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Structural characterization of β-D-(1→3)-glucans from different growth phases of the marine diatoms *Chaetoceros mülleri* and *Thalassiosira weissflogii*

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Abstract—We have investigated the content and structure of the chrysolaminarans isolated from the two marine diatoms *Chaetoceros mülleri* and *Thalassiosira weissflogii*. Samples were taken from different phases of growth, and the structure of the chrysolaminaran was seen in relation to the specific growth rate of the diatoms. The structure determined for the glucan from *C. mülleri* was found not to vary with different specific growth rates. *T. weissflogii* showed some variance in the structure, both throughout the different stages of growth and between samples taken from the stationary phase. *C. mülleri* was found to have a chrysolaminaran with a degree of polymerization (DP) of 22–24 and a degree of β-(1→6) branching of 0.006–0.009. These results corresponded well with previous results obtained in our laboratories. The chrysolaminaran isolated from *T. weissflogii* was found to have a DP of 5–13 and no β-(1→6) branching. This is to our knowledge the first characterization of the chrysolaminaran from *T. weissflogii*.

Keywords: β-Glucan; Diatom; Chrysolaminaran; *Chaetoceros mülleri*; *Thalassiosira weissflogii*

1. Introduction

One major task within the international aquaculture industry is to deal with the poorly developed immune system of newly hatched fish larvae, which is one of the reasons for high mortality rates following hatching. Increasing problems with viral and bacterial infections call for strategies other than conventional antibiotics to ensure high yields, and one such strategy would be the use of immunostimulants.[1–4] Glucans with β-(1→3,1→6)-linked anhydroglucose units (AGUs) are well established as immunostimulants. They have the ability to enhance the immune system of mammals and the effect of glucans from different sources have been documented on fish.[5,6] Chrysolaminarans are the storage polysaccharides of marine diatoms. Characterized chrysolaminarans have been found to consist of β-(1→3)-linked AGU chains with reported chain lengths of 20–60[7,8] and branches in the β-(1→6) and β-(1→2) positions. Laminaran is a immunoactive (1→3)-β-D-glucan with a degree of polymerization (DP) of 20–33 and a degree of β-(1→6) branching (DB) of 0.05–0.07.[5,9] These structural features closely resemble those of the chrysolaminarans which up to now have been characterized.[8] Several studies have been done by Dalmo et al. documenting the effect of laminaran on Atlantic cod, *Gadus morhua* L., and Atlantic salmon, *Salmo salar* L. Different methods for administration of laminaran have given increased production of superoxide anion, and the activity of acid phosphatase has been seen to be elevated.[5] Injection of laminaran have also increased survival against experimental bacterial infections.[10] A review of β-glucans as immunostimulants and the immune responses observed in fish is given by Sakai,[11] and Bohn and BeMiller.[12]

The structural similarities between laminaran and glucans isolated from diatoms make diatoms interesting as sources for screening for new immunostimulants.

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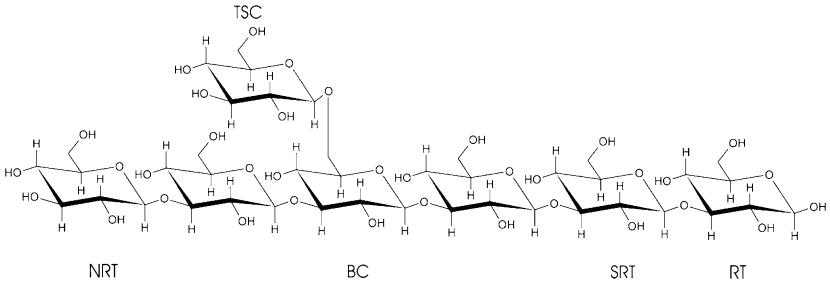

Figure 1. Structure elements of the β-(1→3, 1→6)-linked glucan isolated from *C. mülleri* and *T. weissflogii*. RT: reducing terminal group, BC: backbone chain, SRT: second to the reducing terminal group, NRT: nonreducing terminal group, TSC: terminal side-chain group.

Diatoms and other microalgae may be grown in a variety of different types of culture, for example, batch, continuous and semi-continuous. (1→3)-β-D-Glucans from diatoms may be produced in large quantities using either of these culture techniques. It would then be of importance to know how the structure of a chrysolaminaran varies during the different growth phases of a microalga. The chrysolaminaran of *C. mülleri* (C-glucan) has previously been described in our laboratories.[13] The DP and DB were found to be 19 and 0.005, respectively. In this work we wanted to further study the structure of the C-glucan. In addition, we wanted to check for variations in the structure during the growth phase. Another diatom, *Thalassiosira weissflogii*, was also chosen for the study. To our knowledge this is the first study done of the structure of the *T. weissflogii* glucan (T-glucan).

Publications where structural properties of (1→3)-β-D-glucans have been investigated have used ^{1}H NMR methodology based on work done by Kim et al.[9] This method yields the DP and DB of the glucan structure.[13,14] These parameters are calculated from integrated resonances of different AGUs in the β-D-glucan (Fig. 1). Good agreements between the ^{1}H NMR and other methods have been reported.[9,14] We have investigated the chrysolaminaran structure in different phases of growth of the two diatoms *C. mülleri* and *T. weissflogii* by using this protocol for ^{1}H NMR.

2. Results and discussion

We cultivated the two diatoms, *C. mülleri* and *T. weissflogii* in the Guillard f/2 medium to characterize their chrysolaminarans and to examine variations in the DP/DB ratio throughout the growth of the culture. Two batch cultures were set up of *C. mülleri*; One culture which was harvested on in the stationary phase, and one culture which was set up as a batch culture and harvested when the culture was in the exponential phase of growth, and in early and late stationary phase. The C-glucan had been characterized in our laboratories prior to this investigation.[13] *T. weissflogii* was set up in three batch cultures; Two cultures were harvested in the stationary phase and one culture was harvested in exponential and early and late stationary phase.

The isolated C-glucan from the batch culture of *C. mülleri* gave a ^{1}H NMR spectrum (Fig. 2) with the anomer resonances of the different sub units in the C-glucan chain well resolved. The resonances were found at 4.25–5.05 ppm and corresponded with those of a laminaran standard.[9] The α-anomer of the reducing terminal glucopyranosyl (RT) was found at 5.02 ppm while the β-anomer was found at 4.42 ppm. The resonance from the nonreducing AGU (NRT) was found at 4.44 ppm while the resonances from the backbone chain (BC) was found at 4.56 ppm in partial overlap with the unit second to the RT (SRT) at 4.50 ppm. The resonance from the terminal β-(1→6)-linked groups (TSC) was found at 4.27 ppm. The laminaran standard gives a resonance belonging to side chain β-(1→6)-linked AGUs (SC) not found in the C-glucan. The assignments are consistent with the results published by Kim et al.[9] This suggests that the branch points in the C-glucan consist of a single β-(1→6)-linked AGU. The assignments of the resonances were helped by ^{1}H,^{1}H COSY (Fig. 3), which resolved the anomeric resonances well. From the described resonances a DP/DB of 23/0.006 was calculated, which gives one β-(1→6) branch point for every seven chains of C-glucan. These results are similar to previously published values in which the chrysolaminaran from *C. mülleri* grown under the same conditions and with the same extraction method was found to have a DP/DB of 19/0.005.[13]

The *T. weissflogii* chrysolaminaran isolated from the two cultures harvested in the stationary phase gave a simpler NMR spectrum than the spectrum of the C-glucan. The same chemical shifts were observed for the different AGUs in the T-glucan as for the C-glucan except for the TSC-resonance which was not found. Thus, no β-(1→6) branching was observed for the T-glucan. The DP was calculated to be 7 and 13 for the two cultures.

From the growth curve of the two diatoms the specific growth rates were calculated for each day. Samples for extraction of chrysolaminaran were drawn from the cul-

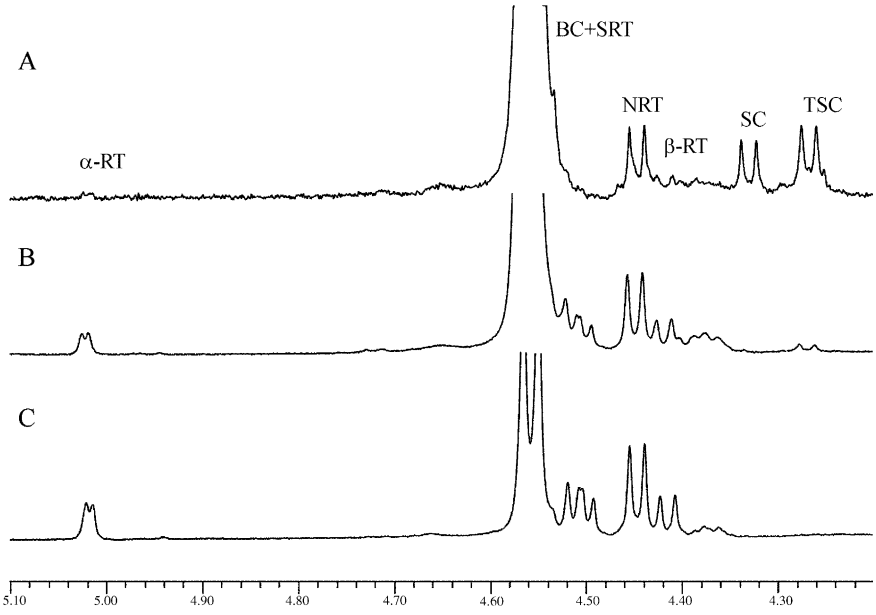

Figure 2. ^{1}H NMR of the anomeric region of (A) laminaran, (B) C-glucan and (C) T-glucan in 6:1 Me_2SO-d_6–D_2O at 353 K.

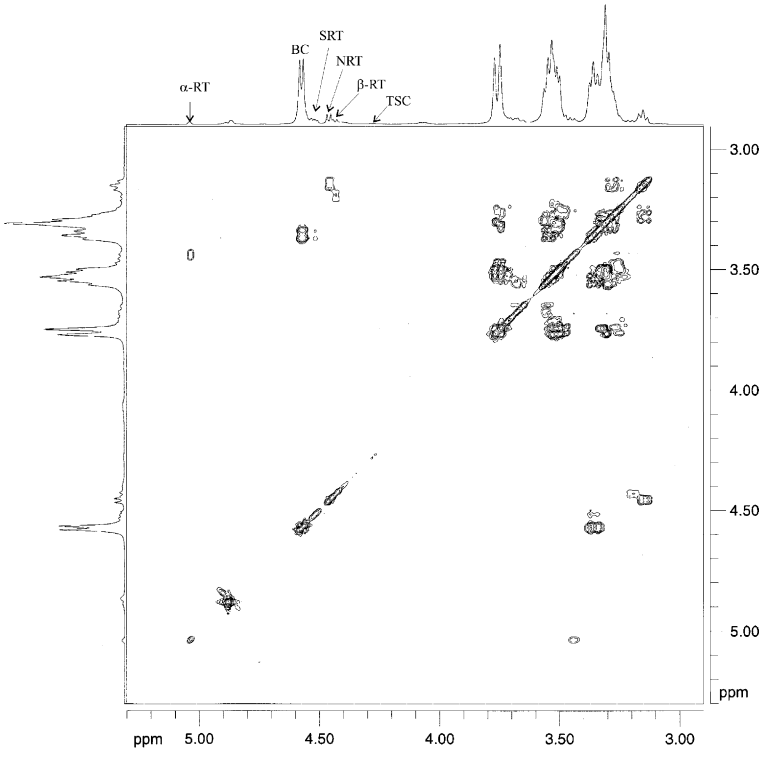

Figure 3. ^{1}H,^{1}H COSY of the C-glucan in Me_2SO-d_6 added with CF_3CO_2D at 333 K.

ture at days 2, 4 and 6. The *C. mülleri* culture was inoculated with a starting cell concentration of 1.4×10^6 cells/mL. The specific growth rate, μ, of the inoculum was high between day 1 and 2 at 0.45. Between day 2 and 3, μ was 0.27, and 0.16 between day 3 and 4. As the growth curve shows the culture reached stationary phase between day 4 and 5 ($\mu = 0.03$). The μ values at which the samples were drawn for *C. mülleri* spanned from 0.45 to 0.03. The isolated C-glucans all had DP/DBs of $23/0.008 \pm 1/0.001$ (Table 1). This

Table 1. DP/DB for the extracted glucans from *C. mülleri* and DP for the glucans extracted from *T. weissflogii*

Alga	DP/DB			
	Day 2	Day 4	Day 6	Batch
C. mülleri	22/0.008	24/0.007	22/0.009	23/0.006
T. weissflogii[a]	8	5	8	7

[a] Branching was not detected for *T. weissflogii*.

showed that the DP/DB of the culture stayed constant within 2/0.002 regardless of the growth phase and μ under our chosen conditions.

Lower cell concentrations were observed for the *T. weissflogii*, and the starting cell concentration was 37,000 cells/mL and on day 2 the concentration was 39,000 cells/mL ($\mu = 0.05$). The culture was thus in the lag phase between day 1 and 2. On day 3 the concentration had risen to 80,000 cells/mL ($\mu = 0.72$). Day 2 was then found to be a good sampling point for the exponential phase. At day 3 the culture was at the start of the stationary phase, as μ was 0 between day 3 and 4 between day 5 and 6 μ was -0.08. None of the glucans isolated at day 2, 4 or 6 for *T. weissflogii* showed any β-(1→6) branching and the DP was constant within three glucopyranosyl units, ranging from 5 to 8 as shown in Table 1.

The chrysolaminaran content of the cultures sampled on days 2, 4 and 6 (Fig. 4) was measured (Table 2). The *C. mülleri* culture was found to have a maximum chrysolaminaran content of 71.57 mg L^{-1}. This was found on day 6 and corresponded with the maximum glucan concentration per cell, which was 18.29 pg cell^{-1}. The *T. weissflogii* culture was found to have a maximum content of 31.72 mg L^{-1} on day 4. The maximum content per cell was found on day 6, and was 411.95 pg cell^{-1}. *C. mülleri* was thus found to give the higher glucan yield due to higher glucan cell concentrations, not concentration of cells.

The ^{1}H NMR method used in this work was found to be a suitable method for analyzing chrysolaminarans. By using this method in this work we have added to the knowledge of the structure of the storage polysaccharide of *C. mülleri*. We have also characterized the chrysolaminaran from *T. weissflogii*. The results obtained from this work showed that the structure of chrysolaminaran structure did not vary markedly during the growth of *C. mülleri*. The variations that were observed were highest in the T-glucan which had the lowest DP of the two. Seen together with the previous data for *C. mülleri*, the observed DP/DB span of *C. mülleri* is 19–24/0.005–0.009, and *T. weissflogii* has no branching and a DP of 5–13.

It was found that there can exist large differences in the structure of chrysolaminarans isolated from different diatoms. The highest DP found for the C-glucan (24) was almost five times that of the smallest DP found for the T-glucan (5). β-(1→6)-Linkages are considered as a prerequisite for immunostimulant activity of (1→3)-β-glucans.[2] The findings in this work indicate

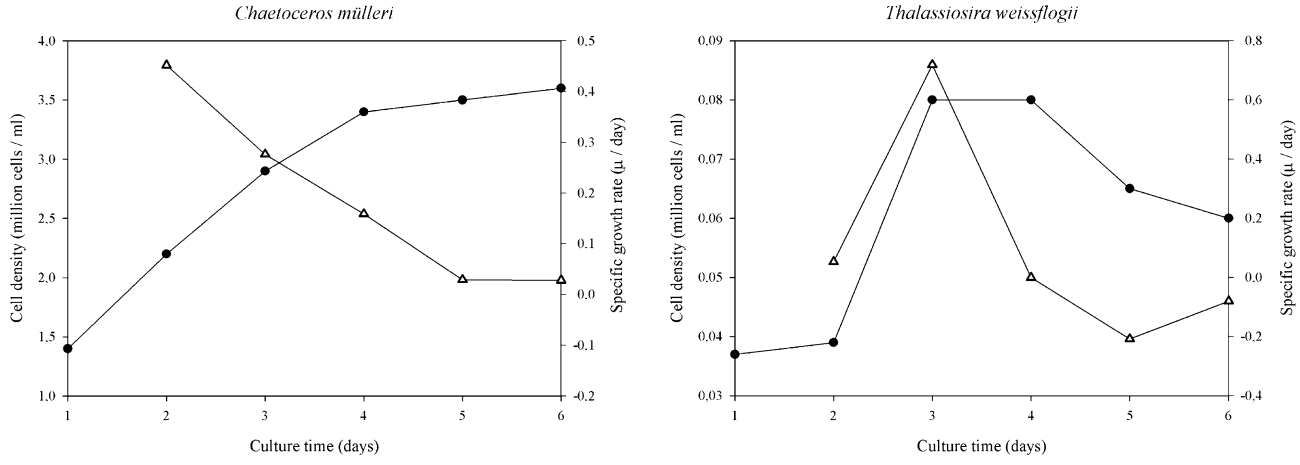

Figure 4. Culture density (●) and specific growth rate μ, (△) curves of *C. mülleri* and *T. weissflogii*.

Table 2. Cell and glucan concentrations for the cultures harvested at day 2, 4 and 6

	C. mülleri			*T. weissflogii*		
	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6
Million cells/mL	2.20	3.40	3.60	0.039	0.080	0.060
mg glucan/L	2.12	61.14	71.57	14.21	31.72	24.72
pg glucan/cell	0.96	17.98	18.29	364.33	396.36	411.95

that some microalgal glucans that possess β-(1→6)-linkages would be more interesting for immunostimulant screening than others like the T-glucan. More diatom, and microalgal polysaccharides, should therefore be examined in detail. Different interactions with glucan receptors due to different conformations caused by variations in DP and DB may be expected.[15] Diatoms and other microalgae could be a convenient source of glucans with different DP and DB for glucan–receptor interaction studies. The simplicity of the ^{1}H NMR analysis would make it an ideal method to further investigate the structures of a vast array of chrysolaminarans isolated from different diatoms.

3. Experimental

3.1. Cultivation of the diatoms

The diatoms *C. mülleri* and *T. weissflogii* were cultivated in batch culture (200 L cylindrical tubes) with f/2 medium with an N/P weight ratio of 10.[16] The cultures were aerated (0.1% CO_2) and the temperature was kept between 18 and 20 °C. Each culture tube was continuously illuminated with four Phillips TL 40W/55 tubes, resulting in an irradiance of 140 μmol photons m^{-2} s^{-1} at the culture surface. The salinity was 24 ppt.

The diatoms were run in batch cultures. One culture (two for *T. weissflogii*) was run until stationary phase, and harvested in the stationary phase. The second culture was also run until stationary phase, and samples of the culture were drawn at day 2, 4 and 6. The turbidity of the cultures were measured daily by use of a spectrophotometer (absorption at 750 nm), and the cell density was calculated using a specific empirical correlation between the absorption and cell density of the species.

The specific growth rate, μ, was calculated as

$$\mu = \ln(N_1/N_0)/t \quad (1)$$

where N_0 is the biomass at time t_0 and N_1 is the biomass at time t.

The algae were harvested by centrifugation at 4000 rpm, and wet algal pellets were freeze dried and stored at −80 °C until extraction and subsequent chemical analysis.

3.2. Measurement of β-D-glucans

For measurement of glucan content in the diatom cells throughout the cultivation, culture aliquots were collected on Whatman GF/C filters and extracted with a modification of the method described by Granum and Myklestad,[17] and then measured by the phenol–H_2SO_4 method of Dubois et al.;[18] 15 mL of culture was filtered onto Whatman GF/C filters and the extracted with H_2SO_4 (2.5 mM, 4 mL) at 60 °C for 10 min. Aliquots (2 mL) of the extracts were transferred to reaction tubes and added aqueous phenol solution (3%, 0.5 mL) and concentrated H_2SO_4 (5 mL). The resulting solution was mixed thoroughly and after 30 min cooled to room temperature. Absorbance of the solution at 485 nm was measured and the glucan content was calculated from a standard curve made using glucose solutions.

3.3. Isolation of β-D-glucans

All cultures were collected by centrifugation and freeze dried before extraction with H_2SO_4 (0.005 M). The extracts were filtered and neutralized with NaOH before they were dialyzed (MWCO 1000) against MQ-water. Freeze drying of the resulting solution gave the glucans as faintly yellow powders, which were used directly for NMR spectroscopy.

3.4. NMR spectroscopy

The isolated chrysolaminarans were compared to laminaran from *Laminaria digitata* purchased from Sigma–Aldrich Norway AS. NMR was run on a Bruker DRX 500-spectrometer fitted with a triple resonance probe at a temperature of 353 K for the 1D ^{1}H-experiments and at 333 K for the ^{1}H,^{1}H COSY-spectrum. For 1D ^{1}H NMR the samples (5 mg) were dissolved in 500 μL 6:1 Me_2SO-d_6–D_2O. Typically a 45° observe pulse of 3.85 μs was used to record 128 or 256 FIDs with an acquisition time of 1.17 s over a spectral region of 7000 Hz into 16k data points. An inter-scan delay of 3 s was used. Zero-filling and an exponential line broadening of 0.3 Hz was applied before Fourier transform. Chemical shifts are referenced to Me_4Si at 0 ppm.

For ^{1}H,^{1}H COSY the sample (5 mg) in 500 μL 6:1 Me_2SO-d_6–D_2O was added a few drops of CF_3CO_2D to shift the residual water resonance downfield to 4.89 ppm where it could be suppressed without affecting the sugar resonances.[19] Suppression was done by presaturation in the inter-scan delay. The polarization-transfer pulse was 45°. Data points (4096/1024) were collected in the F2/F1 dimensions. Before Fourier transform both dimensions were zero-filled and apodized by a squared sine-bell function.

3.5. Calculation of DP and DB

DP and DB were calculated by method of Kim et al.[9] after integration of the anomeric resonances and using the following equations:

$$\text{DP} = \text{integral of all anomeric resonances} / \text{integral of } (\alpha\text{-RT} + \beta\text{-RT}), \quad (2a)$$

or

$$\mathrm{DP} = \text{integral of all anomeric resonances} / \text{integral of NRT} \quad (2b)$$

$$\mathrm{DB} = \text{integral of TSC} / \text{integral of all anomeric resonances} \quad (3)$$

Acknowledgements

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