Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 339 (2004) 421–424
Carbohydrate RESEARCH

Note

Characterization of a β-D-(1 → 3)-glucan from the marine diatom *Chaetoceros mülleri* by high-resolution magic-angle spinning NMR spectroscopy on whole algal cells

Trond R. Størseth,[a,b,*] Karina Hansen,[a] Jorunn Skjermo[a] and Jostein Krane[b]

[a]*Department of Bioresources, SINTEF Fisheries and Aquaculture, 7465 Trondheim, Norway*
[b]*Department of Chemistry, Norwegian University of Science and Technology, 7491 Trondheim, Norway*

Received 12 August 2003; accepted 29 October 2003

Abstract—High-resolution magic-angle spinning (hr-MAS) NMR spectroscopy was used to record NMR spectra of a cell paste from the marine diatom *Chaetoceros mülleri*. This gave information on a cellular storage polysaccharide identified as a β-D-(1 → 3)-linked glucan, using hr-MAS one-dimensional ^{1}H and ^{13}C, two-dimensional $^{1}H,^{1}H$-COSY and $^{13}C,^{1}H$-correlation spectroscopy. The same structural information was deduced from the liquid state NMR data on the glucan extracted from *C. mülleri*. The extracted glucan proved to be a β-D-(1 → 3)-linked glucan with a degree of polymerization of 19 and a degree of β-D-(1 → 6) branching of 0.005. The hr-MAS spectrum of the diatom showed several nonglucan resonances in the carbohydrate region of the NMR spectrum (60–103 ppm) that were shown to be noncarbohydrate resonances by means of two-dimensional $^{13}C,^{1}H$- and $^{1}H,^{1}H$-correlated NMR data.

Keywords: Hr-MAS NMR; β-D-Glucan; Diatom; *Chaetoceros mülleri*

In high-resolution magic-angle spinning (hr-MAS) NMR spectroscopy the sample is spun around its own axis at an angle of 54.7° off the *z* axis of the magnetic field of the NMR magnet, thereby reducing the chemical-shift anisotropy contribution to line broadening in the NMR spectrum.[1] This allows for the study of semi-solids by NMR spectroscopy with a resolution that may be compared to the resolution of liquid samples. There is increasing interest in using NMR spectroscopy for tissue studies,[2,3] and the hr-MAS technique is a promising method that may save laborious and expensive extraction and purification procedures. Sample preparation is simplified; with simple addition of a deuterated solvent to the desired biological sample for field–frequency locking purposes, and loading the sample into the hr-MAS rotor.[2]

β-D-Glucans are well established as fish immunostimulants, as reviewed by Sakai.[4] The stimulatory action of β-D-glucans is connected to the presence of β-D-(1 → 3)-linked side chains, linked to the main β-D-(1 → 3) chain through β-D-(1 → 6) linkages.[5] Commercial glucan products for use in aquaculture, such as the yeast glucan Macrogard® (KS Biotec-Mackzymal, Tromsø, Norway), are available. The glucan laminaran, from the brown algae *Laminaria hyperborea*, has been reported to be immunostimulatory for fish.[6] Laminaran is a low molecular weight β-D-(1 → 3,1 → 6)-glucan with a DP_n 20–30, corresponding to a molecular weight of 3300–5000, and a degree of branching (DB) of 0.05–0.07.[7] Chrysolaminaran, which is the storage polysaccharide of diatoms, has similar structural features, with a reported degree of polymerization (DP_n) of 20–60, and branches have been reported to occur in the (1 → 6) and (1 → 2) positions,[8–10] Several microalgae are presumed to have β-D-(1 → 3)-glucans as food reserves,[11] and are interesting as sources for immunomodulatory β-D-glucans. It is therefore desirable to have a fast and efficient

* Corresponding author. Tel.: +47-73-59-63-83; fax: +47-73-59-56-60; e-mail: trond.r.storseth@sintef.no

0008-6215/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2003.10.021

method for obtaining information on the linkages of β-D-glucans from diatoms and other microalgae.

Here we describe the use of hr-MAS NMR to study the linkages of the chrysolaminaran from a cell paste of the diatom *Chaetoceros mülleri*, and report the comparison of these results with the polysaccharide extract.

1. Results and Discussion

Kim et al.[7] have performed ^{1}H NMR spectroscopy in the liquid phase of a series of glucans to determine DP_n ($n = 2$–600) and DB. By using their method, the DP_n and DB of the chrysolaminaran extracted from *C. mülleri* were in this study determined to be 19 and 0.005, respectively. ^{13}C-DEPT NMR confirmed the β-D-(1→3) linkage but failed to detect the C-6 resonance of the β-D-(1→6) linkage due to the low signal-to-noise ratio of ^{13}C NMR.

Hr-MAS ^{1}H spectra with water suppression by presaturation of the water resonance, and a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence filter with additional presaturation of the water resonance were recorded of the algal cells (Fig. 1A and B). In spectrum A obtained with presaturation only, the broad water peak overlapped the anomeric protons almost completely. These signals could, however, be observed, and the backbone chain (BC), nonreducing end (NRT), and reducing-end (RT) resonances were identified, along with the anomeric peak from β-D-glucose, on the basis of their chemical shifts. These are compared to extract and literature values in Table 1. The broad water peak is due to the chemical shift difference between intra- and extracellular water, for which presaturation of one resonance leaves a broad residual peak. With the combined presaturation–CPMG pulse sequence (spectrum B) anomeric resonances were observed with greater resolution. However, caution should be exercised using data from CPMG-filtered acquisition in a quantitative way on small metabolites because of *J*-coupling modulation. The hr-MAS ^{1}H,^{1}H-COSY[12] (COSY) spectrum of *C. mülleri* gave resolved anomeric proton/H-2 cross peaks in greater detail. The α-anomeric proton of both chrysolaminaran and glucose was found at 5.23 ppm at the anomeric resonance frequency of the crosspeak, but was separated by the resonance frequencies of H-2, which were 3.73 and 3.54 for chrysolaminaran and glucose, respectively (verified by standards). The crosspeak from the unit second to the reducing end (SRT) was observed overlapped with the BC cross peak on the H-2 resonance frequency and the NRT on the anomeric resonance frequency of the crosspeaks. Chemical shifts determined from the COSY spectrum helped confirm and assist the assignments of the 1D spectra. Deviations observed between the hr-MAS results and the liquid-state results can be explained by different dielectric constants and solvation properties. The hr-MAS chemical shifts are remarkably close to those of the extract, which is to be expected because of the intracellular water matrix.

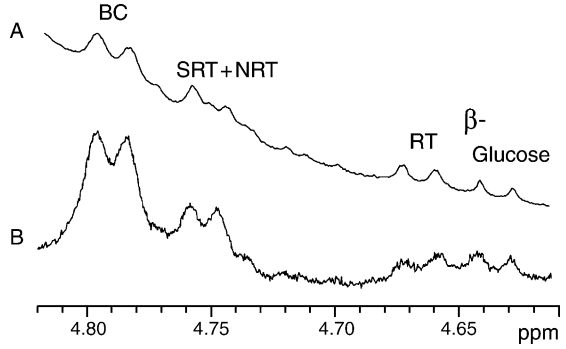

Figure 1. The β-anomeric region of the hr-MAS ^{1}H NMR spectra of *C. mülleri*. (A) Presaturation of water resonance, (B) CPMG pulse sequence with presaturation.

The hr-MAS ^{13}C NMR spectrum (Fig. 2) gave the chrysolaminaran peaks listed in Table 2 along with peaks from noncarbohydrate residues, which could be excluded as originating from carbohydrate by means of a ^{13}C–^{1}H NMR spectrum (HETCORR[13]) together with the ^{1}H,^{1}H COSY spectrum. Glucose is the building block of chrysolaminaran, and the similar chemical shifts of nonbonded and bonded glucose units makes it important to identify the resonances of free glucose from those from the glucopyranosyl resonances of chrysolaminaran. The β-C-5 and C-6 resonances of glucose were shown to overlap with the respective C-5 and C-6 resaonances of

Table 1. δ-Values (ppm) for the anomeric protons detected in the hr-MAS ^{1}H and ^{1}H,^{1}H COSY NMR spectra of *C. mülleri*

Anomeric proton	Glucose α/β	BC	RT α/β	NRT	SRT	SC	TSC
MAS	5.23/4.64	4.79	5.23/4.67	4.75	4.76[a]	[b]	[b]
Extract D_2O	—	4.81	5.24/4.69	4.77	4.77[a]	4.57	4.55
Extract Me_2SO/D_2O	—	4.57	5.03/4.43	4.46	4.53[a]	4.34	4.28
Literature Me_2SO–D_2O[c]	—	4.57	5.03/4.43	4.46	4.53	4.34	4.28

BC: backbone chain, RT: reducing terminal, NRT: nonreducing terminal. SRT: second to reducing terminal, SC: side chain, TSC: terminal side chain. The Me_2SO–D_2O ratio is 6:1.

[a] Partial signal overlap with BC.

[b] Not observed.

[c] Ref. 7.

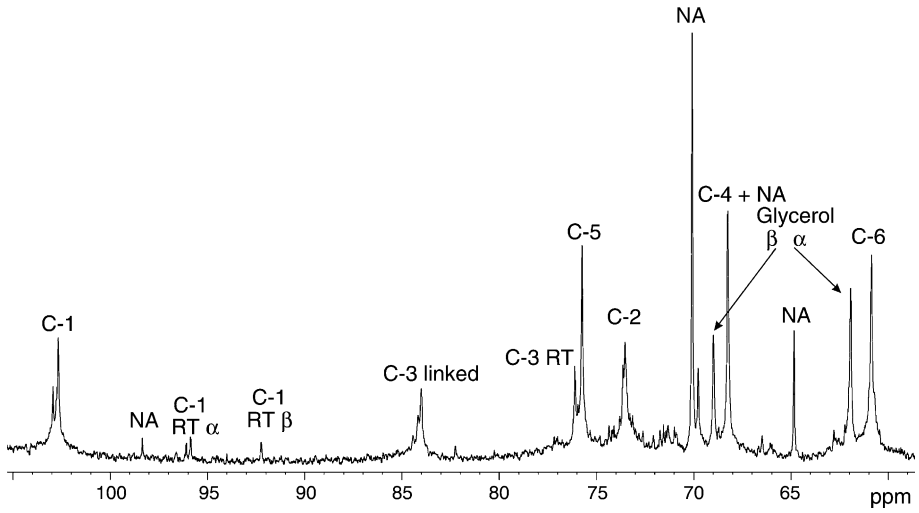

Figure 2. ^{13}C The 59–104 ppm region of the hr-MAS ^{13}C NMR spectrum of *C. mülleri*. NA: Not assigned.

Table 2. ^{13}C NMR δ-values in ppm for the linear β-D-(1→3)-glucan extracted from *C. mülleri*

Nucleus	Hr-MAS δ values	Extract δ values
C-1	102.84	102.96
C-1 linked	102.68	102.68
Reducing-end signal α	95.88	95.68
Reducing-end signal β	92.26	92.16
C-2 RT	74.07	73.98
C-2 NRT	73.68	73.64
C-2 BC	73.57	73.42
C-3	84.44	84.41
C-3	84.19	84.26
C-3	84.03	84.26
Nonreducing end signal	76.14	76.17
C-4	68.27	68.29
C-5[a]	75.77	75.79
C-6[b]	60.88	60.88

[a]Overlaps with C-5 β of glucose.
[b]Overlaps with C-6 of glucose.

the chrysolaminaran. Glycerol/glyceride resonances at 61.97 ppm (α) and 69.01 ppm (β) were readily identified, and other unassigned peaks, two of which were overlapped with chrysolaminaran peaks, were also excluded as carbohydrate peaks by the combination of 2D techniques. The C-4 resonance of the chrysolaminaran and an unidentified peak resonating at the same frequency (68.27 ppm) was detected, and the C-6 resonance at 60.88 ppm was found to have a broad baseline, due to overlap with a unidentified peak at 60.78 ppm.

In addition to the resonances found in the carbohydrate region of the ^{13}C-spectrum, resonances were also found that corresponded with fatty acids. In the low-field region the alkenic and carbonyl signals were observed, and in the high-field region the allyllic, methylene, and methyl resonances were found. The interpretation of these resonances are discussed elsewhere (unpublished data).

Hr-MAS NMR spectroscopy using 1D ^{1}H and ^{13}C and 2D ^{1}H,^{1}H COSY, and heteronuclear correlated techniques (HETCORR, HSQC, and HMBC) opens up the possibility for study of chrysolaminaran structure in *C. mülleri* and possibly other microalgae as well, using small sample quantities readily collected by centrifugation. It is here shown that hr-MAS NMR on microalgal cell paste may be used as a rapid and simple method for obtaining linkage information from storage polysaccharides. As such, this technique may be used to screen microalgae for β-D-glucans of interest for immunostimulation. As compared to liquid-state NMR methods for structure determination of polysaccharides[7,14] the hr-MAS NMR method requires less sample preparation and chemicals, and the time needed for analysis is of the same magnitude. Furthermore sample quantities are substantially decreased, affording the possibility for study of cellular dynamics with respect to carbohydrate production, utilizing small samples from cultures.

Hr-MAS in the study of diatoms and other classes of microalgae has recently been reported, and multivariate analysis of ^{1}H-MAS spectra has been used as a taxonomic tool.[15] The fatty acid composition of *C. mülleri* by ^{13}C MAS NMR spectroscopy has also been studied. The possibility of deducing the structure of storage polysaccharides adds to the utility of hr-MAS NMR as a tool for studying microalgae.

2. Experimental

2.1. Cultivation and collection of *C. mülleri*

C. mülleri was cultivated in 200 L f/2-medium.[16] Six days after medium inoculation (early stationary phase) the culture was collected for extraction. For the hr-MAS sample, *C. mülleri* was cultivated in 10 L f/2-medium and collected at day 9 (late stationary phase).

2.2. Extraction of chrysolaminaran from *C. mülleri*

Freeze-dried algal paste was extracted with 5 mM H_2SO_4 for 15 min at 60 °C. The extract was neutralized with NaOH, filtered (Whatman No.1), and dialyzed (MCWO 1000) against MQ-water and freeze dried to give chrysolaminaran as a white/yellow powder.

2.3. Sample preparation

For MAS, the algal paste with added D_2O was loaded into a 50 μL 4-mm MAS rotor. For liquid state experiments in 6:1 Me_2SO–D_2O samples where prepared according to the literature.[7] For ^1H experiments in D_2O, 6 mg of the chrysolaminaran and of the standards were dissolved in 600 μL D_2O. For ^{13}C experiments, 20 mg was used. Standards used in the experiment were laminarin from *Laminaria digitata*, and gentiobiose, and glucose purchased from Sigma–Aldrich Norway AS.

2.4. NMR spectroscopy

MAS experiments were recorded on a Bruker DRX 600 MHz magnet equipped with a 4-mm MAS probe at 278 K using standard Bruker pulse programs for recording of ^1H-presaturated, ^1H-CPMG-presaturated, ^1H,^1H-presaturated COSY, and HETCOR spectra, spinning the sample at the magic angle with a frequency of 5000 Hz. For liquid-state experiments a 5-mm BBO variable-temperature probe was used to record ^{13}C and DEPT135 spectra at 298 K. The ^1H spectra were recorded in 6:1 Me_2SO–D_2O by the method of Kim et al.[7] ^1H,^1H-COSY of extracts and standards were recorded on a Bruker DRX 500 MHz instrument equipped with a TXI probe head, using a gradient assisted COSY pulse sequence (Bruker, cosygp), and on a Bruker DPX 400 MHz instrument equipped with a QNP probehead, using a Bruker cosy45 pulse sequence at 298 K for both magnets.

Acknowledgements

This work was supported by the Norwegian Research Council project 143450/140. The Bruker Biospin, GmbH company, Rheinstetten, Germany, is thanked for kind collaboration in the development of the 600 MHz hr-MAS probes.

References

1. Gawrisch, K.; Eldho, I. V.; Polozov, I. V. *Chem. Phys. Lip.* **2002**, *116*(1), 135–151.
2. Sitter, B.; Sonnewald, U.; Spraul, M.; Fjösne, H. E.; Gribbestad, I. S. *NMR Biomed.* **2002**, *15*, 327–337.
3. Cheng, L. L.; Ma, M. J.; Becerra, L.; Ptak, T.; Tracey, I.; Lackner, A.; Gonzalez, R. G. *FEBS Lett.* **1997**, *94*, 6408–6413.
4. Sakai, M. *Aquaculture* **1999**, *172*, 63–92.
5. Engstad, R. E.; Robertsen, B. *Dev. Comp. Immunol.* **1993**, *17*, 319–330.
6. Dalmo, R. A.; Seljelid, R. *J. Fish Dis.* **1995**, *18*, 175–185.
7. Kim, Y.-T.; Kim, E.-H.; Cheong, C.; Williams, D. L.; Kim, C.-W.; Lim, S.-T. *Carbohydr. Res.* **2000**, *328*, 331–341.
8. Beattie, A.; Hirst, E. L.; Percival, E. *Biochem. J.* **1961**, *79*, 532–537.
9. Paulsen, B. S.; Myklestad, S. *Carbohydr. Res.* **1978**, *62*, 386–388.
10. McConville, M. J.; Bacic, A.; Clarke, A. E. *Carbohydr. Res.* **1986**, *153*, 330–333.
11. Granum, E. Dr. Ing. Thesis, Norwegian University of Science and Technology, 2002.
12. Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976**, *64*, 2229–2246.
13. Bax, A.; Morris, G. A. *J. Magn. Reson.* **1981**, *42*, 501–505.
14. Vaarum, K. M.; Myklestad, S. *Carbohydr. Res.* **1986**, *152*, 243–248.
15. Chauton, M. S.; Optun, O. I.; Bathen, T. F.; Volent, Z.; Gribbestad, I. S.; Johnsen, G. *Mar. Ecol. Progr. Ser.* **2003**, *256*, 57–62.
16. Guillard, R. R. L.; Ryther, J. H. *Can. J. Microbiol.* **1962**, *8*, 229–239.