Folia Microbiol. **52** (3), 246–252 (2007) **http://www.biomed.cas.cz/mbu/folia/**

Structural Analysis of a Polysaccharide from *Chlorella kessleri* by Means of Gas Chromatography–Mass Spectrometry of Its Saccharide Alditols

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Received 28 August 2006
Revised version 24 January 2007

ABSTRACT. Interpretation of gas chromatographic–mass spectrometric data of oligosaccharide alditols was used to determine their structures and to derive the structure of a water soluble polysaccharide isolated from *Chlorella kessleri*. ^{1}H- and ^{13}C-NMR was employed to assess the configuration of glycosidic bonds and individual monosaccharides were assigned to the L or D series by means of gas chromatography of the acetylated (*S*)-2-butyl glycosides.

Abbreviations

GC gas chromatography MS mass spectrometry SIM simultaneously monitor(ed)

The structure of polysaccharides has been studied primarily in red and brown algae (Percival and McDowell 1981) and in blue-green algae (Dembitsky and Řezanka 2005; Řezanka and Dembitsky 2006). Data concerning the structure of polysaccharides of green algae (*Chlorophyceae*) are much less abundant. Three components (starch, hemicellulose and cellulose) were described in a polysaccharide from *Chlorella pyrenoidosa* (Northcote *et al*. 1958) and the structure of a trisaccharide was proposed after a previous acetolysis of hemicellulose (Olaitan and Northcote 1962).

In spite of the fact that green algae are divided into 3 types (Blumreisinger *et al*. 1983), *viz*. rhamnose–galactose type (Becker and Shefner 1964), mannose–glucose (Loss and Meindl 1982) and glucosamine type (Takeda and Hirokawa 1978), certain heterogeneities of this classification, *e.g*., *C. saccharophila* or *C. kessleri* cultivated heterotrophically (Řezanka *et al*. 1983), have been noted.

The first mention of an acidic polysaccharide that was isolated from *C. pyrenoidosa* and contained rhamnose (52 %), galactose (13 %) and arabinose (12 %) appeared >30 years ago (White and Barber 1972). A different polysaccharide has been isolated from *C. ellipsoidea* which contained glucose as the major component and arabinose and galactose as a minority (Kojima *et al*. 1973). Still another polysaccharide contained fucose or rhamnose with lesser amounts of galactose and other sugars but not arabinose (Ukai *et al*. 1990). An α-D-glucan and an α-D-*arabino*-α-L-*rhamno*-α-D-galactan were isolated from *Chlorella* (Mizuno *et al*. 1980). A polysaccharide from *Chlorella* sp. containing rhamnose, arabinose, mannose and uronic acids (Matsubayashi 1984), extracellular polysaccharide from *Chlorella* that contained 80 % galactose (Noda *et al*. 1993), extracellular polysaccharide from *Chlorella* sp. containing glucuronic acid and arabinose as major components (38 and 32 %, respectively; Yalcin *et al*. 1994), acidic polysaccharides from *C. vulgaris* with the structure α-D-Glc*p*A(1→3)-L-Rha (Ogawa *et al*. 1998) and α-D-Glc*p*A(1→3)-α-L-Rha*p*(1→2)-L-Rha (Ogawa *et al*. 1999) have also been described. A polysaccharide from *C. pyrenoidosa* that contained both arabinose (32 %) and galactose (26 %) plus traces of many other sugars has been reported (Pugh *et al*. 2001).

Methylation analysis is the basic method used to determine the structure of polysaccharides. The methylated polysaccharide is hydrolyzed and the resulting mixture of methylated saccharides is separated by means of GC. In order to suppress anomeric forms the saccharides are converted to alditol acetates that are identified by GC–MS. Packed columns have previously been used for the separation and it has not been possible to separate all positional isomers of partially methylated alditol acetates (Lindberg and Lonngren 1978). Therefore, perfectly separating capillary columns are used (Lomax and Conchie 1982; Klok *et al*. 1982).

The GC–MS method is convenient when determining the structure of complex saccharides of biological origin that are often of complex structure and available in low quantity. This method makes it possible to determine the structure of oligosaccharides and hence also polysaccharides under optimum conditions and as little as tenths to several mg of the polysaccharide are sufficient (Aringman *et al*. 1981; Lindberg 1972). In this paper, based on our earlier work (Řezanka *et al*. 1983, 1986*a*,*b*; Řezanka and Podojil 1984), we describe the isolation, purification and structural studies of a water-soluble polysaccharide from heterotrophically cultivated cells of *C. kessleri*.

MATERIAL AND METHODS

Heterotrophically cultivated *C. kessleri* was obtained in the form of lyophilized biomass from the *Department of Autotrophic Microorganisms, Institute of Microbiology*, Třeboň (Czechia). Ten g of material yielded 168 mg of a mixture of polysaccharides (Řezanka *et al*. 1983) after extraction with water, precipitation with ethanol and removal of glucans by exo-1,4-α-D-glucosidase (EC 3.2.1.3). Chromatography (Laurent and Granath 1967) on Sephadex G-100 (*Pharmacia*, Sweden) and subsequent methylation, hydrolysis (90 % formic acid), $NaBD_4$ reduction and acetylation (Lindberg 1972) yielded partially methylated alditol acetates; these were then separated and identified by GC–MS in the HP 5995 B apparatus (*Hewlett Packard*, USA) with a fused silica capillary column 0.25 mm ID × 25 m with Carbowax 20M phase. Working regime: ionization energy 11.2 aJ (70 eV), scan speed 690 Da/s, mass range 40–800 Da, programmed temperature 165–215 °C at a rate of 2 K/min and further isothermically, splitless injection, injector temperature 250 °C, helium flow 1.1 mL/min. Oligosaccharide alditols were obtained by partial hydrolysis of permethylated polysaccharide, $NaBD_4$ reduction and trideuteriomethylation with CD_3I (Lindberg and Lonngren 1978). GC–MS was performed in the same apparatus with a fused silica capillary column 0.25 mm ID × 25 m with OV-1 phase. Working regime of the apparatus: ionization energy 11.2 aJ, scan speed 690 Da/s, mass range 85–800 Da; column injection with programmed temperature 120–210 °C at a rate of 30 K/min and further at 4 K/min up to 350 °C, helium flow rate 0.9 mL/min.

NMR spectra of the polysaccharide as 2 % solution in D_2O were recorded on a Bruker AMX 500 spectrometer (*Bruker Analytik*, Germany) at 500.1 MHz (^1H) and 125.7 MHz (^{13}C).

The identification and the D or L configuration of sugars was determined using GC according to Gerwig *et al*. (1978), with some modifications (Dembitsky *et al*. 1992).

RESULTS AND DISCUSSION

A uniform fraction, yielding only a single peak even on repeated chromatography was obtained after gel chromatography on Sephadex G-100. In contrast to Northcote *et al*. (1958) and Olaitan and Northcote (1962), glucose and mannose were not detected in our polysaccharide fraction. It can be explained by a more effective enzymic hydrolysis in the case of glucose. Water-soluble heteropolysaccharide is characterized primarily by an unusually high number of monosaccharides (Ara, Xyl, Gal, Rha). This makes it impossible to derive a complete structure only on the basis of identification of its methylation products. Degradation of completely methylated polysaccharide indicates that the polysaccharide is highly branched. The main chain consists of Gal, Rha and Ara (Table I). Terminal sugars are Gal*f* and Xyl*p*. The use of the capil-

Table I. Identification of methylated alditol acetates from the polysaccharide

Peak no.	Molar ratio	Position of *O*-Me	RRT[a]	Monosaccharide[b]
1	0.98	2,3,4-Xyl	0.383	D-Xyl*p*-
2	1.02	2,4-Rha	0.475	-4-L-Rha*p*-
3	1.03	2,3,5,6-Gal	0.527	D-Gal*f*-
4	1.09	2,4-Ara	0.607	-3-L-Ara*p*-
5	1.06	2,3,6-Gal	0.827	-4-D-Gal*p*-
6	2.07	2,6-Gal	0.974	-3,4-D-Gal*p*-

[a]Relative retention time related to penta-*O*-acetylarabinitol.
[b]Moiety in polysaccharide; assignment to the D or L series was determined by means of GLC of the acetylated (*S*)-2-butyl glycosides (*see Material and Methods*).

lary column made it possible to separate all methylated alditol acetates and SIM *m*/*z* 118 ion, helped to identify spectra of individual compounds as secured detection of spectra at the maximum ion current and also excluded all peaks (compounds) that have no D atom in position 1, *i.e.* the structure $[CH_3COO–CHD–CHOCH_3]^+$.

Partial acid hydrolysis of the polysaccharide with aqueous formic acid, $NaBD_4$ reduction and realkylation with CD_3I afforded a mixture of partially methylated, partially trideuteriomethylated oligosaccharide alditols that were separated and identified by GC–MS. CD_3 groups in the oligosaccharide alditol determine the position in which residues of saccharides in the polysaccharide are bound, and, in the case of alditol, also the ring size (pyranoside or furanoside). When the oligosaccharide alditol contains saccharides of various classes, *e.g.*, pentoses (Xyl, Ara), hexoses (Gal) or deoxyhexoses (Rha), the sequence of these saccharides may be determined on the basis of MS; however, it is not possible to discriminate among sugars of the same class (*i.e.* Glu, Man, Gal).

We interpreted our results based on the data of Aringman *et al*. (1981), Laurent and Granath (1967), Kochetkov and Chizhov (1966), and Karkkainen (1970, 1971). Ion at *m*/*z* 88 is present in all spectra of methylated saccharide alditols having 1→4 bonds, in the case of substitution with CD_3 in position 3, *m*/*z* is shifted by 3 Da, *i.e.* to *m*/*z* 91; when a glycosidic residue is bound in position 3, the H^+ ion is not formed (Table II). The main types of fragmentation products are formed by sequences A and J. For the structures of these ions *see* Fig. 1 and Table II. Within the mass range of 40–300 Da, ions J_1, H_1, aA_3, aA_2, and aA_1 are the most important, to be followed by ions bJ_2 and bJ_1. For values within the *m*/*z* range of 300–700 Da we found again important ions of the A and J series. Ions with *m*/*z* > 700 occur only exceptionally; they have a low intensity and are formed by splitting off the individual carbons from alditol, often with an isotopic increase (Table II, peaks I and J). Molecular ion M^+ was not detected in any alditol. Association of glycosidic residues **a**–**b**, **b**–**c**, and **c**–**d** was detected on the basis of ion *m*/*z* 136. In the case of 1→4 bond, a series of ions 45, 49, 92, 93, and 137 (deuterium effect) is formed by reduction of the semiacetal while a series of ions 45, 49, 92, 93, and 136 (without any effect) is produced in the case of 1→3 bond. If the alditol is pentitol (Ara-ol) or the alditol carries two glycosyl residues, ions *m*/*z* 136 or 137 are missing. Ions *M*–136 (*M*–137) may also help to interpret the ring combinations.

Ring associations can also be deduced from the presence of bcJ_1, $abcJ_1$ and cdJ_1 ions. Saccharides bound by 1→4 and 1→6 bonds have very simi-

Table II. Fragments of alditols

Compound	CD_3-3-Ara-1›	4-Rha-ol	CD_3-4-Gal-ol-3›	1-Xyl	CD_3-4-Gal-1›	3-Ara-ol	CD_3-4-Rha-1›	4-Gal-ol | 3-CD_3	CD_3-4-Gal-1›	4-Gal-ol | 3-CD_3	CD_3-4-Gal-1›	4-Gal-ol | 3-CD_3
Peak	**A**		**B**		**C**		**D**		**E_1**		**E_2**	
RRT[a]	0.51		0.58		0.59		0.61		0.62		0.62	
H_1	91		88		88		88		91		91	
aA_1	178		175		222		192		225		225	
aA_2	146		143		190		160		193		193	
aA_3	–		–		158		128		161		161	
bJ_1	275		305		258		305		308		305	
bJ_2	212		245		198		245		245		242	
C-1–C3	137		–		–		140		140		137	
Other ions	62 C^{5-6}		139 C^{4-6}		48 C^6		–		–		–	

[a]Relative retention time related to penta-*O*-acetylarabinitol.

contd.

Table II – *contd.*

Compound	CD_3-4-Gal-1› 4-Gal-ol | 3 3-CD_3 ˇ 1-Xyl	CD_3-4-Gal-1› 4-Gal | 3 3-CD_3 ˇ 1-Ara-ol	CD_3-4-Gal-1› 3-Gal-ol 3 ˇ 1-Xyl	CD_3-4-Gal-1› 4-Gal-1› 4-Gal-ol | | 3-CD_3 3-CD_3	CD_3-4-Gal-1› 4-Gal-1› 4-Gal | | 1 3-CD_3 3-CD_3 ˇ 3-Ara-ol	CD_3-4-Gal-1› 4-Gal-1› 4-Gal-ol 3 | ˇ 3-CD_3 1-Gal
Peak	**F**	**G**	**H**	**I**	**J**	**K**
RRT[a]	0.97	0.99	1.01	1.09	1.91	2.07
H_1	91+88	91+88	88	91	91+88	91
aA_1	175	225	175	225	225	225
aA_2	143	193	143	193	193	193
abA_1	–	429	382	432	432	–
abA_2	–	397	350	400	400	–
cbJ_1	–	465	509	512	–	–
cbJ_2	–	402	449	449	–	–
cJ_1	–	258	–	305	–	–
cJ_2	–	198	242	242	–	–
$abcA_1$	–	–	–	–	636	589
$abcA_2$	–	–	–	–	604	557
$bcdJ_1$	–	–	–	–	672	–
$bcdJ_2$	–	–	–	–	609	606
cdJ_1	–	–	–	–	465	–
cdJ_2	–	–	–	–	402	–
dJ_1	–	–	–	–	258	–
dJ_2	–	–	–	–	198	242
C_{1-3}	–	–	137	137	–	–
Other ions	225 bA_1; 193 bA_2; 452 bcJ_2; 402 acJ_2	48 C^6	–	isotop. abA_2 (abA_1–CD_3OH)	–	175 bA_1; 143 bA_2

lar spectra. However, they are practically absent in the polysaccharide from *C. kessleri* (Table I). Thus, the interpretation of spectra is greatly facilitated. The presence of pentose or deoxyhexose and localization within the oligosaccharide was determined according to the characteristic shift by 44 or by 30 Da toward lower values of series A and J (Table II). Branching of the saccharide chain is demonstrated by the absence of cA_x ions in the spectrum. Also the absence of ion *m*/*z* 136 (137) is in agreement with the structure, in which two glycosyl residues (**a**, **b**) are bound to alditol (**c**) in positions 3 and 4 (Table II, peak F).

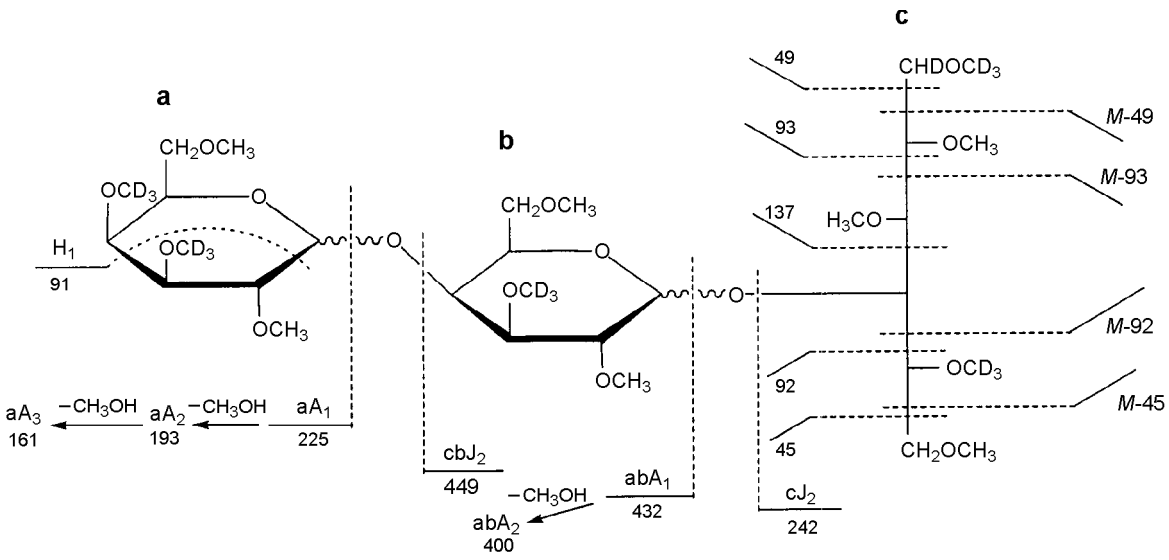

Fig. 1. Fragmentation of trisaccharide alditol in mass spectrum; for symbols *see* Table II.

It is possible to propose a structure of a heptasaccharide repeat unit of the polysaccharide (Tables I and II). This unit (Fig. 2) can be reconstructed from 6 mutually overlapping disaccharide alditols obtained by hydrolysis. Tri- and tetrasaccharide alditols provided another proof of its structure.

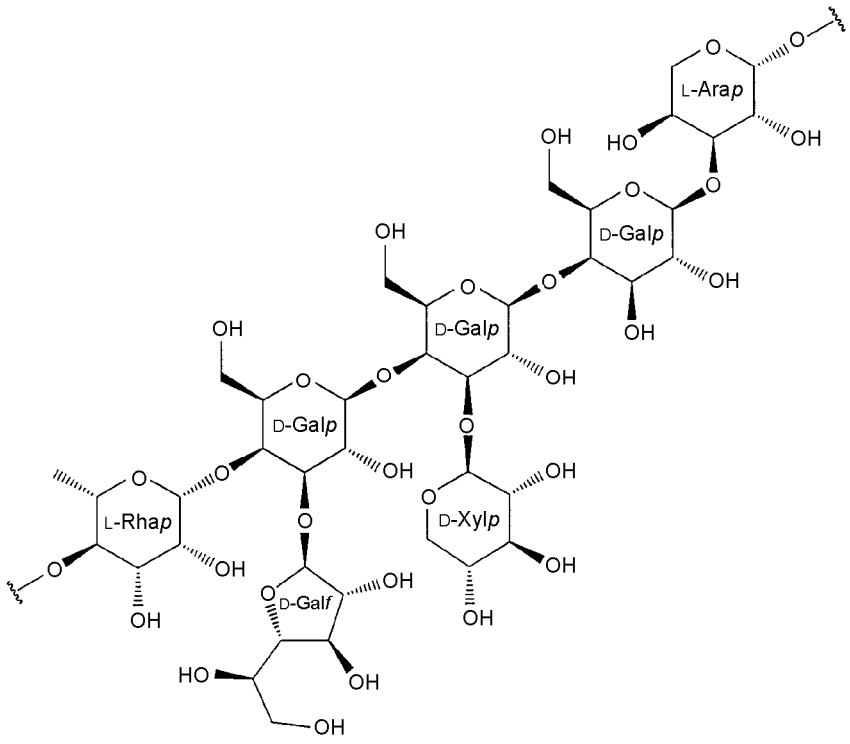

Fig. 2. Repeat unit of oligosaccharide in the polysaccharide.

^{13}C-NMR analysis of the nonderivatized polysaccharide offers further information (Shashkov and Chizhov 1976) about its structure. On the basis of values of 7 signals (109.1, 105.2, 103.2, 103.1, 103.0, 102.8, 102.6 ppm) it can be deduced that the first signal belongs to C-1 D-Gal*f*. From values of the other signals it can be only determined whether a sum of signals and forms of Gal, Rha, Xyl and Ara is involved, with Gal*p* predominating.

^{13}C-NMR examination of the product showed that it had a Gal*f* ring, as a typical low field signal at δ 108.1 was present (Gorin and Mazurek 1975). Its ^{13}C–^{1}H-coupled spectrum contained C-1 doublets centered at δ 108.8 with J = 172.0 Hz from β-Gal*f*. These values differed from those of a standard mixture of β-Gal*p* and α-Gal*p* (Sassaki *et al*. 2005). The J values are consistent only with those of Chambat *et al*. (1978), who found C-1 of Me β-Gal*f* to have 172.5 Hz and C-1 of Me α-Gal*f* to have 175 Hz and Perlin and Casu (1969), who reported 169 Hz for α-Glc*p* and 160 Hz for β-Glc*p*.

The coupling constant between C-1 and H-1 $^{1}J_{CH}$ in pyranose derivatives of saccharides is useful in the assignment of anomeric configurations since pyranoses with an axial H-1 have a $^{1}J_{CH}$ value which is ≈10 Hz lower than the corresponding value in compounds with an equatorial H-1 (Bock *et al*. 1973). The anomeric configuration can be seen to be β in all the other 6 cases (Podlasek *et al*. 1995).

The ^{1}H-NMR spectra of the polysaccharide revealed a coincidence in the position of the signals differing in their intensity, and showed 7 signals of anomeric hydrogens at δ 5.19, 5.03, 4.98, 4.95, 4.93, 4.91, and 4.89 ppm.

The absolute configurations of monosaccharides were determined to be L and D, respectively, by GLC of the acetylated (*S*)-2-butyl glycosides (*see* Table I).

The sensitivity of the GC–MS method used to investigate the complex heteropolysaccharide was further increased by using SIM, which made it possible to separate and identify fragments of saccharides and reduced the amount of material required for the structural analysis of the complex heteropolysaccharide with a simultaneous significant reduction of time necessary for the analysis. The use of ^{1}H–^{13}C spin coupling constants ($^{1}J_{CH}$) makes it further possible to determine the configuration of anomeric hydrogens.

The work was supported by the Research Concept of the *Institute of Microbiology* AV 0Z 5020 0510.

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