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Carrageenan from *Sarconema scinaioides* (Gigartinales, Rhodophyta) of Indian waters

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ABSTRACT

Carrageenan was extracted from the red seaweed *Sarconema scinaioides* of Indian waters and was characterized. The crude carrageenan as well as its alkali modified derivative was composed of 3,6-anhydro galactose, 6-*O*-methyl galactose as well as galactose moieties in various proportions. Linkage analysis exhibited that these two carrageenan samples consisted of 4-linked 3,6-anhydrogalactose residue sulphated at position 2, and 3-linked galactose residue sulphated at position 4. The physicochemical and rheological data along with molecular weight data, FT-IR, 1D and 2D NMR (^{1}H, ^{13}C, COSY and HSQC) spectrometry suggested that the polysaccharide was composed predominantly of iota- along with a small amount of its precursor nu (ν)-carrageenan, unlike the hybrid carrageenans (iota-, pyruvated- and kappa-carrageenans) from this seaweed reported in the literature. This Indian seaweed species would be a potentially important source of iota-carrageenan.

1. Introduction

Sulphated galactans, agars and carrageenans are the main cell wall components of the red seaweeds, are composed of repeating dimeric unit of (1 → 3)-linked β-galactopyranose (Gal*p*) and (1 → 4)-linked 3,6-anhydro α-Gal*p* (Craigie, 1990). The (1 → 4)-linked galactose units have L and D configuration in agar and carrageenans, respectively (Rees, 1969). The number and position of sulphated ester (S), (1 → 3)-linked β-galactopyranose (G unit) and the occurrence of 3,6-anhydro bridges in the (1 → 4)-linked residues (DA unit) determine the type of the carrageenan unit (Knutsen, Myslabodski, Larsen, & Usov, 1994). The presence of one, two and three ester-sulphate groups per repeating disaccharide unit is the characteristic feature of three main carrageenans *viz.* kappa-(κ, DA–G4S), iota-(ι, DA2S–G4S), and lambda-(λ, D2S,6S–G2S), respectively (Knutsen et al., 1994). Besides sulphated esters and 3,6-anhydro bridges, red algal galactans may also bear pyruvate acetal substitutions as well as glycosyl substitutions, most commonly known as single xylopyranosyl (Xyl*p*) or mono-*O*-methyl galactopyranosyl (Me-Gal*p*) residues branching out from the main galactan chain (Chiovitti et al., 1997; Falshaw et al., 1996; Painter, 1983; Usov, 1998). The extraction procedures of the polysaccharides, seaweeds sources and life stages, influence the structural complexity and hybrid nature of the carrageenans (Bixler, 1996; Craigie, 1990; Knutsen et al., 1994; Van de Velde, Peppelman, Rollema, & Tromp, 2001). The presence of biosynthetic precursor *e.g.* mu-(μ, D6S–G4S, κ-carrageenan precursor) and nu-(ν, D2S,6S–G4S, ι carrageenan precursor) also hinders the gelification of the respective carrageenans (Bellion, Brigand, Prome, Welti, & Bociek, 1983; Van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002). Varieties of industrial and laboratory applications (*e.g.* stabilising and viscosity building agent, etc.) are accounted for these carrageenans due to their viscous nature as well as gelling properties (Villanueva, Mendoza, Rodrigueza, Romero, & Montaño, 2004). κ-Carrageenan forms hard gels with KCl solution which are strong and brittle, whereas ι-carrageenan forms soft and weak gels that are shear reversible (Campo, Kawano, Silva, & Carvalho, 2009; Villanueva et al., 2004). Recently hybrid carrageenans have attracted attention of the researchers world over because of its potential in industrial applications especially in the food and personal care industries (De Vries, 2002; Piculell, 1995). Several red seaweeds have been explored for the presence of κ/ι-hybrid carrageenans in relation to their functional properties along with their carrageenan content and characterized to get novel gelling properties and for providing new resources both for studying structure–property relationships as well as for exploring their commercial applications (Chiovitti, Kraft, Bacic, & Liao, 2001).

The red marine alga *Sarconema scinaioides* Børgesen (Gigartinales, Phylum Rhodophyta), having remiform and cylindrical body, occurs naturally in mid littoral zone, tide pools of Indian coasts in a sporadic distribution (Jha, Reddy, Thakur, & Rao, 2009). This seaweed species occurs at the Indian coast along with other various

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marine algal species indicating that this species is well adopted in its habitat. Carrageenans extracted from *S. scinaioides* have been earlier reported as hybrid of κ/ι carrageenans containing pyruvated moieties on the basis of ^{1}H and ^{13}C NMR data (Van de Velde et al., 2005). The aim of this study is the characterization of the carrageenan extracted from *S. scinaioides* of Indian waters in an ongoing program on value addition of seaweeds.

2. Materials and methods

2.1. Seaweed and chemicals

S. scinaioides (Gigartinales, Rhodophyta) was collected from Veraval (20.54°N, 70.20°E), Gujarat, West coast of India in January 2007. The seaweed thalli were washed with sea water to remove impurities and it was air dried. The voucher specimen (AL-II-104-03) was deposited with the herbarium of the CSMCRI, Bhavnagar. Borane 4-methyl morpholine complex (MMB), iota carrageenan (Type V; from *Eucheuma spinosa*), and α-amylase (from *Bacillus amyloliquefaciens*, Enzyme Code 3.2.1.1) were purchased from Sigma–Aldrich. Other chemicals used in this study were of AR grade except for isopropyl alcohol (LR grade), which was purchased from M/s S D Fine Chemicals, Mumbai.

2.2. Extraction of sulphated polysaccharide

Dried seaweed (50 g) was soaked in demineralized (DM) water for 1 h followed by extraction with 0.05 M $NaHCO_3$ (1:30, w/v) at 110 °C for 2 h in an autoclave (Craigie & Leigh, 1978). The cooked seaweed was then homogenized, centrifuged and sulphated polysaccharide (SS_{Native}) was isolated by precipitation from the supernatant with isopropyl alcohol (1:2, v/v). The sulphated polysaccharide (SS_{Native}) was treated with α-amylase to digest floridean starch present (detected by iodine test, GC–MS and ^{13}C NMR), reheated, centrifuged then dialysed and precipitated with isopropyl alcohol (1:2, v/v) to get SS_{Crude} (Falshaw & Furneaux, 1998). SS_{Crude} was alkali modified using $NaBH_4$/NaOH system to give SS_{AM} (Falshaw & Furneaux, 1998; Viana, Noseda, Duarte, & Cerezo, 2004).

2.3. Desulphation of SS_{AM}

Solvolytic desulphation of pyridinium salt of SS_{AM} was done according to the method described by Falshaw and Furneaux (1998). Briefly, the pyridinium salt of SS_{AM} (200 mg) was dissolved in 100 ml of a mixture of anhydrous DMSO–MeOH–Pyridine (89:10:1) and heated at 100 °C for 4 h. After completion of the reaction the content was cooled to room temperature, distilled water (100 ml) was added and the mixture was dialysed overnight against distilled water and freeze-dried to afford desulphated polysaccharide (SS_D).

2.4. Chemical analysis

Total carbohydrate content was determined by the method described by Dubious, Gilles, Hamilton, Rebers, and Smith (1956). Sulphate contents were estimated on a Perkin-Elmer inductively coupled plasma-emission spectrophotometry (ICP)-OES Optima 2000DV machine following the method reported in the literature (Wolnik, 1998). The sulphur content (%), which was obtained from the experiment, was multiplied by 3 to get sulphate content (%). All analyses were done in triplicate and the mean values were considered. Apparent viscosities of SS_{Crude} (1.0%, w/v) and iota-carrageenan in 1% salt solutions (KCl and $CaCl_2$) were measured on a Brookfield viscometer (DV II + Pro), using LV3 spindle at 30 rpm at 80 °C. The flow behaviour (0–1000 s^{-1} shear rate), frequency dependence G' (at constant 10% strain) and strain dependence G' and G'' (at constant 1.0 Hz frequency), of the SS_{Crude} and iota-carrageenan in 1% salt solutions (1%, w/v) were studied on an Anton-Paar Physica MCR 301 Rheometer, Germany, employing plate/plate geometry (50 mm diameter) at 25 °C (Kumar, Mehta, Prasad, Meena, & Siddhanta, 2011).

2.5. Monosaccharide analysis

Monosaccharide composition in SS_{Native}, SS_{Crude}, SS_{AM} and SS_D was determined quantitatively as their peracetylated alditols obtained by reductive hydrolysis followed by acetylation as described by Stevenson and Furneaux (1991). The resulting alditol acetates were analyzed by GC–MS on a capillary column (length 25 m, thickness 0.25 μm, diameter 0.22 mm, BP 225 on a Shimadzu GCMS-QP2010 machine) using a temperature program of 160 °C (3 min hold) to 230 °C (10 °C/min), split ratio 1:30 and a flame ionization detector (FID) (Kumar, Mehta, et al., 2011; Kumar, Nandan, Meena, Prasad, & Siddhanta, 2011; Siddhanta, Goswami, Ramavat, Mody, & Maihr, 2001). Quantification was carried out from the peak area calculation. Helium was used as carrier gas at flow rate 1 ml/min.

2.6. Methylation and ethylation analysis

Permethylation of SS_{Native}, SS_{AM} and SS_D was done as described by Ciucanu and Kerek (1984). Partially methylated alditol acetates (PMAAs) were obtained by reductive hydrolysis and acetylation (Stevenson & Furneaux, 1991). The resulting partially methylated alditol acetates (PMAAs) were analyzed by GC–MS using the above mentioned programme. The identifications of PMAAs were done on the basis of retention time and mass fragmentation patterns (Kumar, Nandan, et al., 2011; Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005). To determine the nature of 6-*O*-Me galactose residue, ethylation analysis of SS_{AM} and SS_D was done using ethyl iodide. The resulting partially ethylated alditol acetates (PEAAs) were analyzed by GC–MS using the above mentioned programme (Cases, Stortz, & Cerezo, 1994; Kolender & Matulewicz, 2002; Matsuhiro et al., 2005).

2.7. Molecular weight determination

Molecular weights of SS_{Native}, SS_{Crude}, SS_{AM} and SS_D were determined by high-performance gel-permeation chromatography (HP-GPC) on a Waters HPLC (Waters, MA, USA) equipped with an Ultrahydrogel column, a refractive index detector (model 410) and a Millennium 32 Workstation. A 20 μl aliquot of 0.5% solutions were injected on to the column and 0.1% aqueous $NaNO_3$ was used as the mobile phase with a flow rate 0.6 ml/min (cf. Yamamoto, Nunome, Yamauchi, Kato, & Sone, 1995). Dextrans having different Mws were used as standards.

2.8. FT-IR, ^{1}H, ^{13}C, 2D $^{1}H/^{1}H$ COSY and $^{1}H/^{13}C$ HSQC NMR

FT-IR spectra of SS_{Native}, SS_{Crude}, SS_{AM}, SS_D and iota-carrageenan were recorded on a Perkin-Elmer Spectrum GX (FT-IR System, USA), using the KBr disk method. ^{13}C NMR spectra of SS_{Native}, SS_{Crude}, SS_{AM} and SS_D were recorded on a Bruker Avance-II 500 (Ultra shield) spectrometer, Switzerland, at 125 MHz, in D_2O (50 mg/ml). The ^{13}C NMR spectra were recorded at 70 °C with 6000–6500 accumulations, pulse duration 11.25 μs; acquisition time 1.048 s and relaxation delay 6 μs using DMSO as the internal standard (ca. 39.4 ppm). ^{1}H NMR as well as 2D $^{1}H/^{1}H$ COSY (Homonuclear correlation spectroscopy) and $^{13}C/^{1}H$ HSQC (Heteronuclear Single Quantum Coherence) correlation spectrum analysis of SS_{AM} was recorded on a Bruker Avance-II 500 (Ultra shield) spectrometer,

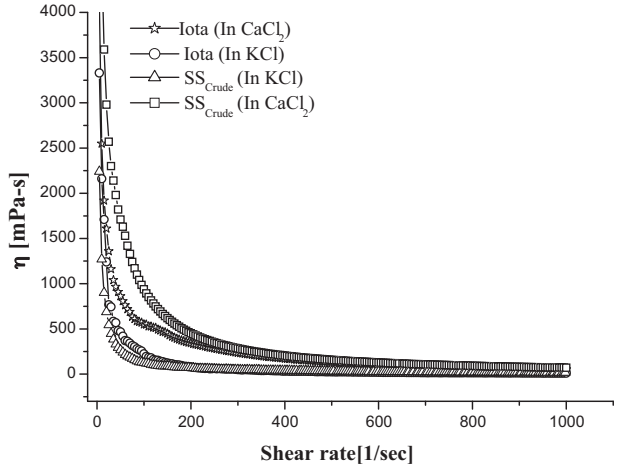

Fig. 1. Flow behaviour of SS_{Crude} and iota carrageenan (1%, w/v) in salt solutions.

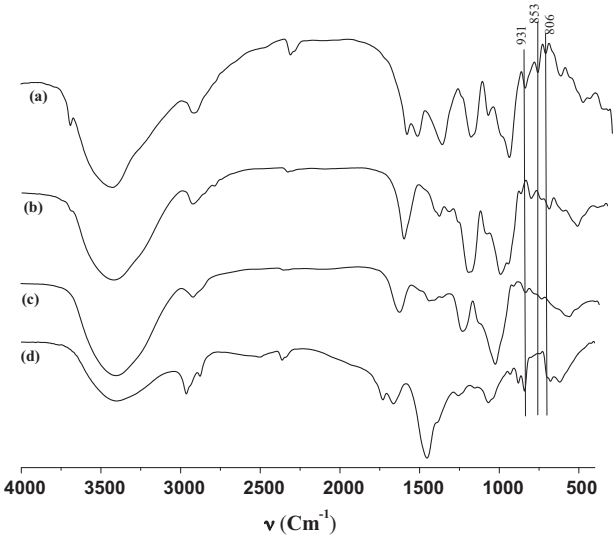

Fig. 2. FT-IR spectra of (a) SS_{Native}, (b) SS_{Crude}, (c) SS_{AM} and (d) SS_D.

Switzerland, at 500 and 125 MHz (for ^1H and ^{13}C, respectively) at 70 °C. Default programs of Bruker were used for COSY and HSQC spectra using DMSO as the internal signal (ca. 2.68 ppm for ^1H and 39.4 ppm for ^{13}C).

3. Results and discussion

3.1. Physicochemical properties

The yields of the native, crude, alkali modified and desulphated carrageenan of *S. scinaioides* were 31.0%, 27.8%, 24.2% and 16.0% with respect to dry seaweed, respectively (Table 1). Their total sugar, sulphate contents as well as weight average molecular weights are presented in Table 1. Further the flow behaviour of SS_{Crude} exhibited reduction of shear viscosity with shear rate in the following order, $KCl < CaCl_2$ indicating existence of a stronger gel network formed due to the cross linking with $CaCl_2$ (Fig. 1). It validates the fact that the carrageenan was of ι-variety, showing greater increase in viscosity and cross-over point of G' and G'' (at constant frequency of 1.0 Hz), in the presence of Ca^{2+} ions compared to K^+ ions (Morris & Belton, 1982; *vide* ESI). High viscosity of SS_{Crude} in the presence of salts makes it potentially useful for various applications.

3.2. Monosaccharide analysis

The GC–MS profile of alditol acetates of SS_{Crude}, SS_{AM} and SS_D revealed that sulphated polysaccharides were mainly composed of galactose, 3,6-AnGal, 6-*O*-methylated galactose along with trace amounts of xylose in various molar proportions (Table 1). The native carrageenan (SS_{Native}) contained galactose, 3,6-AnGal, 6-*O*-methylated galactose along with glucose (22.5 mol%), the latter being solely due to floridean starch (Table 1, Falshaw & Furneaux, 1998; Freile-Pelegrín, Azamar, & Robledo, 2011). Alkali modification of SS_{Crude} resulted in an increase of 3,6-AnGal content by 4.0 mol%, by cyclization of D2S,6S (ν-carrageenan unit) in to DA2S units, associated with the increase (ca. 2.1%) in sulphate content as well as with a decrease in 6-*O*-methyl galactose content (Table 1), presumably because of leach out of polysaccharides during alkaline modification. The ratios of AnGal:Gal, which is assumed to be 1:1 for ideal carrageenan, for SS_{Crude} and SS_{AM} were considerably less, which may be due to the incomplete recovery of AnGal by reductive hydrolysis of ι-carrageenan having sulphate ester substitution at *O*-2 of the AnGal residues, resulting in higher amount of galactose residues (cf. Falshaw et al., 1996; Stevenson & Furneaux, 1991). There was significant amount of 6-*O*-methyl galactose units in all the samples (Table 1).

3.3. Linkages analysis

Linkage analysis of SS_{Native} and SS_{AM} showed the presence of 2,4-linked 3,6-AnGal; 2,4,6-linked Gal; 3,4-linked Gal; 4-linked Gal; and 3-linked Gal units in various molar proportions (Table 2). The main linked units were 2,4-linked 3,6-AnGal and 3,4-linked-Gal accounted for DA2S and G4S of ι-carrageenan units, respectively. The presence of 2,4,6-linked Gal was accounted for D2S,6S units of ν-carrageenan. The presence of significant amount of 4-linked glucose (24.2 mol%) in SS_{Native} was due to floridean starch, which was removed by α-amylase treatment. The other linked galactose units were 4-linked Gal and 3-linked Gal (<5 mol%, Table 2).

The positions of sulphate ester group were determined by the linkages analysis of desulphated derivative SS_D. Linkage analysis of SS_D showed the presence of 4-linked 3,6-AnGal; 2,4-linked 3,6-AnGal; 2,4,6-linked Gal; 3,4-linked Gal; 4-linked Gal and 3-linked Gal units (Table 2). The presence of higher amount of 4-linked 3,6-AnGal and 3-linked Gal contents in SS_D suggested that all the 4-linked 3,6-AnGal units were sulphated at *O*-2 and 3-linked Gal were sulphated at *O*-4, as found in DA2S and G4S units of the ι-carrageenan. The presence of 4-linked Gal units was attributed to the desulphated derivative of D2S,6S units of ν-carrageenan. Other linked minor sugar units were 2,4-linked 3,6-AnGal and 3,4-linked Gal (<4 mol%; Table 2). The ethylation analysis of SS_{AM} and SS_D revealed that all the 6-*O*-Me residues were attached to C-6 to 3-linked Gal residues (ESI Table S4).

3.4. FT-IR analysis

The appearance of the strong IR band at 1255 cm^{-1} indicated the presence of sulphated ester (Fig. 2 and ESI), the bands at 931, 806, and 853 cm^{-1} confirmed the presence of 3,6-AnGal, axial sulphate ester at *O*-2 of 4-linked 3,6-AnGal and sulphate ester at *O*-4 of 3-linked Gal residues, respectively (Matsuhiro & Rivas, 1993; Pereira, Sousa, Coelho, Amado, & Ribeiro-Claro, 2003; Yermak et al., 2006). Other characteristic bands at 1637, 1154, 1079, and 1024 cm^{-1} were due to the bending of water molecule, C–O–C stretching of 6-*O*-methylated Gal, C–O stretching of secondary and primary alcohols of pyranose ring residues, respectively (Kacuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000; Matsuhiro & Rivas, 1993;

Table 1
Physicochemical properties of native, crude, alkali modified and desulphated carrageenan of *Sarconema scinaioides*.

Extract	Yield[a] (%)	Total sugar[b] (%)	Sulphate[b] (%)	Mw ($\times 10^5$)	Monosaccharide compositions				
					Xyl (mol%)	3,6-AnGal (mol%)	6-*O*-Me Gal (mol%)	Gal (mol%)	Glc (mol%)
SS_{Native}	31.0	45.4	25.1	9.5	2.3	18.9	15.1	41.2	22.5
SS_{Crude}	27.8	46.1	26.2	12.5	3.2	24.4	12.7	59.7	–
SS_{AM}	24.2	47.2	28.3	5.1	–	28.4	9.3	62.3	–
SS_D	16.8	ND	ND	1.5	–	22.2	10.8	67.0	–

[a] Yield was calculated on the basis of as received dry seaweeds; and are the mean values of the three replicates.
[b] Yield is with respect to polysaccharide.
ND, not done.

Table 2
Linkage analysis of SS_{Native}, SS_{AM} and SS_D.

Sugar	PMAA of sugar	Deduced linkage	SS_{Native} (mol%)	SS_{AM} (mol%)	SS_D (mol%)
3,6-Anhydro Gal*p*	1,4,5-Tri-*O*-acetyl-2-*O*-methyl-3,6-anhydrogalactitol	→4)-3,6-Anhydro Gal*p* (1→	–	–	24.9
	1,2,4,5-Tetra-*O*-acetyl-3,6-anhydrogalactitol	→2,4)-3,6-Anhydro Gal*p* (1→	20.5	34.8	1.3
Galactose including	1,2,4,5,6-Penta-*O*-acetyl-3-*O*-methyl-galactitol	→2,4,6)-Gal*p* (1→	2.9	1.2	–
6-*O*-methyl galactose	1,3,4,5-Tetra-*O*-acetyl-2,6-di-*O*-methyl-galactitol	→3,4)-Gal*p* (1→	48.1	57.4	3.6
	1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol	→4)-Gal*p* (1→	4.3	3.8	5.8
	1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol	→3)-Gal*p* (1→	–	2.8	64.4
Glucose	1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol	→4)-Glc*p* (1→	24.2	–	–

Pereira et al., 2003). The absence of the band at 897 cm^{-1} confirmed that there was no pyruvate acetal substitution on the galactose residues (Chiovitti et al., 1997; Kumar, Mehta, et al., 2011). The presence of sulphate group at *O*-6 of 4-linked galactose units of ν-carrageenan was observed at 869 cm^{-1} (Tuvikene et al., 2010). Therefore, the presence of IR bands at 931, 853 and 806 cm^{-1} confirmed that galactan obtained from *S. scinaioides* was indeed ι-carrageenan along with its precursor ν-carrageenan (Chiovitti et al., 1997; Matsuhiro & Rivas, 1993; Pereira et al., 2003; Yermak et al., 2006).

3.5. ^1H, ^{13}C, 2D ^1H COSY and $^1H/^{13}C$ HSQC NMR analysis

The ^1H NMR spectrum of SS_{AM} exhibited the anomeric proton signals at 4.85, 5.33 and 5.50 ppm of G4S, DA2S (of iota carrageenan) and D2S,6S (of ν carrageenan), respectively (Pereira & Van de Velde, 2011; Villanueva, Montaño, & Romero, 2009, Fig. 3). The signal at 3.43 ppm was assigned to the methyl proton of 6-*O*-methyl Gal. The proton resonances in the range of 3.63–4.91 ppm were attributed to the remaining methylene and methine hydrogens of the carrageenan units. The anomeric and as well other proton signals were assigned unambiguously from the ^1H COSY NMR experiment. The correlations of protons of G4S-2,3; G4S-3,4; G4S-6,6′; DA2S-2,3 and DA2S-4,5 were deduced from the COSY spectrum (Kumar, Mehta, et al., 2011; Fig. 4).

The ^{13}C NMR resonances of SS_{AM} were assigned and are depicted in Fig. 5. The chemical shifts were identical to those of ι-carrageenan are in good agreement with the values reported earlier (Chiovitti et al., 1998; Van de Velde et al., 2002; Van de Velde, Pereira, & Rollema, 2004). The absence of ^{13}C resonances at 176.49, 102.25 and 26.26 ppm indicated that there were no carboxyl, acetal and methyl carbons of the pyruvate unit (Chiovitti et al., 1998; Kumar, Mehta, et al., 2011), which were present in the galactan of the same seaweed species reported earlier by Van de Velde et al. (2005). The ^{13}C resonances of 6-*O*-Me residue of 3-linked galactose 4-sulphate were observed at 59.7 ppm in SS_{AM}. The ^{13}C NMR resonances of SS_{Native} and SS_{Crude} were assigned to G4S and DA2S

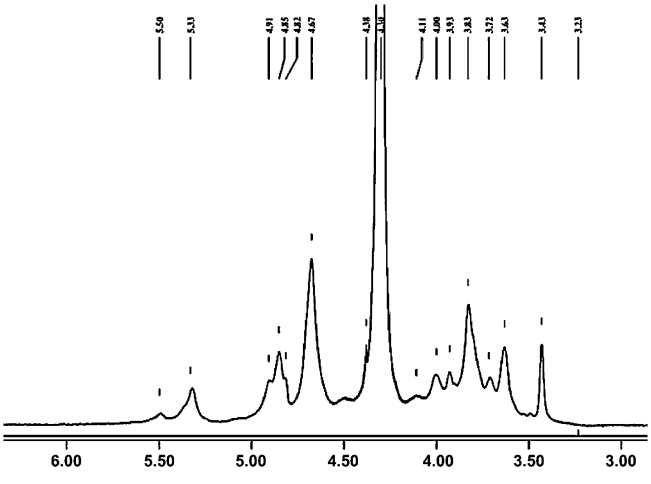

Fig. 3. ^1H NMR spectrum of SS_{AM}; H-1 of D2S,6S (5.50 ppm), DA2S (5.33 ppm) and G4S (4.85 ppm) and H of -*O*-CH_3 (3.43 ppm).

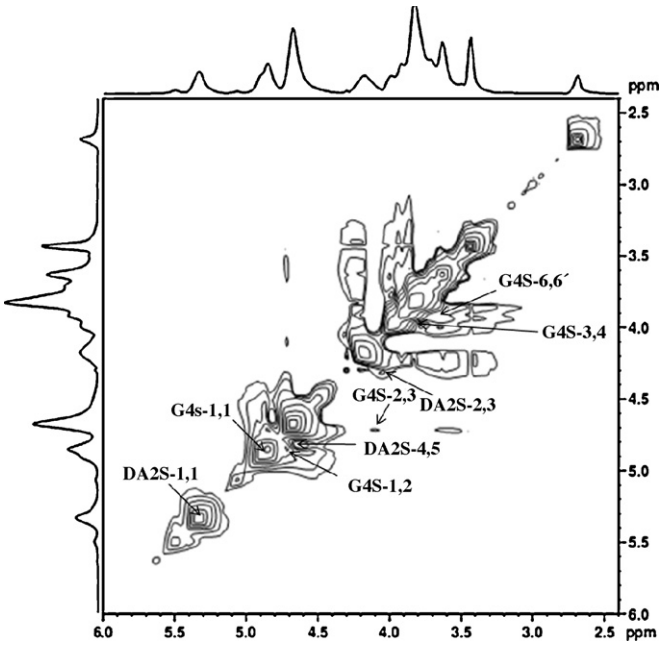

Fig. 4. 2D $^1H/^1H$ COSY spectrum of SS_{AM}.

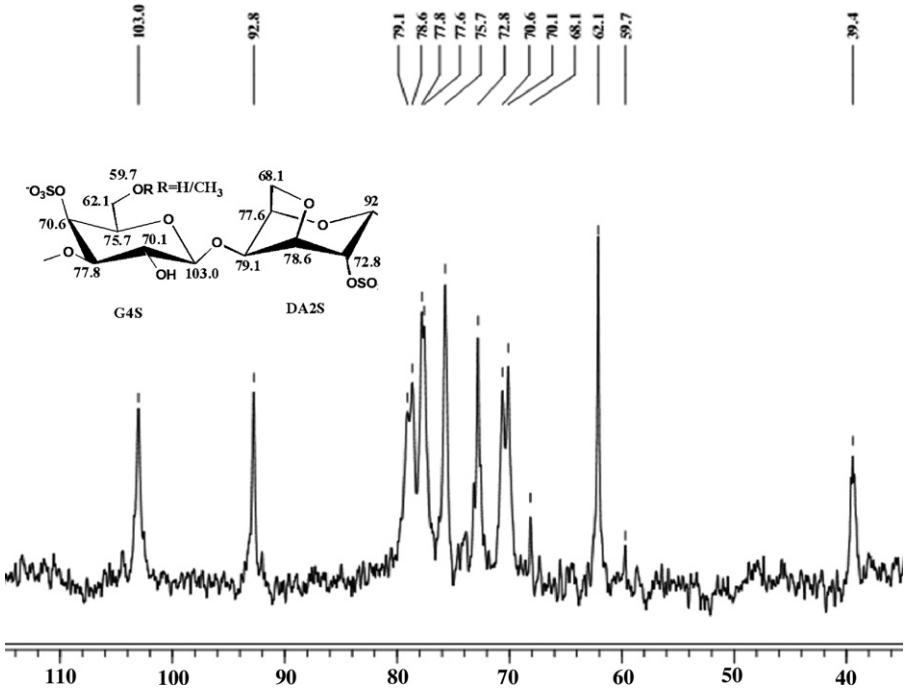

Fig. 5. ^{13}C NMR spectrum of SS_{AM}.

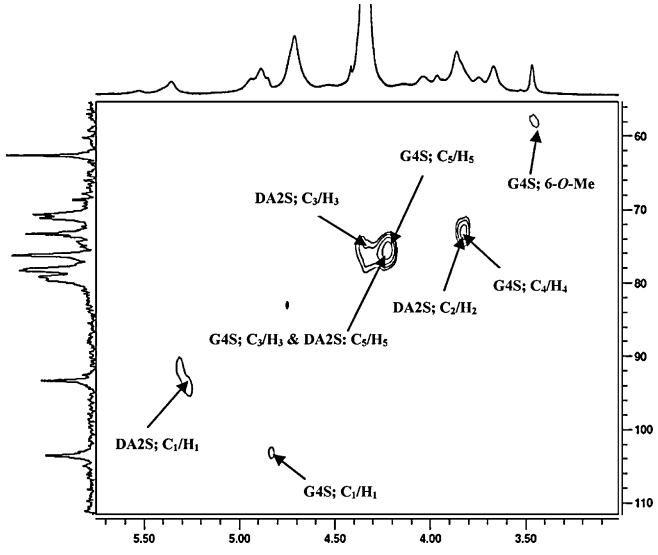

Fig. 6. ^{1}H–^{13}C HSQC spectrum of SS_{AM}.

units of ι-carrageenan while of the desulphated derivative (SS_D) were assigned to G and DA units of β-carrageenan, respectively along with the presence of 6-*O*-methyl residue attached to 3-linked galactose (Van de Velde et al., 2002; ESI Figs. S5–S7). In addition the presence of floridean starch, a storage polymer of red seaweeds species, was also detected in the SS_{Native} and assigned by ^{13}C NMR (ESI Fig. S5). The correlation of C/H of 6-*O*-Me (59.7/3.43 ppm), G4S (C_1/H_1 – 103/4.85 ppm) and DA2S (C_1/H_1 – 92.8/5.33 ppm) as well as other C/H correlations were assigned ambiguously by HSQC analysis of SS_{AM} (Fig. 6).

4. Conclusions

The carrageenan of *S. scinaioides* of Indian waters was constituted predominantly of iota-carrageenan along with a small amount of its precursor ν-carrageenan. No κ-carrageenan or pyruvated units were detected in this Indian seaweeds species unlike the carrageenan of *S. scinaioides* reported by Van de Velde et al. (2005). To our knowledge this is the first report of the sole occurrence of iota-carrageenan along with its precursor (ν) in *S. scinaioides*. The results of this study would be useful in bioprospecting of carrageenophytes.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.09.062.

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