MALDI MS (both in native and methylated forms) and FAB MS (as permethylated derivatives). The existence of M- and G-chains was confirmed for *Alaria angusta*, *Chorda filum*, *Laminaria cichorioides*, *L. digitata*,

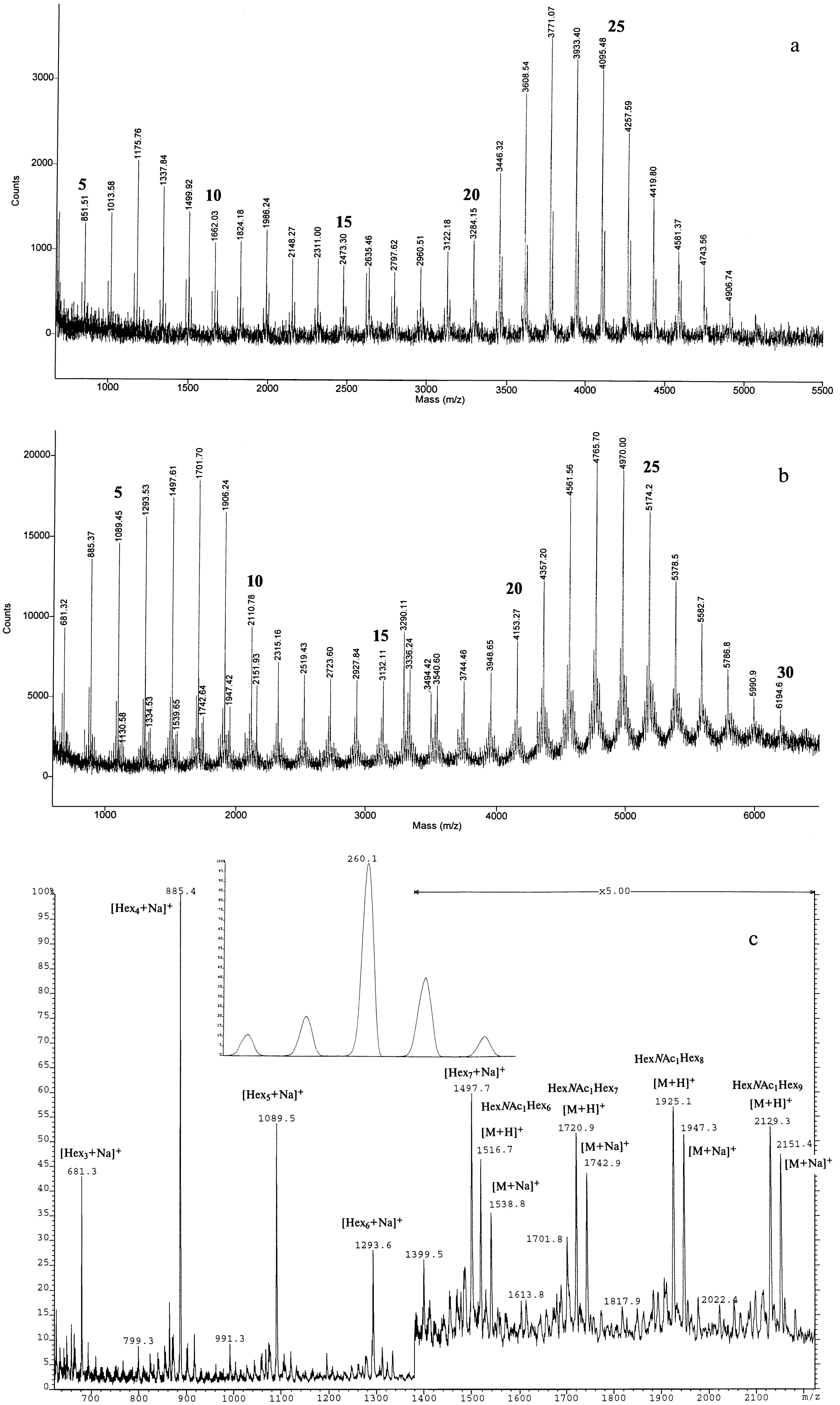

Fig. 4. MS analyses of *C. crinita* laminaran: (a) MALDI-TOF mass spectrum of native sample; (b) MALDI-TOF mass spectrum of the methylated derivative; (c) partial FAB mass spectrum of the 50% acetonitrile fraction from Sep-Pak C18 purification of the methylated derivative; the inset shows the A-type fragment ion at *m*/*z* 260.

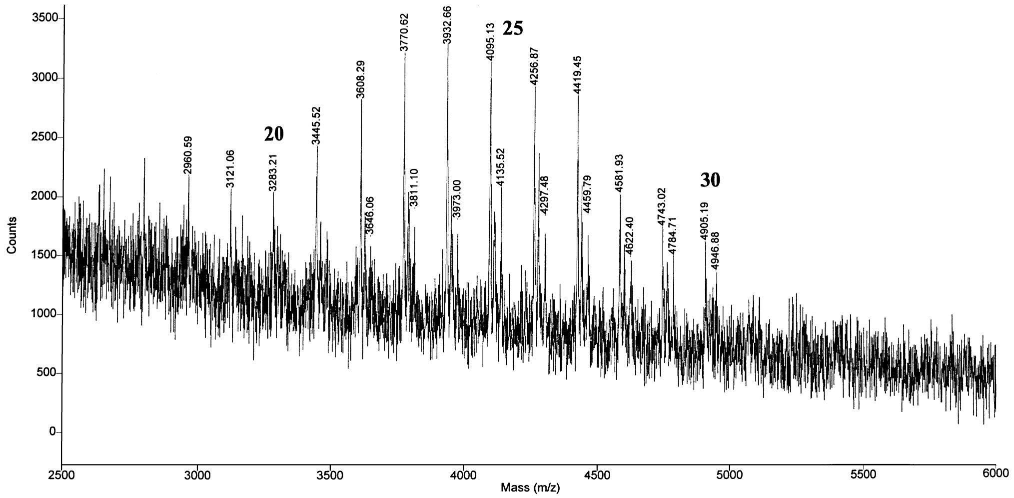

Fig. 5. MALDI-TOF mass spectrum of native laminaran from *C. barbata*.

L. hyperborea, and *Sphaerotrichia divaricata*, and the absence of M-chains in laminarans from *Cystoseira barbata* and *C. crinita* was demonstrated unambiguously. The MS experiments provided evidence for the existence of minor amounts of cyclic oligomers in some samples. In addition, the glucans from *Cystoseira* sp. were unexpectedly found to contain a small percentage of N-acetylhexosamine-terminated chains.

The data in this paper illustrate the power of modern MS technology for defining d.p. profiles of polysaccharides and for revealing the presence of unexpected constituents such as the cyclic and HexNAc-containing oligomers observed in this work. MALDI-TOF is the technique of choice for analysing native laminarans but FAB-MS is a valuable complement to MALDI for the study of derivatives and is particularly useful for establishing the location of HexNAc residues via A-type cleavage [9] which reliably occurs at HexNAc in the FAB experiment. The main drawback of FAB-MS for the investigation of d.p. profiles is the drop of signal intensity in the high m/z range and, hence, overestimation of lower-mass components. Thus, the d.p. patterns obtained by FAB-MS do not accurately reflect relative concentrations of low and high molecular weight components and the higher molecular weight cut-off should be interpreted with caution. The d.p. distribution defined from MALDI mass spectra is in more close agreement with that found by HPAEC [14].

It should be noted that peaks due to derivatisation artefacts were observed in FAB mass spectra if long reaction times were employed in order to minimise undermethylation (see Experimental). For example, "minus 46" signals were present in some methylated samples which did not exhibit corresponding "minus 18" signals prior to methylation. The components which afford these signals are probably produced by alkaline degradation ("peeling", ref. [2], pp 28–31) resulting in permethylated 6-*O*-glycosylated 3-*O*-deoxyhexonolactones. At least in aqueous media, the formation of 6-*O*-glycosylated 3-deoxyhexonic (metasaccharinic) acids has been established for alkaline peeling of β-(1→3, 1→6)-glucans [15]. Whilst the presence of artefact peaks does not interfere with d.p. and/or M and G assignments from permethyl derivatives, caution needs to be exercised when assigning possible artefact peaks since genuine dehydrated and/or cyclic components give the same molecular ions. Although artefacts can complicate the spectra of derivatives, it is nevertheless important to acquire data from derivatives, as well as their native counterparts, because (i) very minor components might be revealed due to the extra sensitivity conferred by derivatisation, and (ii) derivatisation permits fragment ions, for example those derived from elimination of water from native samples, to be distinguished from genuine molecular ions. Furthermore, structurally useful fragment ions, e.g., the HexNAc A-type ion, may be present in the FAB spectra of derivatives.

Acknowledgements

A.O.C. is a recipient of the NATO/Royal Society Postdoctoral Research Programme Fellowship Award. This work was supported by the

Biotechnology and Biological Sciences Research Council and the Wellcome Trust (Grant 030825).

References

[1] E. Percival and R.H. McDowell, *Chemistry and Enzymology of Marine Algal Polysaccharides*, 1967, Academic Press, New York and London.

[2] D.J. Manners and R.J. Sturgeon, Reserve carbohydrates of algae, fungi, and lichens, in F.A. Loewus, and W. Tanner (Eds.), *Encyclopedia of Plant Physiology*, New Series, Vol. 13A, Springer-Verlag, Berlin, Heidelberg, New York, 1982, pp 472–514.

[3] B.A. Stone and A.E. Clarke, *Chemistry and Biology of (1–3)-β-Glucans*, La Trobe University Press, Melbourne, Australia, 1992, pp 1–803.

[4] a. A.I. Usov and A.O. Chizhov, *Izv. Akad. Nauk, Ser. Khim.*, (1993) 1160–1164 [Engl. transl. *Russ. Chem. Bull.*, 42 (1993) 1597–1601]; b. *Bioorgan. Khim.*, 15 (1989) 208–216 [Engl. trans. *Sov. J. Bioorgan. Chem.*, 15 (1989) 118–126].

[5] A. Kobayashi, A. Tai, and K. Kawazu, *J. Carbohydr. Chem.*, 14 (1995) 819–832.

[6] T.N. Zvyagintseva, L.A. Elyakova, and V.V. Isakov, *Bioorgan. Khim.*, 21 (1995) 208–216.

[7] S.M. Read, G. Currie, and A. Bacic, *Carbohydr. Res.*, 281 (1996) 187–201.

[8] P. Chen, A.G. Baker, and M.V. Novotny, *Analyt. Biochem.*, 244 (1997) 144–151.

[9] A. Dell, *Adv. Carbohydr. Chem. Biochem.*, 45 (1987) 19–72.

[10] A. Dell, A.J. Reason, K.-H. Khoo, M. Panico, R.A. McDowell, H.R. Morris, *Methods Enzymol.*, 230 (1994) 108–132.

[11] A.I. Usov and A.O. Chizhov, *Izv. Akad. Nauk, Ser. Khim.*, (1993) 1813–1817 [Engl. transl. *Russ. Chem. Bull.*, 42, 1738–1742].

[12] T. Nishino, C. Nishioka, H. Ura, and T. Nagumo, *Carbohydr. Res.*, 255 (1994) 213–224.

[13] P.E. Pfeffer, S.E. Osman, A. Hotchkiss, A.A. Bhagwat, D.E. Keister, and K.M. Valentine, *Carbohydr. Res.*, 296 (1996) 23–37.

[14] B. Stahl, A. Linos, M. Karas, F. Hillenkamp, and M. Steup, *Analyt. Biochem.*, 246 (1997) 195–204.

[15] G.O. Aspinall, T.N. Krishnamurthy, I. Furda, and R. Khan, *Canad. J. Chem.*, 53 (1975) 2171–2177.