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Sugar compositional determination of polysaccharides from *Dunaliella salina* by modified RP-HPLC method of precolumn derivatization with 1-phenyl-3-methyl-5-pyrazolone

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ABSTRACT

A modified high-performance liquid chromatography method of pre-column derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) has been established for high resolution separation and high sensitivity determination of ten monosaccharides simultaneously, which frequently occur in algae polysaccharides. The effects of volume proportion of acetonitrile and pH value of mobile phase (0.1 M phosphate buffer–acetonitrile) on retention and separation of the monosaccharide derivatives were investigated with Eclipse XPB-C18 column screened out. The hydrolyzation condition of polysaccharides and derivatization procedure of hydrolysates were also optimized. The modified analysis method was used for the determination of monosaccharide compositions in five polysaccharide fractions isolated from *Duanaliella salina*. The results showed that PD1 and PD4a were acidic heteropolysaccharide mainly containing glucose and galactose respectively, and PD4a contained sulfated groups; PD2 and PD3 all were a glucan; while PD4b was a complex of polysaccharide linked with nucleic acids by covalent bonds.

1. Introduction

Dunaliella salina (*D. salina*) is a unique unicellular species of chlorophyta with no cell wall found in saline environments, which is known for its carotenoids accumulation (mainly beta-carotene), having various applications in the health and nutritional products (Borowitzka, Borowitzka, & Moulton, 1984; Murthy et al., 2005). Meanwhile, rich polysaccharides from the residues after β-carotene extraction from *D. salina*, their content being 12–40% of *D. salina* (Brown, 1991), also have been studied for biological activities (Fabregas, Garcia, & Fernandez-Alonso, 1999; Sun, Wang, Xue, & Wang, 1997; Zheng, Wang, Shi, & Chu, 2004) such as anti-tumor, antivirus and immunomodulating properties. However, the study on monosaccharide compositions of the polysaccharides from *D. salina* was rarely reported. Zheng, Wang, Zhang, and Shen (1997) first reported one polysaccharide isolated from *D. salina* residue after extraction of β-carotene and its monosaccharide composition was identified as glucose, galactose, xylose, mannose, and rhamnose by paper chromatography. Later, Xue, Yin, Wang, and Xu (2003) and Xie, Yin, Chen, Xie, and Wang (2005) obtained three polysaccharide fractions from *D. salina* by hot basic water extraction and purification with DEAE-32 ion-exchange column and Sephadex G-100 gel filtration column. The three fractions were determinated as a glucan, a sulfated proteoglycan and a sulfated heteropolysaccharide mainly containing glucose by GC, IR and barium sulfate turbidimetry.

For monosaccharide composition analysis of polysaccharides, which is of fundamental importance for the research on polysaccharide structure and its characteristics, GC, HPLC, GC–MS and CE have been applied respectively according to different separation model and detection method. HPLC has been accepted as one of the major techniques for the analysis of sugars, especially in the form of anion-exchange chromatography coupled with pulsed amperometric detection of the underivatized sugars (Hardy, Townsend, & Lee, 1988; Quigley & Englyst, 1994). Owing to requiring more specialized equipment and fall of the electrochemical response arisen from the presence of peptides and proteins in sample for the anion-exchange techniques (Rohrer, Thayer, Weitzhandler, & Avdalovic, 1998), RP-HPLC (reverse phase-high performance liquid chromatography) method of pre-column derivatization was often choosed for use of sugar analysis with highly sensitive detection and high separation selectivity, which may be used to simultaneous determination of neutral, acidic and basic carbohydrates whereas GC could not do usually (Huie & Di, 2004). The reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) with strong UV absorbance at 245 nm is one of the popular labels for the HPLC

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method that can react with reducing carbohydrates under mild conditions, requiring no acids catalyst and causing no desialylation and isomerization (Fu & O'Neill, 1995; Fu & Zopf, 1999; Honda et al., 1989; Strydom, 1994; Zhang, Xu, Zhang, Zhang, & Zhang, 2003). Honda et al. (1989) first reported the application of PMP labeling for analysis of component monosaccharides of glycoproteins and optimized the PMP derivatization chemistry using glucose as a model compound by independently changing reaction parameters, including the concentrations of PMP and sodium hydroxide, the reaction temperature, and the reaction time. They optimized mobile phase (a mixture of pH 5 phosphate buffer and acetonitrile) on Capcell Pak C18 column by isocratic elution to make seven neutral sugars resolved basically except two acetyl-amino sugars. Later, Strydom (1994) established the condition for reversed-phase chromatographic analysis of glycoproteins hydrolysates containing eleven sugars with NovaPak column and gradient elution of triethylamine buffer (pH 4.86 with phosphoric acid)–acetonitrile, using PMP derivatization procedure described by Honda et al. (1989), and most of sugars were separated aside from glucuronic and galacturonic acids. In recent years, the HPLC method of PMP derivatization has been widely used for the analysis of monosaccharides and oligosaccharides in many biology samples such as rat plasma (Aghazadeh-Habashi, Carran, Anastassiades, & Jamali, 2005), exopolysaccharides including hyaluronic acid (Izawa et al., 2009), and some factors of the derivatization procedure were improved, for instance, omitting of drying steps (Fu & Zopf, 1999; Zhang et al., 2003) or selection of extraction solvents (Fu & O'Neill, 1995). In the present study, a RP-HPLC method for simultaneous determination of twelve sugars labeled with PMP using UV detection and isocratic elution on ZORBAX Eclipse XDB-C18 column was established, and the procedure of the pre-column derivatization reaction was optimized by examination of reaction time and neutralization condition of the reaction system for eight neutral and two acidic sugars. At the same time, the optimized method and procedure were successfully applied in sugar compositional analysis of polysaccharide fractions isolated from *D. salina*.

2. Experimental

2.1. Chemicals and reagents

Monosaccharides (glucose, galactose, arabinose, xylose, mannose, rhamnose, fucose, ribose, glucuronic acid and galacturonic acid) used for standards were purchased from USA Sigma–Aldrich Co. 1-Phenyl-3-methyl-5-pyrazolone (PMP), glucosamine and galactosamine were obtained from USA Acros Organics Co. and HPLC-grade acetonitrile were purchased from USA Tedia Co. Water used throughout the experiments was double distilled and purified on a Milli-Q system (Millipore Inc., Milford, MA, USA). All other chemicals and solvents used were of analytical grade or HPLC-grade unless otherwise specified. *D. salina* powder was provided by Lantai Biological Engineering Co. Ltd. (Inner Mongolia, China).

2.2. Extraction, purification and relative molecular mass analysis of polysaccharides from D. salina

After carotenoids were extracted from dried *D. salina* powder using acetone, other polar organic compounds with low molecular weight in the extraction residue were removed by reflux with ethanol. Then the organic solvent was vaporized and the residue was dried in a vacuum state. The pretreated dry powder was extracted twice with alkaline water (pH adjusted by 2.0 M NaOH to 9.0) under constant stirring for 3.5 h in an 80 °C water bath. After cooling, the mixture was adjusted pH to 7.0 by 2.0 M HCl, then was centrifuged (4000 × *g*, 20 min), and the supernatant was adjusted pH to 4.0 for precipitating the proteins and centrifuged. Adjusted again back to pH 7.0, the supernatant was then concentrated and mixed with three times volume of ethanol. After standing for 12 h at 4 °C, the sample was centrifuged and the precipitates were rehydrated. In succession the free proteins were removed by Sevag method (Wang et al., 2007): the polysaccharide solution and the Sevag reagent (chloroform:n-butanol = 4:1, v/v) were mixed (polysaccharide solution:Sevag reagent = 5:1, v/v) and shaken acutely for 30 min, then centrifuged for removing the denaturalized proteins, and the process was repeated 5–6 times. Finally, the polysaccharides were dialyzed against distilled water (Molecular weight cutoff 3500 Da), then concentrated and lyophilized. Crude polysaccharide extract (PD) was obtained.

PD dissolved in water to a concentration of about 10 mg/ml, was fractionated by anion-exchange chromatography on DEAE-Sepharose Fast Flow column (*D* 3.5 cm × 30 cm) and eluted by a linear gradient of NaCl concentration (0.01–1.0 M) in 0.02 M Tris–HCl buffer at a flow rate of 8.0 ml/min. Four fractions (PD1, PD2, PD3 and PD4) were collected with a fraction collector and concentrated, dialyzed for 3 days and lyophilized. PD4 were then loaded onto a Sepharose CL-6B gel filtration column (*D* 2.6 cm × 100 cm), and eluted with 0.1 M NaCl at a flow rate of 0.5 ml/min. The eluate obtained (PD4a and PD4b) was pooled, concentrated, dialyzed and lyophilized. The eluting fractions were monitored for the presence of carbohydrate using phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

Relative molecular mass analysis of polysaccharide fractions were carried out according to the HPSEC procedure as described by Sun, Tang, Gu, and Li (2005).

2.3. Hydrolysis of polysaccharides

4 M trifluoroacetic acid (100 μl) was added into the polysaccharide solution (100 μl, 3–4 mg/ml) in a small ampoule. The ampoule was sealed under nitrogen atmosphere and kept for 2 h in an oven at 110 °C. After the ampoule was cooled to room temperature, it was opened and 200 μl of methanol was added into it for the reaction mixture to be evaporated to dryness by blowing of nitrogen stream and heating of water bath. Then the same amount of methanol was again added and dried by the same method as above, and the procedure was repeated thrice for TFA to be removed. The dried hydrolyzed polysaccharide sample was dissolved in 100 μl of water for subsequent derivatization.

2.4. Derivatization of monosaccharides with PMP

100 μl of solution of hydrolyzed polysaccharide sample or monosaccharide standard mixture was mixed with 100 μl of 0.6 M sodium hydroxide. 50 μl of the mixture in a small sample tube with lid was added a 0.5 mol/l methanolic solution (50 μl) of PMP and mixed thoroughly by a vortex mixer. The whole mixture was heated to 70 °C and incubated for 100 min. After the reaction mixture was cooled to room temperature, and was neutralized with 50 μl of 0.3 M hydrochloric acid and the resultant solution evaporated to dryness. Water and chloroform (1.0 ml each) were added to the residue, and the mixture was shaken vigorously. The chloroform layer was discarded, and the extraction process was repeated three times. The aqueous layer was filtered through a 0.45 μm pore membrane filter for HPLC analysis. The derivatization procedure of hydrolysate samples and standard samples must be carried out simultaneity under the same condition.

3. Analysis of monosaccharide composition of polysaccharide fractions from *D. salina*

3.1. HPLC analysis of monosaccharide-PMP derivatives

The HPLC system (Agilent1100, USA) used consisted of a G1311A liquid chromatograph pump, G1379A online degas unit, G1313A automatic injector with a 20 μl loop, and G1315B diode array detector. The data were collected using an Agilent1100 ChemStation system provided by Agilent Company. A special ZORBAX Eclipse XDB-C18 HPLC column (250 mm length, 4.6 mm i.d., and 5 μm particle size) (Agilent, USA), optimized for the separation of PMP derivatives, was used at ambient temperature of 30 °C. The PMP derivatives elution was performed with a mixture of 0.1 M phosphate buffer (pH 6.7) and acetonitrile in a ratio of 83:17 (v/v, %) at a flow rate of 1 ml/min, and UV absorbance of the effluent was monitored at 245 nm. The PMP derivatives were quantified by comparing their integration values of peak area to a calibrated standard curve.

3.2. LC–MS analysis of PMP derivatives of hydrolysate of PD4b

LC-ESI/MS measurements were performed using a Waters Alliance 2690 HPLC coupled with Platform ZMD 4000 single quadrupole mass spectrometer. Because the mobile phase for LC–MS only can contain volatile salt, the mobile phase used to liquid chromatography was 20 mM ammonium acetate buffer–acetonitrile (volume proportion is 84:16). The same chromatographic column as HPLC-UV was used. The flow rate was 0.8 ml/min, but eluent from the column was split to interface of liquid chromatography–mass spectrometry with 20 μl of injection volume and a post column split ratio of 1/3. The experiment was carried out in positive ion mode. The capillary voltage and sample cone voltage were 4.4 kV and 67 V respectively. The temperature of ion resource was 100 °C and desolvation 250 °C, mass range: 200–1000 *m*/*z*; photomultiplier voltage: 700 V; analyser vacuum: 2.6e−5 mbar; gas flow: 4.2 l/h.

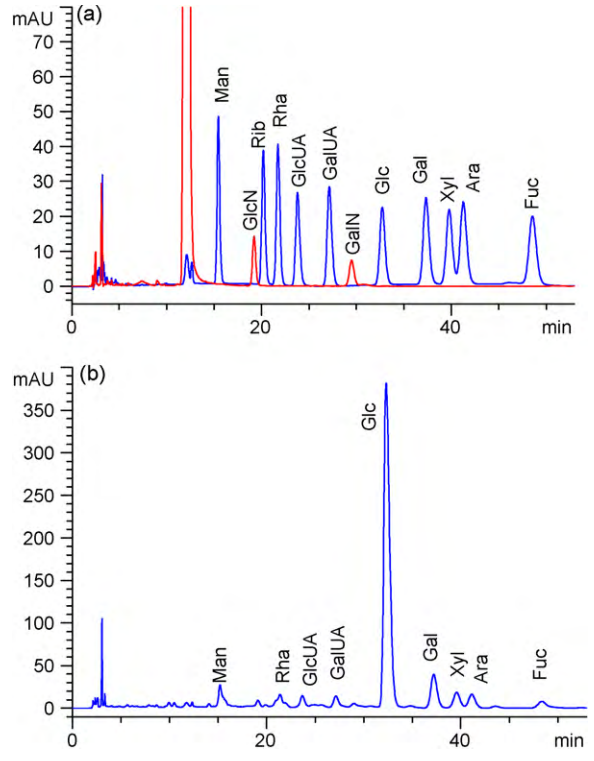

Fig. 1. Chromatograms of PMP derivatives of monosaccharide standard samples (a) and hydrolysate of fraction PD1 from *D. salina* (b).

3.3. Ion chromatography analysis of hydrolysate of PD4a

Except the method of precolumn derivatization with PMP, monosaccharide composition of polysaccharide fraction PD4a isolated from *D. salina* was determined and validated yet by hydrolyzing samples in trifluoroacetic acid, using a Dionex HPAEC system (ICS3000) equipped with pulsed amperometric detection (PAD) and eluting by a gradient of H_2O, 0.25 M NaOH and 1 M NaAC solutons according to the procedure as described by Quigley and Englyst (1994).

3.4. Chemical analysis of polysaccharide components

Total sugar content was determined by the reaction with phenol in the presence of sulfuric acid (Dubois et al., 1956; Sun et al., 2005) using glucose as a standard. Proteins in the solution were quantified by the method of binding of Coomassie Brilliant Blue G-250 to protein (Bradford, 1976) using bovine serum albumin as a standard. The total uronic acid content was colorimetrically determined by the m-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973) using galacturonic acid as a standard. The sulfated group content was determined by barium sulfate turbidimetry (Dodgson, 1961).

4. Results and discussion

4.1. Optimization of separation of monosaccharide-PMP derivatives

Separation of PMP derivatives of monosaccharides was studied by using various stationary and mobile phases. The stationary phase most suitable for the rapid and effective separation of eight neutral, two acidic and two amino sugars labeled with PMP was found to be ZORBAX Eclipse XDB-C18 column compared with Waters Atlantis dC18 (Waters, USA), Waters Symmetry C18 (Waters, USA), ZORBAX Extend-C18 (Agilent, USA), Hypersil AA-ODS (SMI-LabHut Ltd., UK) and Venusil XBP-C18 (Agela Technologies Inc., USA). The separation result obtained with the most suitable column was shown as Fig. 1.

The retention behaviors of the PMP derivatives were examined by using mixtures of 0.1 M phosphate buffer with various pH values and acetonitrile at varying proportions as mobile phase. The effects of acetonitrile proportions on the retention time in the separation of eight neutral, two acidic sugars labeled with PMP was given in Fig. 2(a) (buffer pH 6.0). The result showed with the increase of acetonitrile proportion the elution process of ten PMP derivatives became faster but the elution order of derivatives keep changeless. 17% (v/v) of acetonitrile in the mobile phase presented better separation effect. Because affecting charge state of PMP-sugars with alkaline group, pH also has obvious effect on retetion and separation of PMP-sugars. At 17% of acetonitrile proportion, the effects of phosphate buffer pH on the retention time were investigated, as shown in Fig. 2(b). The result showed an increase of phosphate buffer pH resulted in rapid elution and the elution order of derivatives remained changeless. The phosphate buffer of pH 6.7 brought better separation and relatively rapid elution simultaneously. Furthermore, when methanol was used as organic solvent in the mobile phase, the separation effect of the monosaccharide derivatives was obviously poorer than acetonitrile. As a result, the mixture of 0.1 M phosphate buffer (pH 6.7) and acetonitrile to a concentration of 17 (v/v%) gave optimal separation condition for PMP derivatives of eight neutral, two acidic sugars. Two aminosugars shown in Fig. 1 was not detected in polysaccharide samples

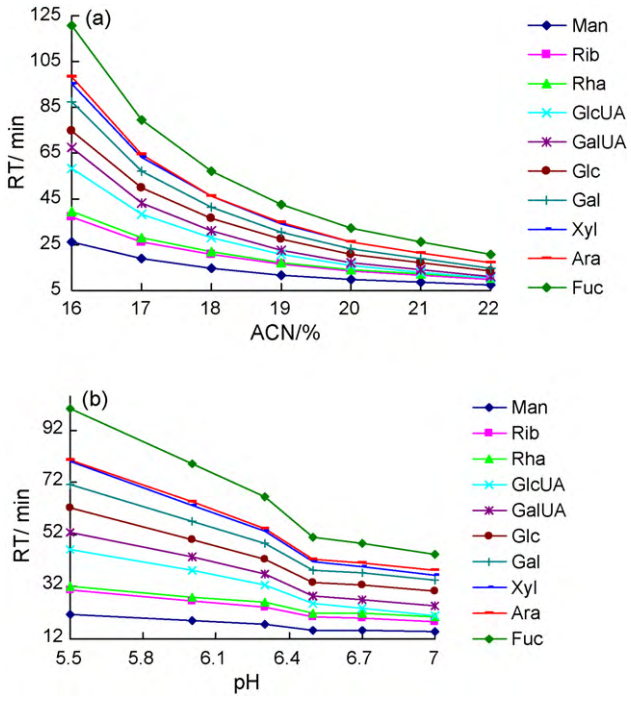

Fig. 2. Effects of volume proportions of acetonitrile (a) and phosphate buffer pH values (b) in mobile phase on retention time of PMP derivatives of ten monosaccharides.

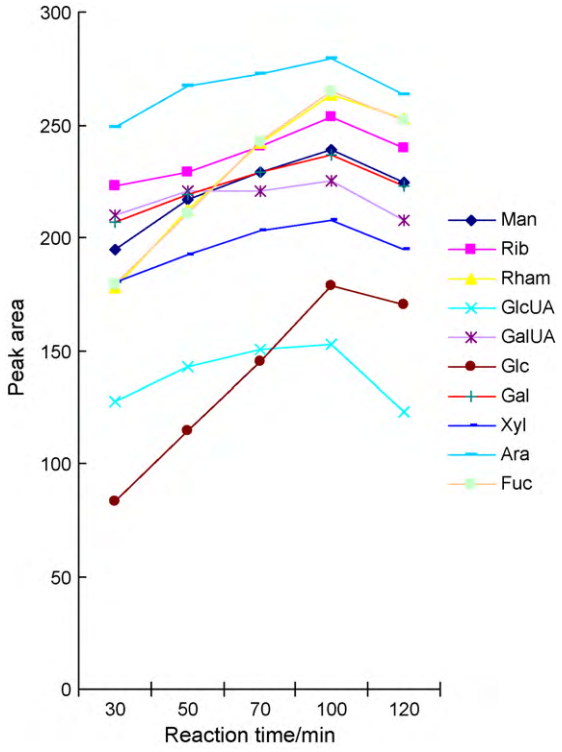

Fig. 3. Effects of derivatization reaction time on peak area of PMP monosaccharide derivatives.

from *D. salina*, so retention behaviors of the two sugars at different mobile phase composition were not investigated.

4.2. Optimization of PMP derivatization reaction

PMP derivatization reaction time was usually selected to be 30 min (Honda et al., 1989; Strydom, 1994) or 2 h (Fu & O'Neill, 1995) according to the literatures. The effects of derivatization reaction time on peak area of derivative products in HPLC were presented in Fig. 3 (the peak area is in proportion to yields of derivative products). The graph data were average of thrice repeated determination, and the RSD were 2.1–4.8%. The result showed the peak area of derivative of each sugar was not the same at different derivatization reaction time. While the reaction time was 100 min the peak area of derivative of each sugar exhibited the maximum, which was higher by 30% at least than that at reaction time of 30 min. PMP derivatization reaction time was determined to be 100 min with highest sensitivity.

A large excess PMP was used for derivatization reaction, therefore removal of residuary reagent by chloroform extraction before HPLC analysis was necessary. PMP with alkaline group in a strongly acidic solution, were hardly extracted completely by chloroform, due to protonation of PMP, whereas at high pH significant amounts of the PMP-sugars were also extracted (Strydom, 1994). Because quantitative recovery of monosaccharides was essential for accurate monosaccharide composition analysis, pH of PMP derivative solution before extraction was a important parameter. Honda et al. (1989) neutralized the reaction solution using equal mol hydrochloric acid with sodium hydroxide, and Strydom (1994) and Fu et al. (1995) selected excess hydrochloric acid for neutralization of solution (pH 3–4 or 4–5). The effects of different volume of HCl (0.3 M) for neutralization on peak area of PMP derivatives were examined in the present study. The result indicated the peak areas of PMP derivatives decreased with increasing HCl volume, perhaps because the derivatives were instable in the acidic solution. When using HCl volume of 50 μl (equal mol hydrochloric acid used to neutralization) most PMP derivatives exhibited maximal peak areas and highest detective sensitivity except glucose.

4.3. Validation of the analysis method

To achieve accurate analysis results of the monosaccharide composition, the detection linearity of PMP derivatives was verified by the analysis of seven data points ($n = 3$, take the means) of different concentration of mixture standard sugars, and the linear regression parameters of the calibration curves were shown in Table 1. Excellent response linearity was obtained for all ten monosaccharides over tested concentration ranges. The limits of detection at the signal-noise ratio of 3 were determined to be 0.081–0.271 nmol (listed as Table 1) by successive dilutions of PMP-labeled monosaccharide mixture.

The method repeatability was also determined by measuring relative standard deviations (RSD) of retention time and peak area for each tested monosaccharide derivatives. Results of repeated determination ($n = 5$) of mixture standard sugars (including derivatization and HPLC analysis) showed the relative standard deviations were less than 1.15% for the retention time and 2.89% for the peak areas, indicating that the method precision was satisfactory.

In the experiments, known amounts of each monosaccharide at a close concentration with the sample were added to the hydrolyzed polysaccharide sample (PD4a), and the resulting spiked sample in triplicate were subjected to the derivatization reaction and HPLC determination.The recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. The results showed that the recoveries of all the ten monosaccharides ranged between 95.3% and 103.2% and the RSD values fell within 2.7–4.9% (Table 2).

Table 1
Calibration curves and detection limit of ten monosaccharides.

Sugar	Regression equation[a]	Determination coefficient R^2	Liner range (μmol/ml)	Detection limit (nmol)
Man	$Y = 373.81X + 2.07$	0.9999	0.0104–8.33	0.081
Rham	$Y = 371.06X - 5.54$	0.9998	0.0117–9.38	0.12
Rib	$Y = 373.40X + 5.05$	0.9998	0.0114–9.09	0.17
GlcUA	$Y = 342.33X - 21.73$	0.9983	0.00964–7.72	0.20
GalUA	$Y = 406.76X - 12.71$	0.9996	0.0101–8.06	0.20
Glc	$Y = 336.34X - 6.54$	0.9998	0.0102–8.15	0.13
Gal	$Y = 406.73X - 7.07$	0.9998	0.0103–8.21	0.16
Xyl	$Y = 312.22X - 10.08$	0.9995	0.0124–9.91	0.27
Ara	$Y = 389.78X - 6.50$	0.9999	0.0122–9.76	0.14
Fuc	$Y = 375.58X - 3.03$	0.9998	0.0114–9.08	0.10

[a] Y: Peak area; X: sugar concentration (mmol/l).

Table 2
Recoveries of the ten monosaccharides in process of the derivatization reaction and HPLC determination ($n = 3$).

Sugar component	Man	Rib	Rham	GlcUA	GalUA	Glc	Gal	Xyl	Ara	Fuc
Content in sample (nmol)	280.2	39.5	200.2	134.0	292.5	299.1	2337.5	642.7	1083.1	251.3
Spiked amount (nmol)	100	50	200	100	200	200	1000	500	1000	200
Found amount (nmol)	375.4	90.7	397.4	230.7	487.3	494.7	3328.5	1158.7	2066.1	448.7
Recovery (%)	95.2	102.4	98.6	96.7	97.4	97.8	99.1	103.2	98.3	98.7
RSD (%)	4.9	4.3	2.7	4.1	3.8	3.5	3.2	4.5	3.8	2.9

4.4. Chemical components of polysaccharide fractions from D. salina

The crude polysaccharides (PD) from *D. salina* were fractionated to five fractions (PD1, PD2, PD3, PD4a and PD4b) by anion exchange and gel filtration chromatography as stated above. Total carbohydrate, protein, uronic acid and sulfated group content of the polysaccharide fractions and their relative molecular mass were determined using aforementioned analysis methods. The results showed that total carbohydrate contents of PD1, PD2, PD3, PD4a and PD4b are 91.67, 94.21, 85.01, 80.35 and 57.09% (w/w) respectively, PD1, PD4a and PD4b contained uronic acids of 5.28, 8.53 and 3.57% (w/w), and the three fractions and PD3 all contained small amounts of proteins (respectively 1.92, 5.17, 6.90 and 10.34%, w/w). PD4a containing sulfated group (8.36%, w/w) belonged to sulfated polysaccharides. Weight mean molecular mass (M_w) of PD1, PD2, PD3, PD4a and PD4b were determined to be 1548.3 kDa, 33.5 kDa, 66.8 kDa, 424.2 kDa, 10.3 kDa respectively.

The UV spectra of PD4b and PD4b treated by nuclease (and dialyzed) have been measured. The results showed PD4b had the remarkable absorbance at the wavelength of 260 nm, and however, the absorbance of PD4b after hydrolysis by nuclease at 260 nm disappeared, indicating PD4b contained the nucleic acids. The nucleic acids content determined by measuring phosphate (Alexander, Griffiths, & Wilkinson, 1985) was 49.26%. In addition, PD4b was obtained by anion exchange and gel filtration chromatography, and the analysis results of HPSEC and agarose gel electrophoresis (Motlagh, Ravines, Karamallah, & Ma, 2006) of PD4b only presented a single peak and a remarkable spot respectively, so PD4b was a complex of polysaccharide linked with nucleic acids by covalent bonds.

4.5. Monosaccharide composition of polysaccharide fractions from D. salina

The results of sugar compositions of five polysaccharide fractions from *D. salina* obtained by the HPLC analysis based on precolumn PMP derivatization are shown in Table 3, indicating PD2 and PD3 were all glucan but PD4a, PD4b and PD1 were found to be acidic heteropolysaccharides. The HPLC profile of PMP derivatives of hydrolysate from PD1 are shown in Fig. 1, chromatograms of other fractions are not shown. The data in Table 3 also shown monosaccharide composition of PD4a by RP-HPLC was in good agreement with the result obtained by HPAEC-PAD detection.

Based on Table 3, the ribose content in PD4b was much higher than any other monosaccharide. In addition, PMP derivatives of PD4b were detected by selective ion monitoring using LC–MS, and the result showed there was no deoxyribose in fraction PD4b.

Table 3
Sugar composition of three polysaccharide fractions from *D. salina* (mol%, $n = 3$).

Fractions	Methods	Man		Rib		rha		glcUA		galUA	
		Average	RSD (%)	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
PD4a	PMP	5.04	3.54	0.71	3.17	3.60	3.22	2.41	5.22	5.26	4.80
	IC	4.18	2.72	0	–	3.06	3.98	3.33	4.87	5.89	4.32
PD4b	PMP	3.48	4.87	67.8	2.92	1.34	3.04	2.36	4.06	2.70	3.95
PD1	PMP	2.32	3.87	0	–	3.67	4.35	1.95	3.24	2.00	2.83
Fractions	Methods	glc		gal		xyl		ara		fuc	
		Average	RSD (%)	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
PD4a	PMP	5.38	3.31	42.04	3.24	11.56	2.93	19.48	3.29	4.52	2.87
	IC	5.95	2.16	41.49	2.57	12.24	2.84	19.35	3.05	4.51	3.19
PD4b	PMP	4.69	2.07	8.40	2.22	4.25	3.16	3.61	3.73	1.37	3.45
PD1	PMP	73.1	2.38	7.40	3.04	4.01	3.41	3.35	3.60	2.18	4.02

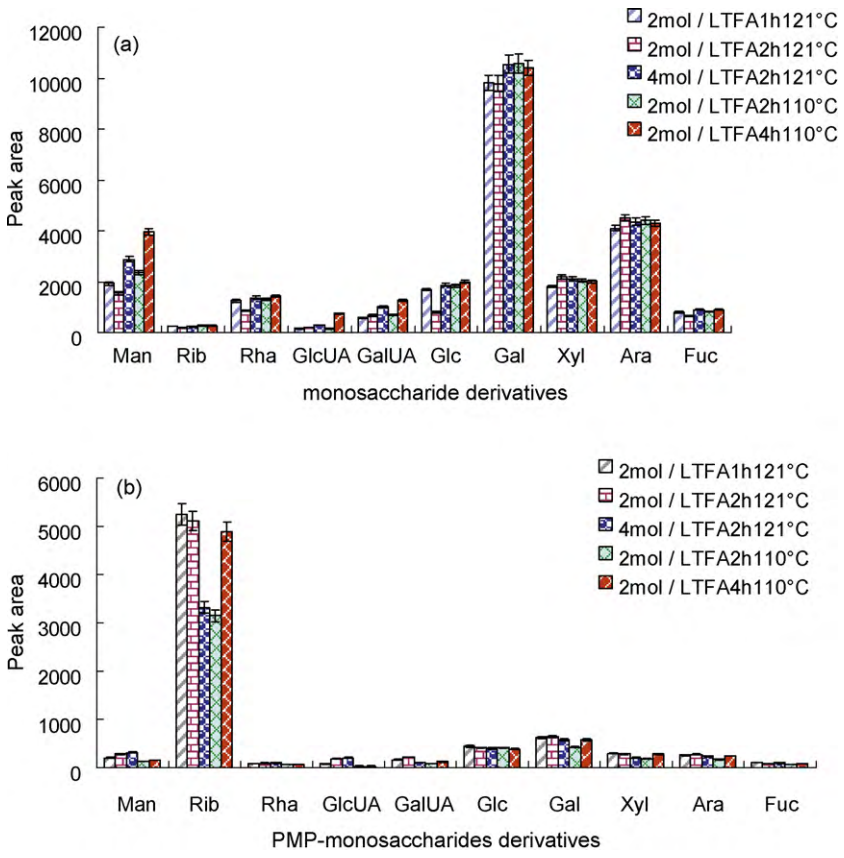

Fig. 4. Peak area change of PMP-monosaccharide derivatives from hydrolysates of PD4a (a) and PD4b (b) by HPLC in different hydrolysis conditions.

Therefore, the nucleic acids contained in PD4b should be ribonucleic acids (RNA).

4.6. Hydrolysis of polysaccharide fractions from D. salina

PD1, PD2 and PD3 with simple carbohydrate chains (see Table 3) could be essentially hydrolyzed completely using 2 M TFA in 110 °C for 2 h (Strydom, 1994). However, PD4a and PD4b containing sulfated group or nucleic acid were difficult to be hydrolyzed completely in above condition. The peak area changes of monosaccharide derivatives of their hydrolysates from HPLC with different hydrolysis conditions (Fu & O'Neill, 1995; Strydom, 1994; Sun et al., 2005) are shown in Fig. 4. The results were presented: (a) for PD4a, to make the hydrolysis complete basically and the loss least, it was appropriate to hydrolyze by 2 M TFA in 110 °C for 4 h; (b) owing to alike reason, PD4b may be hydrolyzed using 2 M TFA in 121 °C for 2 h.

5. Conclusion

The analysis conditions of monosaccharide composition of the polysaccharides using reversed-phase HPLC method based on PMP pre-column derivatization was detailedly examined and optimized. The modified HPLC method of pre-column derivatization was suitable to some minute quantities of polysaccharide samples with high resolution separation and simultaneous determination of many kinds of component sugars. Monosaccharide compositions of the polysaccharide fractions from *D. salina* were determined by the modified method. The results showed that PD1 and PD4a were acidic heteropolysaccharides mainly containing glucose and galactose respectively, and PD4a contained sulfated groups; PD2 and PD3 all were a glucan; while PD4b was a complex of polysaccharide linked with nucleic acids by covalent bonds. Composition of PD4a (M_w = 424.2 kDa) confirmed the deduction made by Fabregas et al. (1999) that water extracts with antivirus activity from *D. salina* contained sulfated polysaccharides with molecular weight of hundreds thousand Da. For the polysaccharide linked with nucleic acids such as PD4b, little result was reported until now.

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