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Enzymatic desulfation of the red seaweeds agar by *Marinomonas* arylsulfatase

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ABSTRACT

Agar and sulfated galactans were isolated from the red seaweeds *Gracilariopsis lemaneiformis* and *Gelidium amansii*. A previously purified arylsulfatase from *Marinomonas* sp. FW-1 was used to remove sulfate groups in agar and sulfated galactans. After enzymatic desulfation, the sulfate content decreased to about 0.16% and gel strength increased about two folds. Moreover, there was no difference between the DNA electrophoresis spectrum on the gel of the arylsulfatase-treated agar and that of the commercial agarose. In order to reveal the desulfation ratio and site, chemical and structural identification of sulfated galactan were carried out. *G. amansii* sulfated galactan with 7.4% sulfated content was composed of galactose and 3,6-anhydro-L-galactose. Meanwhile, *G. lemaneiformis* sulfated galactan with 8.5% sulfated content was composed of galactose, 3,6-anhydro-L-galactose, 2-*O*-methyl-3,6-anhydro-L-galactose and xylose. Data from ^{13}C NMR, FT-IR, GC–MS provided evidence of sulfate groups at C-4 and C-6 of d-galactose and C-6 of l-galactose both in GRAP and GEAP. Data from GC–MS revealed that desulfation was carried out by the arylsulfatase at the sulfate bonds at C-4 and C-6 of d-galactose and C-6 of l-galactose, with a desulfation ratio of 83.4% and 86.0% against GEAP and GRAP, respectively.

1. Introduction

Algae belonging to the red seaweed genera of *Gracilariopsis* and *Gelidium* are important resources in the agar industry [1]. Agar is generally considered to be a mixture of about 70% agarose and 30% sulfated galactan [2]. Agarose can be used as an electrophoresis medium and a chromatographic resin, which are widely used in biotechnology and pharmaceutical industries. Moreover, the gel properties of agar enable its broad application as a gelling agent in processed foods and cosmetics products [3]. In the agar industry, alkaline treatment is the traditional method of removing the primary sulfate groups of the galactopyranose unit by converting it to 3,6-anhydrogalactopyranose [4]. The alkaline treatment could improve the gel strength; however it has many drawbacks, such as decreasing polysaccharide yield, browning of agar product, and generation of environmental pollution [5]. Thus, there is a growing need to develop eco-friendly processing technologies for recovery of products from bioresource. Enzymatic processing technology can solve these problems.

Arylsulfatase (aryl-sulfate sulfohydrolase; E.C.3.1.6.1) can catalyze the hydrolysis of arylsulfate esters to aryl compounds and inorganic sulfate. The purification and characterization of a few arylsulfatases have been reported [6]. However, only arylsulfatase from *Sphingomonas* sp. AS 6330 [7] and arylsulfatase purified in our previous study [8] have been reported to improve agar quality. Our previous study reported that after enzymatic treatment, the sulfate content of agar decreased, and the content of 3,6-anhydrogalactose increased. However, the desulfation mechanism remains unknown.

Similar to agarose, sulfated galactans usually have a linear backbone built on alternating 3-linked β-galactopyranose (G) and 4-linked α-galactopyranose residues [9]. In general, sulfate groups are found in some C-6 of the 4-linked α-L-galactose unit, the biogenic precursor of the 3,6-anhydro-α-L-galactopyranose (LA) unit [10]. The incorporation of sulfate groups into agar usually weakens gel strength because of the avoidance of a cross-linked structure during gelation [11].

This study aimed to evaluate the potential application of arylsulfatase from *Marinomonas* sp. FW-1 to improve agar quality and to reveal its desulfation mechanism. To obtain a relatively purified substrate for this enzyme, sulfated galactans with relatively high sulfate content were isolated from red seaweeds *Gracilariopsis lemaneiformis* and *Gelidium amansii*. Structural characterizations

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of sulfated galactan before and after enzymatic treatment were performed by chemical and spectroscopy methods.

2. Experimental

2.1. Collection of seaweed

G. lemaneiformis was harvested from Fujian Province, China (24.8° N and 118.6° E) in April 2014. *G. amansii* was harvested from Shandong Province, China (35.7° N and 120° E) in July 2014. After collection, the samples were cleaned, air dried, ground in a mill to fine powder, and stored at −20 °C.

2.2. Isolation of agar and sulfated galactan

The dried tissue (20 g) was autoclaved for 2 h at 121 °C in 600 mL of distilled water. The water soluble fraction was separated from the residue by filtration and centrifugation at 12,000 × *g* for 3 min. Agar solution was cooled down to room temperature, gelated, frozen overnight, and thawed. After removing the thawed liquid, the agar gel was dried with hot air.

The isolated agar (10 g) was dissolved in 400 mL of 8.87 mM ethylenediaminetetraacetic acid disodium salt solution and stirred for 4 h at 60 °C. After filtration and concentration of the solution, the sulfated galactan was precipitated with ethanol (1:3, v/v), re-dissolved in distilled water, dialyzed, and lyophilized. Sulfated galactans from *G. lemaneiformis* and *G. amansii* were isolated as described above and designated as GRAP and GEAP, respectively.

2.3. Enzymatic desulfation of agar

The arylsulfatase was used to degrade the sulfate groups of agar from *G. amansii* and *G. lemaneiformis*. Agar powder (5 g) was suspended in 50 mL of 0.1 M Tris-HCl buffer (pH 9.0) and liquefied at 121 °C for 30 min. After cooling down to 45 °C, 200 U of purified arylsulfatase was added to the agar, which was incubated overnight at 45 °C. After incubation, the enzymatic treated agar was extracted and dried through a traditional freezing melting and drying method [12].

2.4. Physical properties of commercial agarose products and agar

The physical properties like gel strength, melting and gelling temperatures, sulfate content and gel electrophoresis were evaluated in commercial agarose and agar from *G. lemaneiformis* and *G. amansii* before and after enzymatic treatment.

The sulfate content was determined by the turbidimetric method [13]. Gel strength was measured on agar discs (5 cm diameter, 6 cm height) using a texture analyzer (TMS-PRO, Food Technology, USA) with a 0.78 cm^2 probe area and operated at a crosshead speed of 0.5 mm s^{-1}. Finally, 1% agar solution was gelated at 25 °C for 15 h before measurement. The gelling and melting temperatures were recorded with a precision thermometer (0.1 °C divisions), in 1.0% agar solution as previously described [14].

Gel electrophoresis was performed using the enzymatically desulfated agar and the commercial agarose was used as a control. The experiment was performed with 1.0% of gel and 100–200 bp DNA ladder using a mini-sub cell GT 8 gel rigs (Bio-Rad) according to the method of Sambrook [15].

IR spectra were recorded from the polysaccharide powder in KBr pellets using a Fourier transform infrared spectrometer (Nicolet Nexus 470, Thermo Electron, USA) between 400 and 4000 cm^{-1}. The FT-IR spectrum was used to compare the structure of agar before and after the enzymatic treatment by purified arylsulfatase and the commercial agarose was used as a control.

Data were expressed as the mean ± standard (SD). Statistical analyses were performed by one-way Turkey (SPSS V 21.0). Group means were considered to be significantly different at $P < 0.05$.

2.5. The desulfation mechanism of sulfated galactan by arylsulfatase

The enzymatic desulfation mechanism was revealed by comparing changes in sulfated galactan structure before and after enzymatic treatment. Hence, the structures of sulfated galactans from *G. lemaneiformis* and *G. amansii* were analyzed before mechanism investigation.

2.5.1. Structure of sulfated galactan

2.5.1.1. General analysis. The monosaccharide compositions of GEAP and GRAP were determined by GC and GC–MS as their alditol acetates. Derivatives were prepared by two-step reductive hydrolysis with N-methylmorpholine-borane followed by acetylation as previously described [16]. GC analysis was performed using Agilent 6890N GC instrument equipped with a fused silica capillary column DB-225 (30 m × 0.32 mm × 0.25 μm; J & M Scientific, Folsom, CA, USA). Samples were detected with a flame ionization detector at 250 °C, whereas the injector and oven temperatures were set at 250 and 210 °C, respectively.

GC–MS of partially methylated alditol acetates was carried out on a spectrometer equipped with DB-225 using helium as carrier gas. The temperature program was the same as in GC analysis.

Sulfate content was measured by the turbidimetric method reported by Dodgson and Price [13] with some modifications after hydrolysis of the samples with 1 M HCl for 4 h at 105 °C. In a typical procedure, 4 mL of barium chloride-gelatin reagent was incubated with 1 mL of K_2SO_4 solution at room temperature for 15 min, with substrate concentrations of 0.03, 0.06, 0.12, 0.18, 0.24, and 0.30 mg/mL SO_4^{2-} ion and then measured at 360 nm. Gelatin solution was performed as described above.

The peak molar masses were estimated by gel-permeation chromatography using an Agilent 1260 instrument equipped with a differential refractometer detector in a TSK-gel GM PWXL column (7.8 mm × 30 cm) at 35 °C and a flow rate of 0.5 mL/min. Polysaccharide concentration was 1 mg/mL in 0.2 M NaCl solvent. Dextran samples of Mw 5.21×10^5, 2.89×10^5, 1.1×10^4, 6.06×10^4, 1.26×10^4, and 4.32×10^3 g mol^{-1} were used as standards.

2.5.1.2. Spectroscopy methods. IR spectra were recorded from the polysaccharide powder in KBr pellets using a Fourier transform infrared spectrometer between 400 and 4000 cm^{-1}.

Samples (20 mg) were dissolved in 0.5 mL of D_2O and freeze dried twice to replace all exchangeable protons with deuterium. ^{13}C and ^1H NMR spectra of the polysaccharide were recorded at 35 °C on a ProPulse NMR 500 MHz. A distortionless enhancement by polarization transfer (DEPT 135°) spectrum was recorded to determine the hydrogenation of each carbon. 2D ^1H and ^{13}C HSQC spectra were carried out using the pulse programs supplied with the apparatus. Acetone-d6 was used as the internal standard.

2.5.1.3. Desulfation and methylation analysis. According to the method of Nagasawa [17], desulfation reaction was carried out in 2 mL of DMSO-methanol (9:1, v/v) at 80 °C for 10 h. The desulfated product was recovered by dialysis and then freeze dried.

Methylation analyses of native and desulfated polysaccharide were carried out by the method of Hakomori [18] after converting to pyridinium salts by using ion-exchange resin [19]. The methylated polysaccharide was hydrolyzed, reduced, and acetylated as described above before being subjected to GC–MS analysis.

Table 1
Physical properties of commercial agarose and agar from *G. lemaneiformis* and *G. amansii* before and after enzymatic treatment.

	Commercial agarose	*G. amansii* agar		*G. lemaneiformis* agar	
		before	after	before	after
Sulfate (%)	0.15 ± 0.01^{c}	1.11 ± 0.04^{b}	0.16 ± 0.01^{c}	1.52 ± 0.06^{a}	0.17 ± 0.07^{c}
Gel strength ($g\,cm^{-2}$)	1129 ± 29.70^{a}	484 ± 20.51^{b}	1080 ± 38.19^{a}	442 ± 15.56^{b}	1035 ± 43.13^{a}
Melting temperature (°C)	95.25 ± 0.64^{ab}	97.05 ± 0.35^{a}	93.15 ± 0.92^{b}	96.9 ± 0.28^{a}	94.75 ± 0.49^{ab}
Gelling temperature (°C)	32.05 ± 0.35^{c}	34.15 ± 0.21^{b}	36.50 ± 0.42^{a}	35.80 ± 0.28^{a}	34.00 ± 0.42^{b}

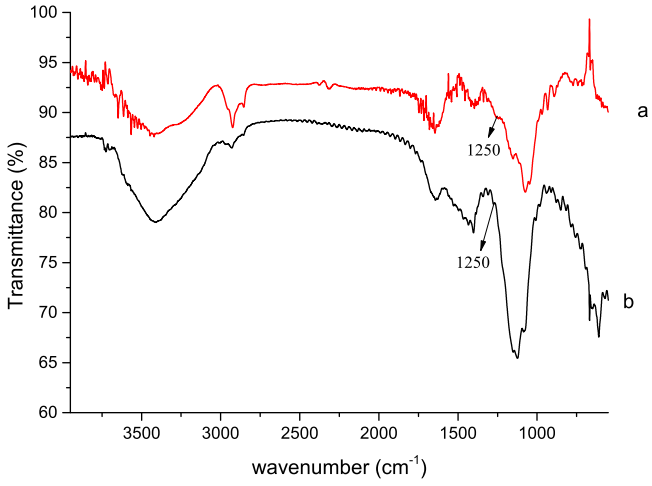

Fig. 1. FT-IR spectra of *Gracilariopsis lemaneiformis* agar before (a) and after (b) enzymatic treatment by the arylsulfatase.

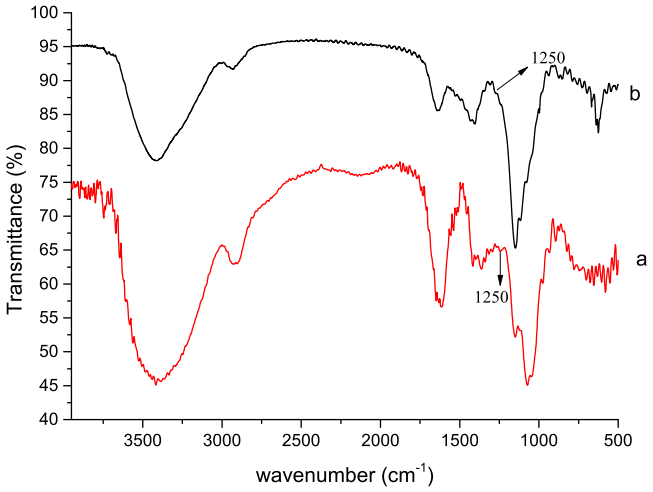

Fig. 2. FT-IR spectra of *Gelidium amansii* agar before (a) and after (b) enzymatic treatment by the arylsulfatase.

2.5.2. Structure of enzymatic treated sulfated galactans

Enzymatic desulfation was carried out by incubating sulfated galactan and arylsulfatase in 100 mM Tris-HCl (pH 9.0) buffer at 45 °C for 12 h. The polysaccharide was precipitated with ethanol (1:3, v/v) and freeze dried. Thereafter, the structure of obtained polysaccharides was analyzed by methylation and acetylation. FT-IR and GC–MS analyses of the desulfated polysaccharide were carried out according to the method described in 2.5.1.

3. Results and discussion

3.1. Application of arylsulfatase in improving agar quality

After enzymatic treatment, the sulfate content of *G. amansii* agar remarkably decreased from 1.11% to 0.16%, whereas that of *G. lemaneiformis* agar remarkably decreased from 1.52% to 0.17%. The sulfate contents of several commercial agarose products purchased from Sigma and Biowest were determined to range within 0.11% to 0.17%. As shown in Table 1, the physical properties like gel strength and sulfate content of the enzymatic treated agar showed significant differences as comparing with those of untreated agar. The gel strength of desulfated agar of *G. amansii* ($1080\,g\,cm^{-2}$) and *G. lemaneiformis* ($1035\,g\,cm^{-2}$) was similar to that of commercial agarose ($1129\,g\,cm^{-2}$) for electrophoresis. Gel strength increased about two folds compared with that of untreated agar. The gelling and melting temperatures of desulfated *G. amansii* agar were 36.5 and 93.2 °C, respectively, and those of *G. lemaneiformis* agar were 34.0 and 94.8 °C, respectively.

The peak of $1250\,cm^{-1}$ in FT-IR spectra was attributed to sulfate groups. The FT-IR spectra (Figs. 1 and 2) indicated that the peak signal in $1250\,cm^{-1}$ significantly decreased after enzymatic treatment using the purified arylsulfatase and the spectra (Figs. 1b, 2b and 3) were similar between enzymatic treated agar products and commercial agarose. The results demonstrated that sulfate groups of *G. lemaneiformis* and *G. amansii* agar could be removed by arylsulfatase and enzymatic treated agar products reached the level of commercial agarose.

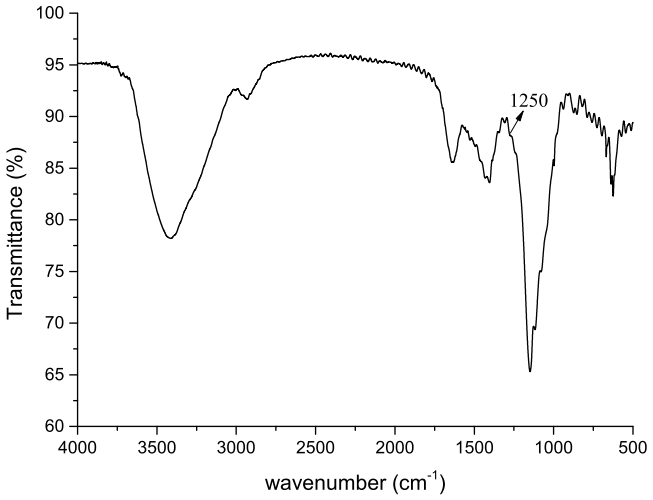

Fig. 3. FT-IR spectrum of the commercial agarose.

Commercial agarose product and *G. amansii* and *G. lemaneiformis* agar before and after enzymatic treatment were applied to agarose gel electrophoresis (Fig. 4). DNA fragments on both gels of enzymatic desulfated agar and commercial agarose showed excellent separation resolution. Hence, the above result indicated that the quality of enzymatic-treated agar was significantly improved by arylsulfatase to values similar to those of commercial agarose.

3.2. General analysis of sulfated galactan

The reductive hydrolysis of GEAP and GC–MS analysis indicated the presence of galactose (58.2%), 3,6-AG (29.8%), 2-*O*-methyl-3,6-

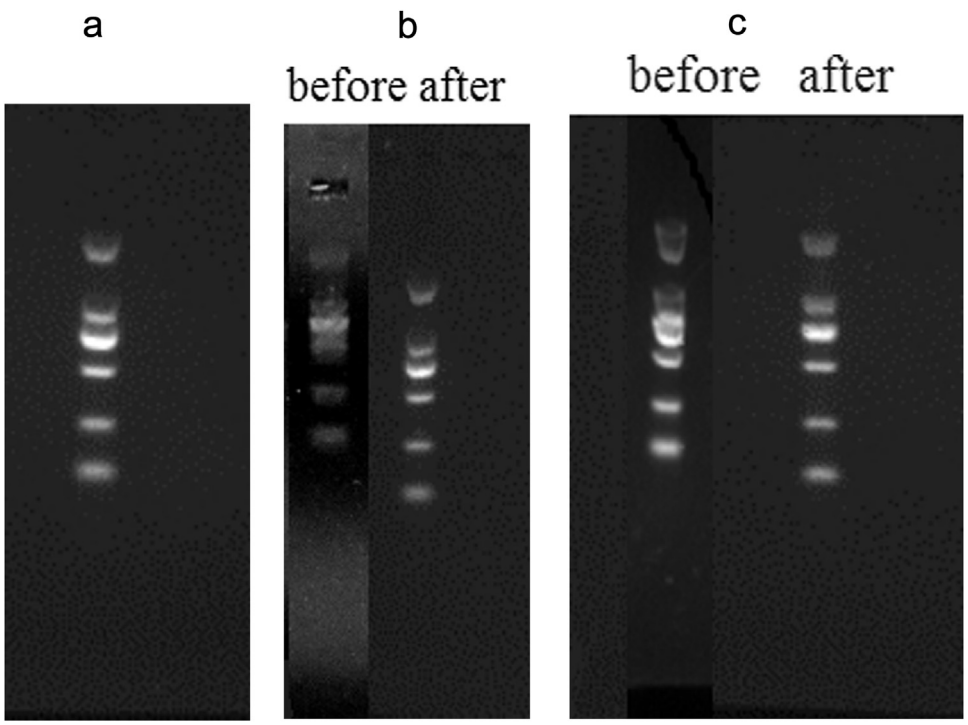

Fig. 4. Agarose gel electrophoresis using the gel of commercial agarose (a)and agar from *Gracilariopsis lemaneiformis*(b) and *Gelidium amansii*(c) before and after enzymatic treatment by the arylsulfatase.

AG (8.5%), and xylose (3.5%). The monosaccharide composition of GRAP was galactose (66.1%) and 3,6-AG (33.9%). The chemical analysis and monosaccharide composition of sulfated polysaccharide are shown in Table 2.

Peaks at 14.502 and 19.227 min of GEAP and at 16.100 and 16.269 min of GRAP were observed in the gel-permeation chromatogram. Both sulfated galactans behaved as a heterogeneous system similar to polysaccharides from *Gracilaria birdiae* [20], *Gracilaria Cornea* [21] and *Botryocladia occidentalis* [22]. The molar masses of the two peaks of GEAP were estimated to be 7×10^4 and 1780 g mol^{-1}, respectively, and those of GRAP were 5×10^5 and 1870 g mol^{-1}, respectively. Polysaccharides were polydisperse, meaning that they generally did not have sharply defined molecular weights, but rather average molecular weights representing a distribution of molecular species nearly identical in structure but varying in chain length [23].

Despite similar sulfate contents and molecular masses, the monosaccharide composition of GEAP was more complex than GRAP. As shown in Table 2, the monosaccharide composition and quantities of major monosaccharide of galactose and 3,6-AG were generally variable depending on species [20,24].

3.3. Structure analysis of sulfated galactose

3.3.1. FT-IR analysis

The FT-IR spectra of GRAP and GEAP are shown in Figs. 5 and 5, respectively. Data revealed the characteristic bands of agar-type polysaccharides (1250, 1072, 931, and 893 cm^{-1}) [20,25]. The absorbance band at 1250 cm^{-1} was attributed to the asymmetric stretching of the sulfate ester group [21,26]. The regions around 1070 and 890 cm^{-1} were equivalent to the skeleton of galactan and agar specific band, respectively [27]. The band at 930 cm^{-1} was assigned to the vibration of the C—O—C bridge in 3,6-AG [28]. The position of the sulfate group of agar-type polysaccharide was identified by the bands at 800–850 cm^{-1} [29]. Several references revealed that the bands at 850 and 830 cm^{-1} were attributed to the 4-O-sulfate and 2-O-sulfate groups, respectively, in d-galactose units, whereas the signals at 820 and 805 cm^{-1} were due to sulfate groups located at C-6 of d-galactose and at C-2 of 3,6-AG, respectively [21,25,27].

The presence of an imperceptible shoulder close to 850 cm^{-1} indicated a small degree of substitution at C-4 of d-galactose sugar units. Several sulfated galactans from *Gracilariopsis* and *Gelidium*

Table 2
The chemical analysis and monosaccharide composition of sulfated polysaccharide.

Species	Molecular mass (g mol^{-1})		Sulfate (%)	Monosaccharide composition (%)				
				Gal	3,6-AG	Methyl-galactose	Glucose	Xylose
G. lemaneiformis[a]	4.8×10^5	1870	8.5	66.1	33.9	–	–	–
G. amansii[a]	7.3×10^4	1780	7.4	58.2	29.8	8.5	–	3.5
G. birdiae[b]	2.6×10^6	3.7×10^5	8.4	65.4	25.1	9.5	–	–
G. persica[c]	2.22×10^5		–	52.1	31.8	10.9	4.1	1.1

[a] This study.
[b] Reported by Souza et al., 2012.
[c] Reported by Salehi et al., 2011.

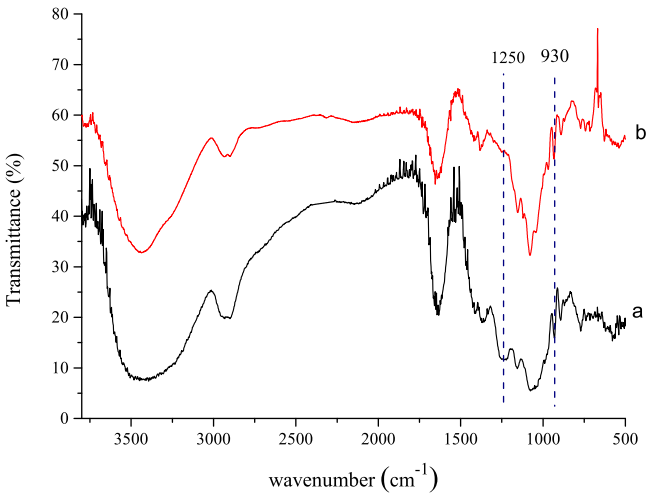

Fig. 5. FT-IR spectra of *Gracilariopsis lemaneiformis* sulfated galactan before (a) and after (b) enzymatic treatment by the arylsulfatase.

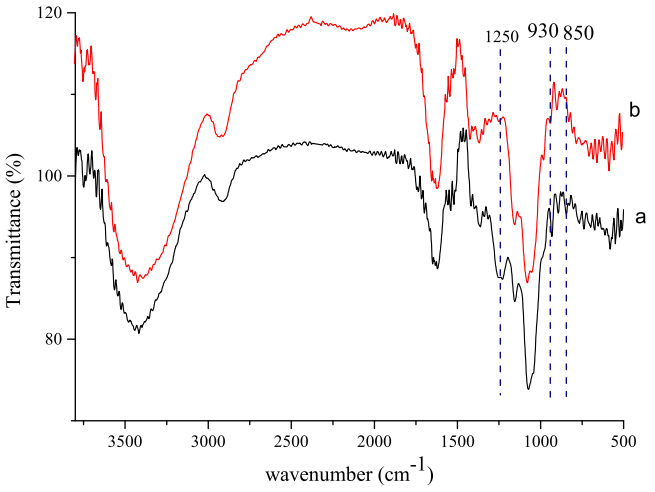

Fig. 6. FT-IR spectra of *Gelidium amansii* sulfated galactan before (a) and after (b) enzymatic treatment by the arylsulfatase.

were found to possess these sulfation patterns [20,24,25,28]. GEAP and GRAP did not show signals resolved at 805 cm^{-1}, indicating the absence of a sulfate group at C-2 of 3,6-AG.

3.3.2. NMR spectroscopy

NMR spectroscopy is a convenient method of obtaining valuable composition information on red seaweed polysaccharides [30]. In the present work, 1D and 2D NMR analyses were used to investigate polysaccharide structure. The NMR spectra of GRAP and GEAP are shown in Figs. 7 and 8, respectively. The ^{1}H NMR spectra of GRAP and GEAP are depicted in Figs. 7a and 8a, respectively. The signals from α anomeric proton at δ 5.13 and 5.27 were assigned to LA and α-L-galactose-6-sulfate (L6S), respectively [31]. H-1 of β-D-galactose (G′) was linked to L6S, and that of G was linked to LA at δ 4.43 and 4.54, respectively [24]. Fig. 7a shows an intense resonance signal at δ 1.44, attributed to methyl protons of the cyclic pyruvate acetal as 4,6-*O*-(1-carboxyethylidene) group, together with a signal at δ 5.20, which was described as H-1 of the l-galactose residue linking to a pyruvated d-galactose residue [28,32]. This spectrum also revealed a methylation pattern with signal resolution at δ 3.50 for protons of methyl groups attached onto O-2 of 3,6-α-L-anhydrogalactose.

The observed resonances were consistent with an alternating structure of the agaran type in the ^{13}C NMR spectrum (Figs. 7b and 8b) of both sulfated galactans, whereas the absence of any anomeric signal below 98 ppm indicated the lack of 4-linked d-galactose residues [33]. Four main signals were found in the anomeric region of ^{13}C NMR (δ 90–110) of GRAP (Fig. 7b), which were assigned based on the data reported by Kolender and Matulewicz [33]. The signal from C-1 at δ 98.2 was assigned to LA, whereas the signal at δ 101 was attributed to L6S. The signals at δ 102.08 and 103.13 were assigned to C-1 of G and G′, respectively. The above signals were also observed in GEAP (Fig. 8b). In addition, the signal at δ 98.42 was attributed to C-1 of the l-galactose residue linked to a pyruvated d-galactose residue.

DEPT 135° experiments were performed to investigate the presence of oxymethylene groups, considering that the pulse sequence signals of carbons bearing two protons had opposite amplitude to the CH and CH_3 carbons [25]. The DEPT 135° spectrum of GRAP (Fig. 7c) showed five CH_2 signals at δ 69.2, 67.66, 67.42, 61.52, and 61.2, which were attributed to LA, L6S, 6-*O*-sulfate-D-galactose (G6S), G′, and G, respectively. In the spectrum of DEPT 135° of GEAP (Fig. 8c), two new peaks were observed apart from the above signals. A signal at δ 65.4 was assigned to a pyruvated d-galactose residue and a small signal at (δ 59) of O—CH_3, indicating that a low amount of O-methyl sugar residues were present in GEAP.

To assign the spin system for GRAP and GEAP, the 2D NMR technique was used. The HSQC spectra showed the correlation of anomeric carbons with their respective protons, i.e., *δ* 98.2/5.13 for LA residue, *δ* 102/4.54 for G residue, *δ* 101/5.27 for L6S residue, and *δ* 103.3/4.4 for G′ residue. A correlation for carbon at *δ* 59/3.4 was observed in the HSQC spectrum of GEAP (Fig. 8d), which indicated the presence of methoxyl group in the galactose residue. Similarly, *δ* 25.4/1.44 indicated the presence of pyruvated d-galactose residues.

2D COSY (Figs. 7e and 8e) was used to determine the proton resonance sequence. The anomeric resonance at 4.54 ppm in the first spin system gave easily identifiable correlations from H-1 to H-2–H-6 of G in the ^{1}H–^{1}H COSY spectrum which demonstrated the presence of several spin systems. For example, the coupling constants between H-1 (*δ* 4.54 ppm) and H-2 (*δ* 3.61 ppm), H-2 and H-3 (*δ* 3.77 ppm) of G were observed. The anomeric proton at 5.13 ppm

Table 3
The chemical shifts for residues of sulfated galactan from *G. lemaneiformis*.

Residue	nucleus	1	2	3	4	5	6
LA	^{13}C	98.20	69.50	79.81	77.14	75.31	69.13
	^{1}H	5.13	4.10	4.55	4.65	4.55	4.19
G	^{13}C	102.12	69.94	81.95	68.50	75.17	61.12
	^{1}H	4.54	3.61	3.77	4.11	3.72	3.80
L6S	^{13}C	101.02	69.82	71.0	79.79	70.55	67.59
	^{1}H	5.27	3.85	3.92	–	–	4.27
G’	^{13}C	103.39	70.73	81.95	68.78	75.68	61.45
	^{1}H	4.40	3.72	–	–	–	4.30
G6S	^{13}C	103.20	69.82	81.95	68.20	73.29	67.35

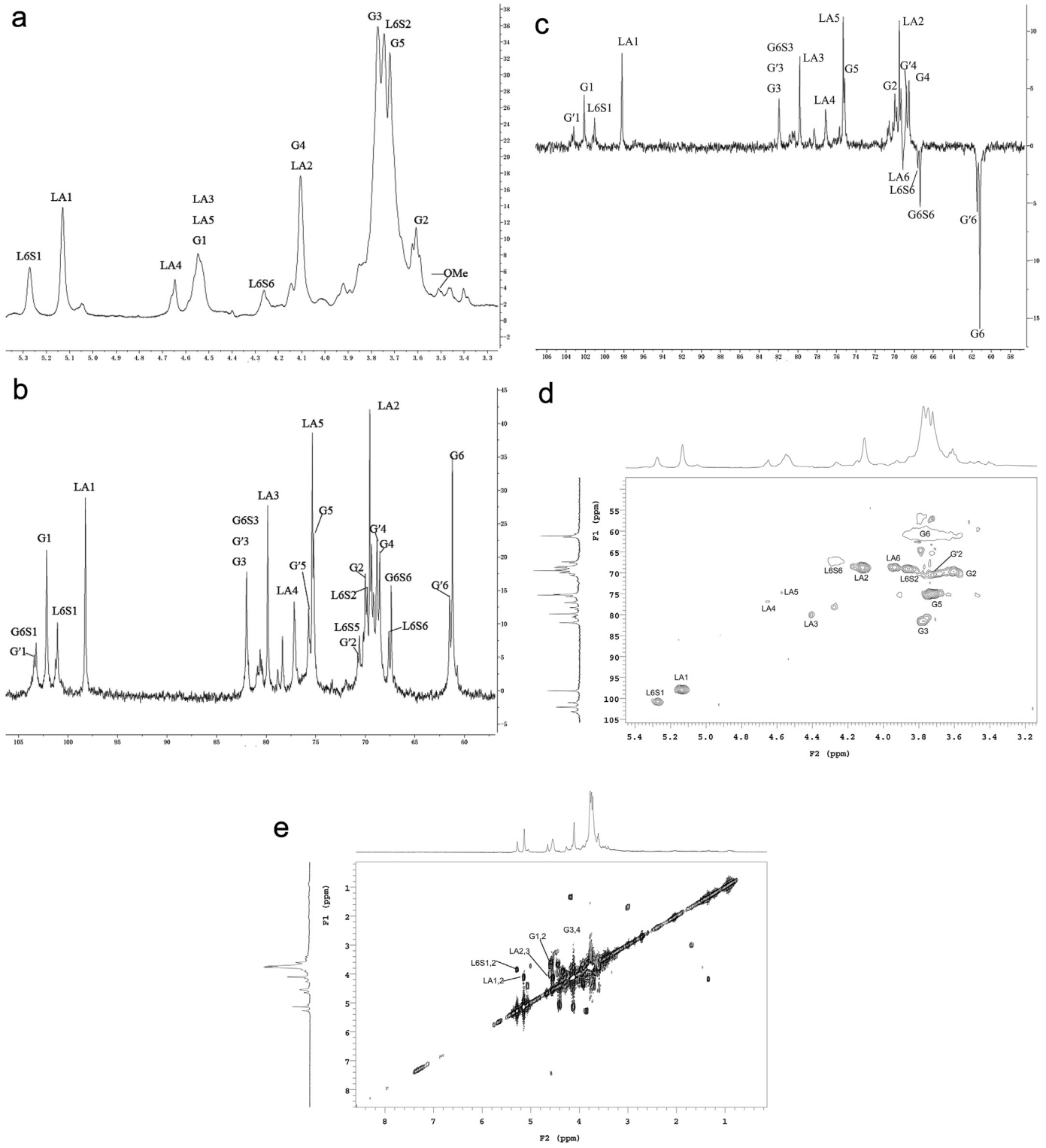

Fig. 7. NMR spectra of *Gracilariopsis lemaneiformis* in D_2O. (a) ^1H NMR spectrum; (b) ^{13}C NMR spectrum; (c) DEPT 135° spectrum; (d) HSQC spectrum; (e) Cosy spectrum.

was the start signal of the next spin system corresponding to H-2 (δ 4.10 ppm) of LA residue. In addition, the H-2 signal was correlated to H-3 at δ 4.55 ppm. The H-2 protons assigned using the COSY spectrum showed a correlation on the HSQC spectrum with the C-2 atom (C-2 of LA δ 69.57, C-2 of L6S δ 69.76, and C-2 of G δ 70.0). Moreover, for L6S, H-1/H-2 was at δ 5.274/3.85 and H-3/H-4 at δ 3.94/4.38. Based on 1D and 2D NMR spectra and literature data, the signal assignments of sulfated galactans from *G. lemaneiformis* and *G. amansii* are listed in Tables 3 and 4, respectively.

3.3.3. *Methylation analysis*

To confirm glycoside linkage position and identify sulfate-group location on GEAP and GRAP, methylation was performed before and after desulfation by DMSO-MeOH. Results of methylation analysis

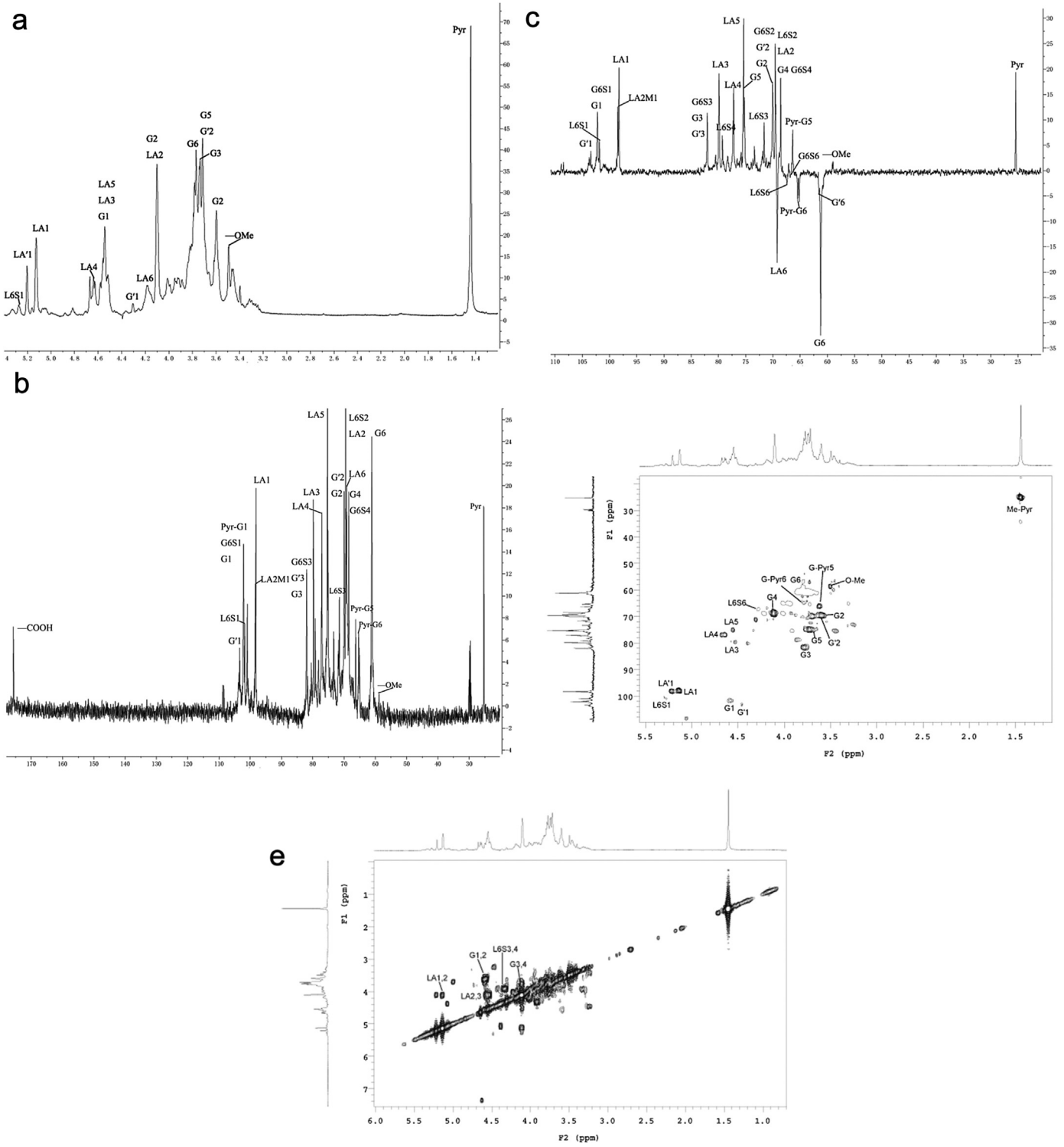

Fig. 8. NMR spectra of *Gelidium amansii* in D_2O. (a) ^1H NMR spectrum; (b) ^{13}C NMR spectrum; (c) DEPT 135° spectrum; (d) HSQC spectrum; (e) Cosy spectrum.

of native and desulfated sulfated galactans are shown in Table 5, which agreed with NMR spectra.

For 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methyl-galactitol (1,4,5-Ac_3-2-Me-3,6-AG) and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol (1,3,5-Ac_3-2,4,6-Me_3-G), their contents significantly increased before and after desulfation. Conversely, the amount of some derivatives related to sulfate groups such as 1,4,5,6-Ac_4-2,3-Me_2-G, 1,3,4,5-Ac_4-2,6-Me_2-G, and 1,3,5,6-Ac_4-2,4-Me_2-G decreased. Hence, sulfate groups of both sulfated galactans were situated at C-4 and C-6 positions of 3-linked d-galactose (G4S and G6S) and C-6 of 4-linked l-galactose (L6S).

IR, NMR, and GC–MS results further revealed the structure of sulfated galactans from *G. lemaneiformis* and *G. amansii* (Figs. 9a and b).

Table 4
The chemical shifts for residues of sulfated galactan from *G. amansii*.

Residue	nucleus	1	2	3	4	5	6	–OMe/-COOH
LA	^{13}C	98.20	69.49	79.80	77.13	75.30	69.13	
	^{1}H	5.13	4.11	4.55	4.65	4.55	4.19	
G	^{13}C	102.12	69.96	81.94	68.49	75.10	61.12	
	^{1}H	4.55	3.60	3.78	4.11	3.72	3.80	
L6S	^{13}C	101.77	69.49	71.53	79.18	70.05	67.33	
	^{1}H	5.28	3.82	3.93	–	–	4.27	
G'	^{13}C	103.36	69.96	81.94	68.76	75.39	61.50	
	^{1}H	4.47	3.71	–	–	–	4.30	
G6S	^{13}C	102.12	69.96	81.94	68.49	73.30	66.72	
LA2M	^{13}C	98.40	78.20	78.10	77.24	75.16	69.13	^{13}C:58.80 ^{1}H:3.50
Pyr-G	^{13}C	102.12	69.96	79.88	71.77	66.30	65.37	^{13}C:25.44;175.62 ^{1}H:1.445

Table 5
Methylation analysis of native and desulfated polysaccharides from *G. lemaneiformis* and *G. amansii*.

Derivative	Linkeages	Proportion (%)					
		GRAP	dsGRAP[a]	dsGRAP[b]	GEAP	dsGEAP[a]	dsGEAP[b]
1,4,5-Ac_3-2-Me-3,6-AG	→4)AnG(1→	29.5	31.4	32.9	30.4	34.3	35.7
1,3,5-Ac_3-2,4,6-Me_3-G	→3)G(1→	55.5	63.1	62.2	57.5	62.4	60.9
1,4,5-Ac_3-2,3,6-Me_3-G	→4)G(1→	0	2.7	2.3	0	1.1	1.2
1,3,4,5-Ac_4-2,6-Me_2-G	→3)G4S(1→	3.0	1.2	0.8	1.9	0.6	0.7
1,4,5,6-Ac_4-2,3-Me_2-G	→4)L6S(1→	8.1	0.7	1.1	7.5	0.3	0.5
1,3,5,6-Ac_4-2,4-Me_2-G	→3)G6S(1→	3.9	0.9	0.7	2.7	1.3	1.0

[a] Polysaccharide treated by DMSO-MeOH.
[b] Polysaccharide treated by the arylsulfatase.

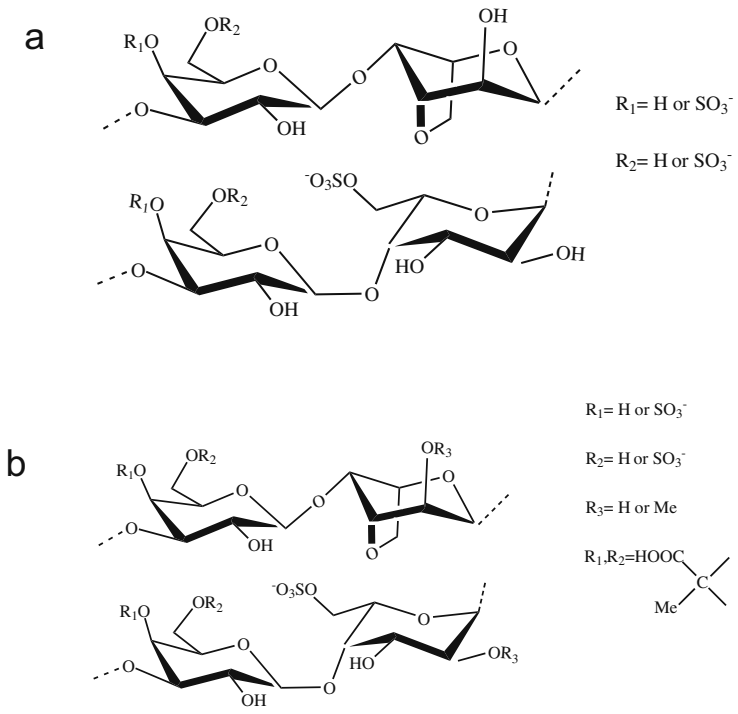

Fig. 9. Polysaccharides Structure. (a) *Gracilariopsis lemaneiformis*sulfated galactan; (b) *Gelidium amansii* sulfated galactan.

3.4. Structure analysis of desulfated galactan and desulfation mechanism by arylsulfatase

After enzymatic treatment, sulfate content was measured by the turbidimetric method. Results indicated that the sulfate content of GEAP remarkably decreased from 7.4% to 1.26% with a desulfation ratio of 82.97%, and that of GRAP remarkably decreased from 8.48% to 1.19% with a desulfation ratio of 86.32%.

As shown in Figs. 5 and 6, the FT-IR peak of 1250 cm^{-1} attributed to the asymmetric stretching of sulfate ester group significantly decreased after enzymatic treatment by arylsulfatase. The absence of band at 850 cm^{-1} indicated that sulfate groups of galactose were eliminated. The above results demonstrated that *Marinomonas* arylsulfatase was an efficient catalyst for removing sulfate groups in sulfated galactans from *G. lemaneiformis* and *G. amansii*. Methylation was performed before and after desulfation by arylsulfatase,

and the products were analyzed by GC–MS. Results of methylation analysis of polysaccharides before and after enzymatic desulfation are given in Table 5.

Significant changes in 1,4,5-AC_3-2-Me-3,6-AG, 1,3,5-Ac_3-2,4,6-Me_3-G, and 1,4,5,6-Ac_4-2,3-Me_2-G contents before and after desulfation were observed in the GC–MS results of both GEAP and GRAP. Methylation analysis of desulfation GRAP showed an increase in 1,3,5-Ac_3-2,4,6-Me_3-G together with a concomitant decrease in 1,3,4,5-Ac_4-2,6-Me_2-G and 1,3,5,6-Ac_3-2,4-Me_2-G, which suggested that the sulfate groups of G4S and G6S were removed by arylsulfatase. Furthermore, given the increase in 1,4,5,6-Ac_4-2,3-Me_2-G content in the premethylated product after desulfation, sulfate groups elimination of L6S were present.

Considering the increase in 1,4,5-Ac_3-2,3,6-Me_3-G content after enzymatic treatment, not all L6S (3,6-AG precursor) were converted to 3,6-AG during enzymatic treatment. Results of methylation analysis for GRAP were similar to those for GEAP, so the desulfation mechanism of arylsulfatase against agar of *G. lemaneiformis* was similar to that of *G. amansii*.

Alkaline treatment is a traditional method widely used in the agar-related industry to remove primary sulfate groups, especially at C-6 position of the galactopyranose unit and to consequently produce 3,6-anhydrogalactose derivatives [10]. Apart from chemical method, enzymatic desulfation can reportedly improve agar quality. Shukla et al. [34] reported that after the treatment of *G. dura* agar by sulfohydrolase, sulfate content decreased, whereas the amount of 3,6-anhydrogalactose was increased. Furthermore, enzymatic desulfation improved gel strength because its value increased from 190 g cm^{-2} to 470 g cm^{-2}. SEM images signified that the enzymatic-desulfated agar was a morphologically distinct agar product with a much stronger network of ordered helical structure than the control agar. After sulfohydrolase reaction, the helical structure of agar cross-linked with one another to form a thick helix. Kim et al. [7] reported that after the treatment of agar by arylsulfatase from *Sphingomonas*, sulfate content decreases from 3.9% to 0.09%, whereas gel strength increases from 462 g cm^{-2} to 1128 g cm^{-2}. These two studies indicated that enzymatic desulfation could improve agar quality. However the changes of structure of agar before and after enzymatic treatment were not studied. In the present work, GC–MS and FT-IR results showed that sulfate groups located at C-6 of α-L-galactose could be removed by arylsulfatase, and parts of formed l-galactose were converted to 3,6-AG. The above conversion procedure was similar to that of alkaline treatment. Superior to alkaline treatment, this arylsulfatase also showed excellent activity for removing sulfate groups located at C-4 and C-6 of d-galactose. Sulfate groups at C-6 or C-4 were stable in alkali [35]. After sulfate-group removal, the helical structure became more regular and intermolecular electrostatic repulsion decreased, thus a much stronger network was formed and the gel strength of agar was greatly improved.

4. Conclusion

Superior to alkaline treatment, arylsulfatase from *Marinomonas* sp. FW-1 showed excellent activity for removing sulfate groups located at C-6 of α-L-galactose and at C-4 and C-6 of β-D-galactose insulfated galactans of the red seaweeds *G. lemaneiformis* and *G. amansii*. The enzymatic treated agar showed characteristics similar with those of commercialized agarose. Our findings revealed the enzymatic desulfation mechanism of sulfated galactan by arylsulfatase and provided an enzymatic processing methodology for agarose production.

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