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Extraction of polysaccharides from a species of *Chlorella*

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ABSTRACT

The objective of this project was to devise a method to recover the total, water-soluble cell-wall polysaccharides of *Chlorella*. It was found that substantial quantities of polysaccharides could be extracted after treatment of the cells with a mildly acidic solution of sodium chlorite (yield of recovered polysaccharide, 19–22%). Water-soluble (13–19% yield) and 2% NaOH-soluble (3–6% yield) fractions were obtained. A second treatment gave a total yield of water-soluble polysaccharides of 24–25%, while reducing the amount of material soluble in 2% NaOH to 0.3%. Each polysaccharide fraction was composed of 6 different neutral sugars (rhamnose, arabinose, xylose, mannose, galactose, and glucose). There was evidence of the presence of uronic acid in all fractions. An alkaline hydrogen peroxide treatment of the cells resulted in a 19% yield of polysaccharides that were not further examined.

1. Introduction

There is a rapidly growing interest in the production of biofuel from unicellular photosynthetic algae, also known as microalgae, as the lipid content of these algae can be as much as 60% of their weight (Sheehan, Dunahay, Beneman, & Roessler, 1998). Fresh-water unicellular algae, such as *Chlorella* sp., are 6–12 times more efficient at creating usable biomass than are terrestrial plants and can be grown in nonarable areas with nonpotable water, which can be recycled. Development of an industry producing hydrocarbon fuels from algae could result in the production of hundreds of millions, if not billions, of tons of residual cells in the U.S. alone. It is, therefore, important to determine if there are useful components to be obtained from such a residue.

Previous attempts at extracting and identifying the polysaccharides of *Chlorella* are summarized in Table 1. On the assumption that algae cell walls are composed primarily of polysaccharides (most likely in greater amounts than the maximum of 8.6% previously extracted) and, therefore, that such cell-wall polysaccharides have been extracted rather inefficiently from *Chlorella* sp. heretofore, this project was undertaken to find a means to develop a practical recovery of polysaccharides from such cells.

2. Materials and methods

2.1. Materials

Dried *Chlorella* cells (unknown species) were obtained as a gift from Yaeama Shokusan Co., Ltd, Okinawa, Japan. They were used as received.

2.2. Treatment of Chlorella cells with a slightly acidified solution of sodium chlorite

Chlorella powder (10 g, 4.11% moisture) was suspended in 800 mL of distilled water at 75 °C (water bath) (Fig. 1). To the suspension was added 7.5 g of sodium chlorite and 0.5 mL of gl. acetic acid at hourly intervals. After 4 h (3 additional additions of reagents), the reaction vessel was removed from the water bath and stored overnight (12–15 h) at either 24 °C (giving fractions A, B, and C) or 4 °C (giving fractions D, E, F) for convenience only. After overnight storage, the reaction mixture was centrifuged at 1200 × *g* for 20 min. The residue is labeled as "Insoluble" (Fig. 1). To the supernatant was added 3 volumes of ethanol slowly with rapid stirring. The suspension was then centrifuged at 1200 × *g* for 10 min to recover the precipitate, which is called the "Soluble" fraction (Fig. 1), which was washed with 100% ethanol (twice), and dried in vacuo in a desiccator.

2.3. Fractions of polysaccharide preparations

The insoluble portion and the precipitated soluble fraction were separately extracted twice with 40 mL of distilled water at 90 °C for

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Table 1
Yields and structural information on water- or alkali-soluble polysaccharides (other than starch) of *Chlorella* published to date.

Source	Information	Yield	Refs.
C. pyrenoidosa	"Hemicellulose" (Gal, Man, Ara, Xyl, and Rha)	8.6%	Northcote et al. (1958)
	"Hemicellulose B" (Glc, Gal, Rha, Man, Ara, Xyl, unknown sugar) in molar ratios of 25.0:13.0:12.0:2.0:1.0:20.0)	–	Olaitan and Northcote (1961)
	"Hemicellulose A" (Gal, Rha, Ara, Xyl, Man, and Glc in molar ratios of 7.0, 5.0, 3.0, 3.0, 2.0, and 1.0) termed a branched, β-linked galactorhamnan	–	Olaitan and Northcote (1962)
	Acidic polysaccharide containing 8.5% GlcA and Rha, Ara, Xyl, Man, Gal, and GlcA in molar ratios of 11.5:2.7:2.2:1.0:2.9:1.9	2.3%	White and Barber (1972)
	Rha, Gal, Ara, Glc, and GlcA	0.02%	Umezawa and Komiyama (1985)
	Glc, Fuc, Rha, Gal, Man in molar ratios of 8.8:4.8:1.8:1.5:1.0	–	Ukai, Kiho, Nagai, Tabata, and Hara (1990)[a]
	Glc, Gal, Man in molar ratios of 18.3:4.7:3.0:1.0	–	Ukai et al. (1990)[a]
	Ara (31.6 mole%), Gal (26.8 mole%), Rha (12.4 mole%), Glc (5.4 mole%) plus at least 20 additional sugar components including pentoses, mono-, di-, and tri-*O*-methylhexoses, mono-*O*-methylpentoses, KDO, GlcNAc, GalNAc, and other amino sugars	~1%	Pugh, Ross, ElSohly, ElSohly, and Pasco (2001)[a]
	Gal, Man, Ara, Xyl, Rib, Fuc, Rha	–	Maksimova and Pimenova (1966)[a], Maksimova, Bratkovskaia, and Plekhanov (2004)[a]
	Glc, Gal, Rha, Ara, GlcA	–	Umezawa et al. (1982)[a]
	Glc and any combination of Gal, Rha, Man, and Ara plus GlcNAc and GalNAc	<7.5%	Kralovec (2005)
	Arabinogalactan	0.01%	Suárez et al. (2005)
	1,2-Linked β-galactofuranan	0.01%	Suárez et al. (2006)
	Man (78.0%), Glc (13.2%)	–	Shi, Sheng, Yang, and Hu (2007)[a]
	Man (76.5%), Glc (8.4%)	–	Shi et al. (2007)[a]
	Cyclic and linear 1,2-linked β-glucans in a 64:36 ratio	0.001%	Suárez et al. (2008)
	A phosphorylated β-galactan consisting of a 1,3-linked backbone with one half of the units substituted on *O*-6 with terminal βGal*p* units and the remaining backbone units substituted on *O*-6 with equal amounts of αMan*p* 1-phosphate and 3-*O*-Me-αMan*p* 1-phosphate through diester linkages	<0.19%	Suárez, Kralovec, and Grindley (2010)
	Polysaccharide containing Rha (31.8%), Glc (20.4%), Gal (10.3%), Man (5.2%), Xyl (1.3%)	[b]	Hsu et al. (2010)
C. ellipsoidea	"Hemicellulose" (Rha, Xyl, Ara, Man, Gal)	~4.4%	Takeda and Hirokawa (1978)
C. vulgaris	Gal, Man, Ara, Xyl, Rib, Fuc, Rha	–	Maksimova and Pimenova (1966)[a]
	Rha, Ara, Xyl, Man, Gal, 2-*O*-MeRha, 3-*O*-MeRha (in molar ratios of 25:2.3:1.7:1.0:3.9:2.1:1.2), 3-*O*-MeGal, αGlcA (1 → 3)αLRha (1 → 2)LRha	0.09%	Ogawa, Yamaura, and Maruyama (1994), Ogawa, Yamaura, and Maruyama (1997), Ogawa, Yamaura, Ikeda, and Kondo (1998), Ogawa et al. (1999)
Chorella sp.	Glc, Fru, Man, Gal; Glc, Fru, Man; Glc, Man, Xyl, Rha; Glc, Xyl, Fru; Glc, Xyl, Rha	–	Streshinskaya et al. (1967)[a]
	Rha, Ara, Man, and uronic acid	–	Matsubayashi and Maruyama (1986)[a]
	GlcA (41 wt%), Ara (34 wt%), Glc (20 wt%), Fuc (5 wt%)	–	Yalcin et al. (1994)[a]
	Gal (>80% of neutral sugars)	–	Noda et al. (1994)[a]

[a] Abstract only available.
[b] Total yield of unfractionated polysaccharides = 5.75%.

30 min (Fig. 1). After centrifugation at 1200 × *g* for 15 min, 3 volumes of ethanol were added to the supernatant with rapid stirring, forming precipitates (fractions A, B, D, and E). Each precipitate was recovered by centrifugation, washed 3 times with 100% ethanol, and dried in vacuo in a desiccator.

The residue from the hot-water extraction of the "Insoluble" fraction was extracted twice with 60 mL of 2% NaOH for 1 h at room temperature. The combined extracts were neutralized to pH 6.5. Three volumes of ethanol were added with rapid stirring. Each precipitate was recovered by centrifugation at 1200 × *g* for 15 min

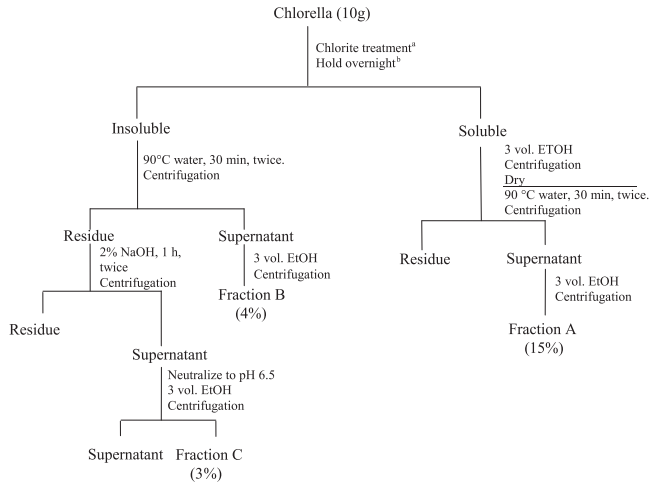

Fig. 1. Isolation of polysaccharide fractions following a single treatment with an acidified solution of sodium chlorite. [a]800 ml water, 7.5 g sodium chlorite, 2.0 ml gl. acetic acid (four 0.5-ml portions at 0, 1, 2, and 3 h), 75 °C, 4 h. [b]Fractions A, B, and C, for which average yields are given, are from an overnight hold at 24 °C. An overnight hold at 4 °C gave corresponding fractions D, E, and F.

(giving fractions C and F), washed 3 times with 100% ethanol, and dried in vacuo in a desiccator.

2.4. Treatment of Chlorella cells with an alkaline solution of hydrogen peroxide

Chlorella powder (1.0 g, 4.11% moisture) was suspended in 10 mL of distilled water at room temperature (rt). Hydrogen peroxide (0.33 mL of a 30% solution) (Sigma–Aldrich, St. Louis, MO) was added to make the mixture 1% in hydrogen peroxide. The pH of the mixture was adjusted to 11.5 with 1 M NaOH, and the mixture was stirred at rt. After 18 h of stirring, the pH of the supernatant was adjusted to 6.5, and 4 volumes of 100% ethanol were added with rapid stirring. The precipitate was collected by centrifugation ($1200 \times g$), dewatered with 100% ethanol, dried, and weighed. The residue was extracted with hot water and 2% NaOH as with the acidified sodium chlorite procedure.

2.5. Hydrolysis for neutral monosaccharide determination

Each fraction (1.0 mg) was placed in a small screw-capped test tube with a Teflon-lined cap. Inositol (20 μg) was added as an internal standard. To each vial was added 0.5 mL of 2 M triflouroacetic acid. The vials were capped tightly and heated to 120 °C for 2 h in a heating block. The tubes were then cooled to room temperature and evaporated to dryness in a stream of nitrogen. The residue was washed twice with 0.25 mL of 2-propanol.

2.6. Preparation of alditol peracetates

After washing with 2-propanol, the residue was dissolved in 1 M NH_4OH (0.1 mL) and reduced by addition of 0.5 mL of DMSO containing $NaBH_4$ (20 mg/mL = 2 mg) at 40 °C for 90 min. The alditols were then peracetylated by addition of 0.5 mL of acetic anhydride and 100 μL of 1-methylimidazole (as a catalyst). The tubes were vortexed, then held 10 min at rt. Dichloromethane (1 mL) and water (4 mL) were added, and the tubes were vortexed. The bottom (CH_2Cl_2) layer was removed to a small tube, and the extraction was repeated. The CH_2Cl_2 solution was washed twice with water (4 mL), then dried in a stream of nitrogen. The residue was dissolved in acetone (0.5 mL). The solution was stored in a refrigerator (4 °C).

2.7. Gas–liquid chromatography (GLC) of alditol peracetates

GLC was performed using a glass capillary column (30 m × 0.2 μm; Agilent HP-88 [Agilent Technologies, Santa Clara, CA], (88% cyanopropyl)-methylarylpolysiloxane); inlet temperature 240 °C, column temperature 100 °C for 2 min, 10 °C/min to 180 °C, 180 °C for 2 min, 4 °C/min to 240 °C, 240 °C for 5 min, 10 °C/min to 255 °C, 255 °C for 5 min; 1.9 mL/min of helium. 1.0 μL of the acetone solution of alditol peracetates was injected.

2.8. Reduction of uronic acid units with sodium borodeuteride

Portions (0.5 g) of fractions (before and after dialysis) were dissolved in 250 μL of distilled, deionized water. 0.25 M NaOH (50 μL) was added, and the mixture was heated at 60 °C for 1 h to remove any acyl groups. The solution was neutralized by addition of 0.25 M HCl (250 μL). 0.05 M pyridinium acetate (pH 4.75, 250 μL) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (20 mg) (Sigma–Aldrich) were added. The contents were mixed, then allowed to stand 1 h at rt. 2.0 M Tris–HCl buffer (500 μL, pH 7.0), 1 drop of octanol (to prevent foaming), and a 70 mg/mL solution of $NaBD_4$ in 0.05 M NaOH (250 μL) were added, and after mixing, the mixture was allowed to stand 1 h at rt. The reaction mixture was desalted by dialysis and freeze-dried. Then the reduction, beginning with addition of the carbodiimide in pyridinium acetate buffer, was repeated. Subsequently, the reduced polysaccharide preparation was hydrolyzed in the same way as described for the native preparation (Section 2.5).

2.9. "Lignin" determination

The *Chorella* cells were subjected to analyses of the type used to determine the lignin content of higher plant tissues in the laboratory of Professor C. Chapple, Department of Biochemistry, Purdue University. The modified Klason method of Huntley, Ellis, Gilbert, Chapple, and Mansfield (2003) was used; absorbance was measured at 280 nm. The modified acetyl bromide method of Chang, Chandra, Berleth, and Beatson (2008) was also used. Values were calculated using the same extinction coefficient value of 23.29 g^{-1} L cm^{-1} used by Chang et al. (2008) for determining the lignin content of *Arabidopsis*.

3. Results and discussion

3.1. Isolation of polysaccharide fractions

Many attempts were made to extract the polysaccharides from *Chlorella* cells. These attempts included both physical means to break the cells and chemical means to solubilize the polysaccharides. Only two procedures were at all successful. Neither was optimized, however, for the extraction conditions used, most successful one was to treat the cells with a solution of sodium chlorite acidified with acetic acid, using essentially the same procedure used to make holocellulose from lignified plant tissue (Whistler & BeMiller, 1963) and a procedure that results in minimal polysaccharide degradation. The result was a light-colored, insoluble residue and a light yellow solution.

The fact that an oxidizing system of the type used to delignify annual plant tissues was required to obtain the polysaccharides suggests that the *Chlorella* wall consisted of polysaccharides crosslinked with a phenolic units similar to those in lignin. The fact that hot alkaline solutions did not extract polysaccharides (data not given) suggests that crosslinkages involving ester bonds were not present. That leaves the possibility that the cell-wall polysaccharides may be crosslinked with phenolics in ether linkages.

Although there was no reason to believe that the *Chlorella* cells contained lignin, two types of lignin analysis were done on them. The modified Klason method of Huntley et al. (2003) gave a value of 2.45 ± 1.14%. The modified acetyl bromide method of Chang et al. (2008) gave a value of 6.35 ± 1.17 wt% (of extract-free cells). Both analyses would seem to indicate the presence of phenylpropane units, but that conclusion has not been confirmed.

After precipitating the polysaccharides from the supernatant (fractions A and D of Fig. 1), the insoluble residue was extracted in succession with hot water (90 °C) (fractions B and E) and 2% NaOH (fractions C and F). The 2% NaOH extracts were neutralized. To both extracts were added 3 volumes of ethanol to precipitate any extracted polysaccharides. The residue remaining after the 2% NaOH extract of the residue was negligible, so no further extractions were made.

Also, essentially the entire amount of the precipitated soluble fraction dissolved in hot water, i.e., the residue was negligible, so further fractionations were unnecessary. Yields are given in Tables 2 and 3. Total yields from the procedure employing an overnight hold at 24 °C averaged 22.4%; those from the procedure employing an overnight hold at 4 °C averaged 18.5%.

A second treatment of the cellular residue in an identical way (24 °C hold) gave another 0.60 g of the soluble fraction (A) for a total yield of 2.5 g (25%) (1.8 g was obtained from the first treatment in this run), another 0.04 g (0.4%) of the hot water extract (fraction B) of the second residue, and 0.03 g (0.3%) of the 2% NaOH extract (fraction C), leaving a remaining insoluble residue of 0.03 g (0.3%). Results from a single treatment only with a slightly acidic sodium chlorite solution are reported in this paper. (*Note*: It has not been established that the overnight hold as used in this research is necessary or even if it may be harmful.)

The cells were also treated with an alkaline solution of hydrogen peroxide as described by Gould and Dexter (1988). This treatment resulted in 12% of crude soluble polysaccharides, 0.3% of crude polysaccharides from a hot-water extract of the residue, 5.5% of crude polysaccharides from a 2% NaOH extraction of the remaining insoluble material, and 34% of remaining insoluble residue. The first two fractions were grey-green; the material precipitated from the neutralized 2% NaOH extract was dark green. A second treatment with the alkaline hydrogen peroxide solution gave a total soluble polysaccharide yield of 19%. As this treatment was less efficient in extracting polysaccharides from *Chlorella* cells, it was not pursued further.The highest yield of polysaccharides previously reported was the ~7.5% reported by Kralovec (2005) (Table 1) (80 °C water extraction of *C. pyrenoidosa* followed by microfiltration and dialysis), but this prepration appears to have 28–38% protein associated with it (Kralovec, Metera, Kumar, Watson, Girouard, Guan, Carr, Barrow, & Ewart, 2007).

It has been reported that the acidified chlorite delignification procedure effects structural changes in polysaccharides (Ford, 1986) and that it depolymerizes cellulose (Kumar, Mago, Balan, & Wyman, 2009). However, it has also been found that the depolymerization is inversely related to the amount of lignin present (Hubbell & Ragauskas, 2010). Alkaline peroxide delignification processes effect more extensive degradation of cellulose (Fang, Sun, Salisbury, Fowler, & Tomkinson, 1999; Sun, Sun, Sun, & Su, 2004). It was not the intent of this work to obtain completely undegraded polysaccharides from *Chlorella* for structural analysis, but rather the intent was to isolate polysaccharides from *Chlorella* as a potential commercial preparation.

Both treatments made extraction of the oil from *Chlorella* cells (using dichloromethane) more effective. In a separate experiment, treatment of cells of *Chlorella vulgaris* with a higher oil content using the alkaline hydrogen peroxide solution resulted in some oil on the surface of the aqueous phase.

Following conclusion of this work, it was reported that intracellular polysaccharides could be obtained from a marine microalgal species using 0.6% sodium hypochlorite solution (Gasljevic, Hall, Chapman, & Matthys, 2008). The same paper reported that the extracellular polysaccharide from *Chlorella stigmatophora* resulted in drag reduction, but not as effectively as did the extracellular polysaccharides from 3 other marine microalgae. Many investigations have been carried out on the physiological effects of *Chlorella* polysaccharides.

3.2. Compositional analysis: Hydrolysis of the polysaccharides and determination of neutral sugars

Acid-catalyzed hydrolysis of the polysaccharides in the extracted fractions was done according to the general procedure first reported by Albersheim, Nevins, English, and Karr (1967).

Reduction of the monosaccharides to their corresponding alditols and their peracetylation was done by the general procedure originally described by Gunner, Jones, and Perry (1961) and perfected by Sawardeker, Sloneker, and Jeanes (1965), Laine, Esselman, and Sweeley (1972), and others.

Identification and quantification of the released monosaccharides of both the native and uronic acid-reduced polysaccharide fractions was done by GLC. Results for the non-reduced fractions are given in Tables 4 and 5. The tables indicate that all fractions contained the same sugars. In the preparation obtained after holding at 24 °C overnight, the three main neutral sugars in the hot-water extract of the residue were Glc > Gal > Xyl. In the preparation obtained after holding at 4 °C overnight, the main neutral sugars in the hot-water extract were Gal > Glc > Man. In the 2% NaOH extract of the residues from both preparations, the three main neutral sugars were Glc > Xyl > Rha. In the soluble fraction of both preparations, the main neutral sugars were Gal > Glc, indicating a substituted (decorated) glucogalactan or a substituted (decorated) galactan. Neither of the two fractions from the insoluble residue had a dominate component sugar.

These data indicate a family of novel polysaccharides. Since a qualitative test for uronic acid was positive, they were acidic polysaccharides. In addition to the water- and/or alkali-soluble polysaccharides listed in Table 1, it has been reported that the cell walls of *Chlorella pyrenoidosa* contain cellulose (Northcote, Goulding, & Horne, 1958), that the cell walls of *Chlorella ellipsoidea* contain chitin (Mihara, 1961; Takeda & Hirokawa, 1978) (as indicated by the presence of glucosamine in a hydrolyzate), and that the cell wall is 13.6% of the dry weight of *C. pyrenoidosa* cells (Northcote et al., 1958). *Chlorella* sp. also accumulates A-type starch when grown in the presence of light or organic carbon sources such as sucrose (Bailey & Neish, 1954; Kobayashi, Tanabe, & Obayashi, 1974; Olaitan & Northcote, 1962; Streshinskaya, Pakhomova, & Kosikov, 1967; Suárez et al., 2008). Finally, it has been found that the carbohydrate composition varies from strain to strain of *Chlorella* (Streshinskaya et al., 1967) and during the cell cycle of *C. ellipsoidea* (Takeda & Hirokawa, 1978).

3.3. Compositional analysis: Hydrolysis of the uronic acid-reduced polysaccharide fractions

Hydrolysis of the carboxyl-reduced polysaccharide fractions and identification and quantification of the released monosaccharides was also done procedure first reported by Albersheim et al. (1967). The greatest change after reduction was a decrease in the amount of glucose present (data not given). The reduction process involves a dialysis step for desalting. It was speculated that the preparations contained low-molecular-weight glucans as reported by Suárez et al. (2008) that were removed by the dialysis.

Table 2
Yields of major fractions after holding at 24 °C overnight.

Extracts	Yields from 10 g of dried Chlorella				
	Run 1	Run 2	Run 3	Average (SD)	% yield
Soluble fraction					
Hot water soluble[a]	1.483	1.484	1.610	1.526 (0.073)	15.3
Residue					
Hot water extract[b]	0.556	0.276	0.354	0.3955 (0.145)	4.0
2% NaOH extract[c]	0.310	0.318	0.315	0.314 (0.004)	3.1
Total					22.4

[a] Fraction A (Fig. 1).
[b] Fraction B (Fig. 1).
[c] Fraction C (Fig. 1).

Table 3
Yields of major fractions after holding at 4 °C overnight.

Extracts	Yields from 10 g of dried Chlorella					
	Run 1	Run 2	Run 3	Run 4	Average (SD)	% yield
Soluble fraction						
Hot water soluble[a]	0.860	0.625	1.302	1.444	1.058 (0.381)	10.6
Residue						
Hot water extract[b]	0.171	0.226	0.2015	0.249	0.212 (0.034)	2.1
2% NaOH extract[c]	0.850	0.735	0.351	0.398	0.584 (0.247)	5.8
Total						18.5

[a] Fraction D.
[b] Fraction E.
[c] Fraction F.

Table 4
Neutral sugar compositions of fractions obtained from non-uronic acid-reduced polysaccharides from preparations obtained after holding at 24 °C overnight.

Extracts	Rhamnose RD[a] (SD[b])	Arabinose RD (SD)	Xylose RD (SD)	Mannose RD (SD)	Galactose RD (SD)	Glucose RD (SD)	Unknown 1[c]	Unknown 2[d]
Soluble fraction								
Hot-water soluble (A)	0.293 (0.044)	0.430 (0.046)	0.428 (0.003)	0.486 (0.051)	3.273 (0.208)	0.987 (0.060)	+	+
MR[e]	1.0	1.6	1.6	1.5	10.2	3.1		
Mole%	5.3	8.5	8.4	8.0	53.7	16.2		
Insoluble fraction								
Hot-water extract (B)	0.384 (0.060)	0.368 (0.093)	0.469 (0.076)	0.478 (0.128)	0.931 (0.119)	1.012 (0.128)	+	+
MR	1.0	1.1	1.3	1.1	2.2	2.4		
Mole%	11.0	11.5	14.6	12.4	24.2	26.3		
2% NaOH extract (C)	1.034 (0.115)	0.431 (0.058)	1.266 (0.106)	0.966 (0.155)	1.067 (0.161)	2.116 (0.344)		+
MR	1.0	0.5	1.3	0.8	0.9	1.9		
Mole%	15.5	7.1	20.8	13.2	14.6	28.9		

[a] RD = raw data = ratio of GLC reading to that of an internal inositol standard to 3 decimal points.
[b] SD = standard deviation.
[c] Unknown 1 = peak preceding that of arabinose (see text).
[d] Unknown 2 = peak between that of unknown 1 and that of arabinose (see text).
[e] MR = mole ratios normalized to rhamnose to 1 decimal point.

Table 5
Neutral sugar compositions of fractions obtained from non-uronic acid-reduced polysaccharides obtained from preparations obtained after holding at 4 °C overnight.

Extracts	Rhamnose RD[a] (SD[b])	Arabinose RD (SD)	Xylose RD (SD)	Mannose RD (SD)	Galactose RD (SD)	Glucose RD (SD)	Unknown 1[c]	Unknown 2[d]
Soluble fraction								
Hot-water soluble (D)	0.360 (0.024)	0.228 (0.010)	0.560 (0.115)	0.687 (0.070)	4.720 (0.812)	1.392 (0.234)	+	+
MR[e]	1.0	0.7	1.7	1.7	11.9	3.5		
Mole%	4.9	3.4	8.25	8.4	58.0	17.1		
Insoluble fraction								
Hot-water extract (E)	0.321 (0.060)	0.317 (0.051)	0.350 (0.045)	0.589 (0.010)	1.086 (0.220)	1.028 (0.294)	+	+
MR	1.0	1.0	1.2	1.7	3.1	2.9		
Mole%	9.2	9.9	10.9	15.3	28.2	26.7		
2% NaOH extract (F)	0.601 (0.051)	0.279 (0.043)	0.659 (0.093)	0.624 (0.112)	0.669 (0.088)	1.249 (0.167)		+
MR	1.0	0.5	1.2	0.9	1.0	1.9		
Mole%	15.3	7.7	18.3	14.4	15.5	28.9		

[a] RD = raw data = ratio of GLC reading to that of an internal inositol standard to 3 decimal points.
[b] SD = standard deviation.
[c] Unknown 1 = peak preceding that of arabinose (see text).
[d] Unknown 2 = peak between that of unknown 1 and that of arabinose (see text).
[e] MR = mole ratios normalized to rhamnose to 1 decimal point.

Another consistent difference was an increase in the percentage of arabinose, and in 5 of the 6 analyses, there was an increase in the percentage of xylose. Previous studies have identified GlcA as the only uronic acid (Ogawa, Ikeda, & Kondo, 1999; Umezawa & Komiyama, 1985; Umezawa, Komiyama, Shibukawa, Mori, & Kojima, 1982; White & Barber, 1972; Yalcin, Hicsasmaz, Box, & Bozoglu, 1994), so the most likely conclusion to be drawn from this data, based on literature information (Table 1), is that the uronic acid constituent is glucuronic acid (the increase in glucose after reduction being masked by the loss of glucan during dialysis) and that hydrolysis of the native fractions released the aldobiouronic acid GlcA → Ara.

Because of the hypothesis formed, the fractions were subjected to dialysis (Mw cutoff 6000–8000) before reduction and hydrolysis. In general, dialysis decreased the mole ratio values for all components except for rhamnose (the component used for normalization), a finding indicating that dialysis removed low-Mw substances, but not exclusively a glucan. The only increases in the molar amounts found were for Man in fractions C, D, and E, Glc in fraction C, and Gal in fraction D. Thus, there was evidence for the presence of mannuronic acid units with lesser evidence for the presence of galacturonic and glucuronic acid units, but the uronic acid constituent(s) in this species of *Chlorella* remain unidentified at this time.

4. Conclusions

Polysaccharides were recovered from *Chlorella* cells via treatment with an acidified chlorite solution. The total yield was 18–22% of the total dry weight of the *Chlorella* cells. The polysaccharides were isolated as fractions, the principal fraction being that which was soluble in the chlorine dioxide/chlorous acid solution and, after being precipitated from the ClO_2/$HClO_2$ solution, in hot water (11–15%); total yield of water-soluble polysaccharides = 13–19%. Treatment of the remaining insoluble residue in the same way released an additional 6.4% of water-soluble polysaccharides for a total yield of about 25%. Although the majority of polysaccharides could not be obtained from the cells without use of a mild oxidant, they were soluble in water once extracted with the acidified chlorite solution. The combination of water-soluble polysaccharides and crosslinking may provide the cells with a tough, but flexible, cell wall that can withstand the osmotic gradient resulting from growth in fresh water.

Treatment of *Chlorella* cells in this way made extraction of the oil from them much easier, as did treatment of the cells with an alkaline solution of hydrogen peroxide. The latter treatment gave a lower yield of polysaccharides, however.

Data was obtained that indicated that the polysaccharide fractions were composed of galactosyl, glucosyl, mannosyl, xylosyl, arabinosyl, rhamnosyl, and uronic acid units. Homogeneity/heterogeneity of the fractions was not determined. It is concluded that the fractions probably contain polysaccharides of complex structure (undoubtedly both polydisperse and polymolecular) crosslinked with bonds that can be cleaved by the oxidizing system of a mildly acidic chlorite solution or that they are present in a matrix of a lignin-like material. The fact that each fraction contains 6 different sugars suggests branched structures. A likely possibility is that the fractions contain mixtures of essentially linear polysaccharides that contain short side chains or are otherwise substituted enough to make them water soluble. The constituent polysaccharides were not separated from each other and their structures were not determined (because it was the objective of this research to isolate a total polysaccharide fraction for potential industrial use). It is hoped that the successful methods of polysaccharide isolation reported here will spur efforts to fractionate and characterize polysaccharides from various species of microalgae.

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