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Structural analysis of kappa-carrageenan isolated from *Hypnea musciformis* (red algae) and evaluation as an elicitor of plant defense mechanism

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ABSTRACT

High molecular weight crude and purified polysaccharide fractions obtained from *Hypnea musciformis*, red algae were evaluated for their elicitor activity in terms of induced browning and phytoalexins production in the cotyledons of Chickpea and Peas. Intense browning was performed from purified fraction of algae that was extracted with water, dilute alkali and acid for elicitor preparations and maximum yield (30.2%) was obtained in aqueous extraction. Chemical composition of these extracted polysaccharides in terms of total sugar, protein, sulfate and uronic acid was performed and found that purified fractions contained sugar content (67.6%) and galactose as a major sugar component. using. Gel Permeation Chromatography of purified fraction identified molecular range ≥70,000 Da. On the basis of FTIR, ^{1}H and ^{13}C NMR studies, the purified fraction was safely characterized as k-carrageenan.

1. Introduction

Hypersensitive responses produced in plants after microbial attack may be triggered by elicitors, the compounds isolated from cell wall, culture filtrate and cytoplasm of parasitic and non parasitic plant pathogens. Elicitors are diverse in nature and are usually polysaccharides, proteins and fatty acids (Rao, Sarada, & Ravishankar, 1996). It is documented that in most cases elicitor activity is associated with polysaccharides fraction of various elicitor preparations when tested in Chickpea and other plant tissues (Melotto & Labavitch, 1994). As a result of infection or stress, plants exhibit some natural resistance responses. Among, induced browning and phytoalexin production have especially gained attention (Nicholson & Wood, 2001). Recently a relationship between Phytoalexin accumulation and defense against pathogenic microorganisms in Peas (*Pisum sativum*), alfalfa (*Medicago sativa*), barrel medic (*Medicago truncatula*) and Chickpea (*Cicer arietinum*) has been reported in literature (Liu et al., 2006).

Chickpea is an important crop plant and have large economic and nutritional importance due to its high content of protein fiber. *Ascochyta rabiei* and *Fusarium oxysporum* are the fungi commonly invade Chickpea and produce *Ascochyta blight* and *Fusarium wilt* diseases in the crop (Jimenez-Gasco, Navas-Cortes, & Jimenez-Diaz, 2004). Phenolics like isoflavones (Formononetin, Biochanin-A, etc.), isoflavanones (homoferreirin, cicerin, etc.) and the pterocarpans (Medicarpin, Maackiain, etc.) are the representative phytoalexins of Chickpea (Barz & Mackenbrock, 1994). Experiments have shown the genetic degradation of Chickpea phytoalexins Medicarpin and Maackiain by the fungal pathogen *Nectria haematococca* and converted them into less toxic compounds (Enkerli, Bhatt, & Covert, 1998).

Seaweeds are generally comprised of 40–69% of carbohydrates. It is reported in literature that kappa-carrageenan is the major polysaccharide obtained from *Hypnea musciformis* (Charles, Ilan, Melvyn, & Wilfred, 1984), therefore we have selected the extract of *H. musciformis* for elicitor activity experiments. Generally elicitors are effective in very small quantities, only micrograms of elicitor are required for protecting the plants from diseases that results in good quality crops. In the present study, our major interest is to exploit seaweed (*H. musciformis*) polysaccharides to evaluate as an elicitor or inducer of plant defense responses in terms of induced browning and phytoalexin production. Purified fraction was characterized by acid hydrolysis and Paper Chromatography carried out for the identification of monosaccharides. Range of molecular weight and the homogeneity was determined by Gel Permeation Chromatography. FTIR, ^{1}H and ^{13}C NMR techniques were used for possible structural characterization of these polysaccharides.

The purpose of the present study is to optimize the extraction of algal plants quantitatively, especially algal polysaccharides using various extracting media. High Molecular Weight Crude

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Elicitor Preparations 'HMWCEP' were obtained by ethanol precipitation of polysaccharides from *H. musciformis* extracts and lyophilization. These preparations were analyzed for total sugar, protein, uronic acid, sulfate and ash contents. The most active elicitor preparations obtained from *H. musciformis* were purified by reprecipitation/dialysis and Anion-Exchange Chromatography. Monosaccharide composition was determined by acid hydrolysis and Paper Chromatography. Range of molecular weight of purified fraction of polysaccharides was determined by Gel-Permeation Chromatography. Crude and purified polysaccharides were evaluated for their elicitor activity in terms of induced browning and production of phytoalexins (low molecular weight antimicrobial compounds) in the treated cotyledons of Chickpea and Peas.

2. Materials and methods

2.1. Plant collection, identification and pretreatment

Plant *H. musciformis* of class rhodophyceae (red algae) was collected from the coastal areas of Karachi such as Hawksbay and Cap Monz, in November to February 2005–2006. Identification of plant was made by Prof. Dr. Mustafa Shameel Department of Botany, University of Karachi-75270 on request. The voucher specimen was deposited at the Herbarium of the Botanical Garden of PCSIR Laboratories Complex Karachi, Pakistan (M.A. R1130.E026) and washed with running tap water and air dried under shade on netted wooden trays.

2.2. Water, moisture and ash contents of H. musciformis

100 g of pure and fresh plant was picked up, thoroughly washed and dried at room temperature till constant weight. Dry yield and water content of plant were determined.1 g of air dried plant was heated at 100 °C in the oven till constant weight and moisture content was determined. 5.0 g of sample was heated on a burner to remove soot and organic matter and then burnt in the furnace at 600 °C till constant weight obtained (Aziza, Givernuad, Chikhaoutikhay, & Bennasser, 2008).

2.3. Extraction and crude elicitor preparation

1.0 g of dried material *H. musciformis* was finally chopped and dipped into 100 ml distilled water, magnetically stirred at room temperature for 16–18 h for an exhaustive cold extraction, filtered through Whatman filter paper No. 1 and filtrate was stored frozen till further processing. 1 g *H. musciformis* was extracted with 100 ml boiling water for 12–16 h; filtered, centrifuged to obtain a clear solution and filtrate was stored at −20 °C. Another 1 g of the substance was subjected to extraction under similar conditions outlined above using 100 ml of 0.1 N NaOH and 0.1 N HCl, the extracts were stored at −20 °C. Aqueous, acidic and alkali extracts were treated with 1:3 (v/v) of distilled ethanol and samples were stored at 4–6 °C for three to four days to precipitate out the polysaccharides. The precipitates were collected by centrifugation at 3000–4000 rpm for 20–30 min. The supernatants were discarded and precipitates were dissolved in minimum quantity of water, dry weights of these High Molecular Weight Crude Elicitor Preparations "HMWCEP" were obtained by lypholization using a bench top model of lab. Conc freeze drier equipped with an Edward high vacuum pump.

2.4. Germination of seeds and elicitor activity of HMW crude and purified fractions of H. musciformis with Chickpea and Peas in terms of visual browning

Chickpea and Peas were purchased from local market. Chickpea seeds were first dipped in water in beaker, next day processed for elicitor activity. Cotyledons of Chickpea were germinated in a tray on filter paper placed on a moist cotton bed and kept at 25 °C in the dark. The excised cotyledons (2–3 days) of Chickpea and fresh cotyledons of Peas were surface sterilized by immersion in 1% sodium hypochlorite solution for 2–3 min then washed extensively with distilled water and finally rinsed with sterile water. Elicitor preparations at a concentration of 100 μg glucose equiv./ml of *H. musciformis* were used for elicitor activity. Treated and control samples were prepared by application of 20 μl of these elicitor preparation and sterile water (control) on the cut surface of cotyledons placed on a moistened filter paper in a Petri dish (10–15 cotyledons) and incubated at 25 °C in the dark for a specified time period of 24 h. Browning was recorded of the treated and control samples in duplicate and estimation of induced browning on UV-Visible Spectrophotometer (Specord-200) of variable wavelengths. The results were recorded in terms of absorption intensity of various alcoholic extracts scanned at wavelength 190–550 nm using ethanol as a blank sample.

2.5. Partial purification of HMWCEP of H. musciformis

2.5.1. Re-precipitation

2.0 g of lyophilized aqueous extract (crude polysaccharides) was dissolved in 400 ml distilled water with constant stirring and kept for 30 min to settled down the solid insoluble particles, decanted the clear solution in 2000 ml beaker and added 1100–1200 ml alcohol in a 1:3 ratio to precipitate the gel. The resulting gel was collected and dried at low temperature in oven.

2.5.2. Dialysis

2.0 g of crude polysaccharides obtained from aqueous extract of *H. musciformis* was partially purified by dialysis. Sample was dissolved in water with constant stirring, dialyzed against distilled water in dialysis sacs of molecular range cut off 8000–12,000 Da. Some of the solid material was settled down in dialysis bags and called as dialyzed insoluble fraction and the material present in the bag in solution form was lyophilized and called as dialyzed soluble fraction.

2.6. Anion Exchange Chromatography of dialyzed soluble fraction

5 mg of a dialyzed soluble fraction was dissolved in 1 ml 10 mM tris–HCl buffer, pH 8.0 (buffer A) and applied to a column (1.6 cm × 7.0 cm) of DEAE-Cellulose which had been equilibrated in buffer A. The column was first eluted with buffer A followed by 1 M NaC1 in the same buffer at a flow rate of 0.5 ml/min. 20 fractions each of 2 ml were collected and carbohydrate contents were determined by phenol/H_2SO_4 method.

2.7. Chemical analysis of HMWCEP and PF-A of H. musciformis

Total carbohydrate contents of HMWCEP and PF-A were determined by phenol/sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). Total protein was determined by the method of Bradford (1976), bovine albumin serum was used as standard. Assay for SO_4 group was carried out by the method of Dodgson (1961). Uronic acid was determined by carbazole method (Bitter & Muir, 1962).

2.8. Acid hydrolysis of PF-A and identification of monosaccharides by Paper Chromatography

100 μl of stock solution (1 mg/ml) of purified fraction-A (PF-A) was taken into air tight tubes and dried over KOH pellets in vacuum desiccator. 100 μl of 95% formic acid was added, flushed with Nitrogen and heated at 90 °C for 3 h. After hydrolysis, sample was left over night in vacuum desiccator over P_2O_5. Dry hydrolysate was resuspended into 50 μl water and applied for Paper Chromatography.

Paper Chromatography was carried out on Whatman paper No 1 with the following solvent, BuOH:Acetic acid:water (4:1:5), shaken well and top layer was used for Paper Chromatography. Chromatogram was run for 18 h and developed with Silver nitrate reagent.

2.9. Molecular weight distribution pattern of PF-A of H. musciformis by Gel Permeation Chromatography

Molecular weight distribution pattern of PF-A was determined by passing through a sequence of Sephadex G-type columns. 5 mg of sample of known sugar content was dissolved in 1 ml of distilled water and applied on sephadex G-25 (29 cm × 1.5 cm) column, eluted with distilled water at a flow rate of 0.5 ml/min. Pooled fractions, A-I of column-I were applied to column-II of sephadex G-50 (27 cm × 1.5 cm). Elution conditions were the same. High molecular weight fraction A-II collected from column-II was passed on column-III of Spehadex G-75 (26 cm × 1.5 cm). Each column was calibrated with relevant size of standard Dextran and glucose.

2.10. FTIR and NMR spectra of crude and purified fraction of H. musciformis

FTIR spectra were recorded on NICOLET AVATAR 370 DTGS (Thermo Electron Corporation, San Jose, CA, USA). The ^1H NMR of crude and purified samples was recorded on Bruker AM 300 and ^{13}C NMR spectra were recorded on Bruker 75.43 in D_20, using TMS as reference.

3. Results and discussion

H. musciformis is very bushy and commonly found at some coastal area of Arabian Sea of Karachi. It has entangled texture some what fragile, fleshy with color dul purplish red or bleached.

Table 1
Characteristic of various extract of *H. musciformis* in different solvent.

Water, moisture and ash contents (%) w/w				
Area of collection	Time of collection	H_2O	Moisture	Ash content
Hawaksbay	November 2005	91.2	10.8	15.3
Cap Monz	November 2006	95.2	11.6	18.4

[0,1-5]Dry weights of HMWCEP obtained after extraction (%) w/w		
Aqueous	Alkali	Acidic
30.2	39.8	4.3

Chemical composition of crude and purified fractions (%) w/w				
Extracts	Sugar[a]	Protein	Uronic acid	Sulfate
H_2O	64.3 (27.6)	1.2	1.7	17.4
NaOH (0.1 N)	33.5 (21.8)	2.3	1.4	44.1
HCl (0.1 N)	40.2 (19.9)	5.5	6.4	41.3
HMWPF-A	67.6 (30.2)	0.6	0.8	14.1

[a] Total sugar, in parenthesis are the values of 3, 6-anhydrogalactose derived from the total sugar.

The basis disc like, ill-defined, erect branches, 10–20 cm tall, about 1–2 mm in diameter, grow on rocks and stones. It is reported that carrageenan are the major sugars of *H. musciformis* (Charles et al., 1984) and our main concerned and focus to use these type of polysaccharides as an elicitor in future. It is reported that the water and moisture contents of algae are generally between 86–88% and 7–9% respectively (Qari, 1988). Results in Table 1 showed that water content of *H. musciformis* is high such as 91.2–95.2%, may be due to their beaded and capsulated structure. Moisture contents of these varieties are also high 10.8 and 11.6%, this suggest that due to their beaded and capsulated structure some of the sea salts were retained and absorbed water from the atmosphere at later stages of storage. Ash contents were found 15.3% and 18.4%, high values of ash content were previously reported in *H. musciformis* i.e. 15–40% (Tuvikene et al., 2006; Mou, Jiang, Liu, & Guan, 2004).

H. musciformis was extracted with water, dilute alkali and acid. Dry weight of High Molecular Weight Crude Elicitor Preparations, HMWCEP was obtained by ethanol precipitation and lypholization (Fig. 1) as mentioned in material and method. Total yield of dry extracts varied in the range 4.3–39.8% depending on the extracting media. Low yield 4.3% in acidic solution suggest that due to acidic media, destruction of galactans occurred and the low molecular products cannot be precipitated by alcohol and remain in the

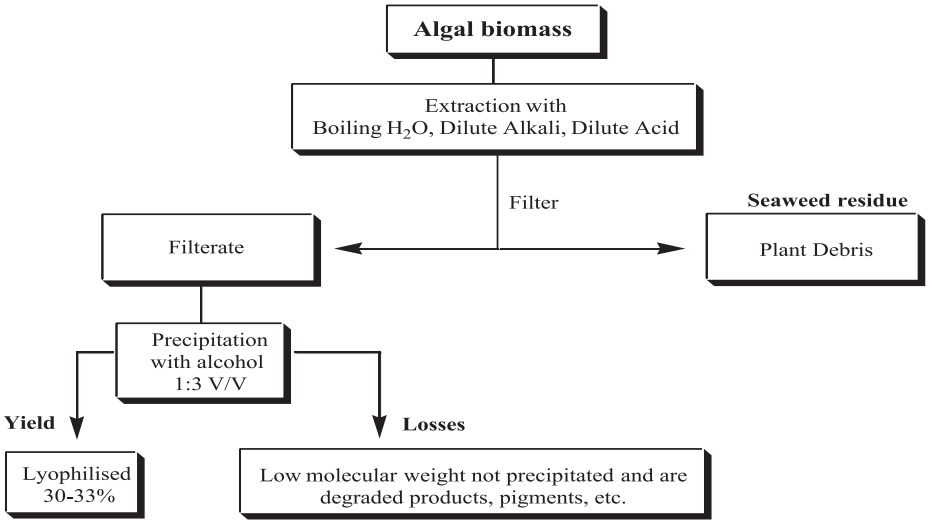


Fig. 1. Isolation process of carrageenan from algal biomass.

Table 2
Elicitor activity of HMW crude and purified fractions of *H. musciformis* in terms of induced browning.

Plants tested	Cotyledons treated with extracts (Crude)	Wavelength (nm)	Preliminary elicitor activity	Cotyledons treated with extracts (purified)	Elicitor activity with purified fraction
Chickpea	Cont. water	254	0.1200 ± 0.0351	Cont. water	0.1000 ± 0.0350
	NaOH (dil)	254	0.2701 ± 0.0353	PF-A	1.5387 ± 0.0352
	HCl (dil)	254	0.3301 ± 0.0355	PF-B	0.1123 ± 0.0349
	H_2O	254	1.1037 ± 0.0352	–	–
Peas	Cont. water	264	0.0900 ± 0.0271	Cont. water	0.0700 ± 0.0254
	NaOH (dil)	264	0.2587 ± 0.0359	PF-A	1.1012 ± 0.0352
	HCl (dil)	264	0.6003 ± 0.0354	PF-B	0.0812 ± 0.0350
	H_2O	264	0.6012 ± 0.0350	–	–

Note: PF-A → purified fraction-A; PF-B → purifird fraction-B.

solution giving a low yield of HMWCEP, the extent of acidic destruction is expressed by losses of sugars. Yields were high in alkali extraction 39.8%, it is quite possible that during extraction and lypholization, some of the NaOH remain intact and showed elevation in yield. Actual yield can be obtained after dialysis to get the real amount of HMW polysaccharides. The amount of HMW polysaccharides obtained by the aqueous extraction was 30.2%, values are in complete agreement with the range (30–32%) normally reported in literature (Mou et al., 2004). Results are presented in Table 1. Chemical composition of HMWCEP obtained from aqueous extraction of *H. musciformis* is reported in terms of total carbohydrate, protein, sulfate group and uronic acid. Results presented in Table 1 showed that reasonably high sugar contents 64.3% were observed in the crude aqueous extract. Sugar content of alkali extract is 33.5% and acidic extract was 40.2%. 3, 6-anhydrogalactose derived from total sugar is 27.6% in aqueous extract, 21.8% in alkali extract and 19.9% in the acidic extract, sugar contents are similar to that described earlier (Mou et al., 2004). Protein found in the aqueous, alkali and acidic extracts of *H. musciformis* is 1.2%, 2.3% and 5.5% respectively. The nature of carbohydrate protein linkages in seaweed is not deduced yet. Small amount of uronic acids have been detected in the aqueous, alkali and acidic extracts of *H. musciformis*, observed values are 1.7%, 1.4% and 6.4% respectively. The presence of uronic acid in *H. musciformis* showed some minor contaminants present in this plant. The SO_4 content of the extracts of *H. musciformis* range from 15% to 40% (Mou et al., 2004; Tuvikene et al., 2006), observed SO_4 content is 17.4% in aqueous extract, 44.1% in alkali extract and 41.3% in the acidic extract. Chemical composition of HMW PF-A is presented in Table 1 showed slightly high as compare to HMWCEP.

Treated cotyledons of Chickpea and Peas exhibited a significant level of elicitor activity (Figs. 2 and 3) on treatment with the HMW crude preparation of aqueous extracts (Table 2). Cotyledons of Chickpea and Peas treated with HMWPF-A produced intense browning as compare to the purified fraction (PF-B) of *H. musciformis*. Reason for producing less browning by HMWPF-B

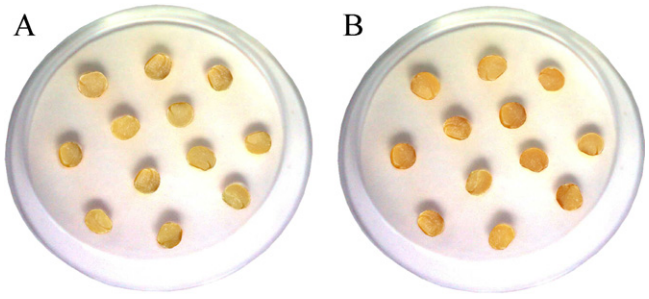


Fig. 2. (A) Control sample (B) browning induced in the elicited cotyledons of Chickpea treated with HMW polysaccharides of *H. musciformis* at a concentration 100 μg glucose equiv./ml.

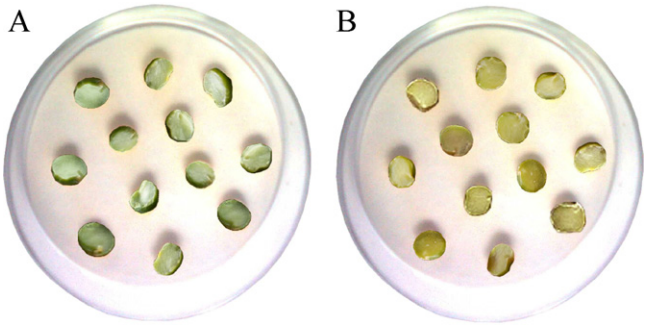


Fig. 3. (A) Control sample (B) browning induced in the elicited cotyledons of Peas treated with HMW polysaccharides of *H. musciformis* at a concentration 100 μg glucose equiv./ml.

could be the heterogeneity in construction or presence of multiple components in purified fraction-B. Overall browning produced by Chickpea was comparatively higher where as browning produced by Peas was low (Table 2).

A white flocculent precipitate was collected. Recovery of reprecipitated insoluble and soluble dry material is shown in Table 3. Polysaccharide obtained from aqueous extraction of *H. musciformis* was also partially purified by dialysis. Some solid material was settled down in dialysis bag and called as dialyzed insoluble fraction. Soluble material of the solution in dialysis sacs was lyophilized and designated as "dialyzed soluble fraction". This fraction was very light weight and white powdered. Amount of dialyzed soluble and insoluble fractions are given in Table 3, recoveries were generally low and suggested that low molecular substances associated with the polysaccharides were dialyzed out. The presence of sulfate group in these carbohydrates provides charge, which can be exploited for the separation of sulfated carbohydrate from neutral sugars and other carbohydrates. The most widely used technique is Anion-Exchange Chromatography using standard liquid

Table 3
Purified fractions obtained from HMWCEP polysaccharides of *H. musciformis*.

Recoveries of partially purified after reprecipitation (%) w/w		
Extract	Insoluble fraction	Re-precipitated fraction
Aqueous	26	74

Recoveries of partially purified after dialysis (%) w/w		
Fraction	Dialyzed insoluble fraction	Dialyzed soluble fraction
Reprecipitated	9.09	68

Recoveries of two fractions A and B, obtained after DEAE-Cellulose Anion-Exchange Chromatography of dialyzed soluble fraction. Column recoveries in terms of sugars (w/v, %)		
Fraction	Fraction-A	Fraction-B
Dialyzed soluble	57.6	17.6

Table 4
Gel Permeation Chromatography of HMWPF-A of *H. musciformis*.

Fraction applied	Column	HMW fraction (A)	LMW fraction (B)	T. recovery
Column recoveries in terms of sugars g % (w/v %)				
HMWPF-A	Sephadex G-25	80.3	15.0	98.0
	Sephadex G-50	48.5	11.6	60.1
	Sephadex G-75	65.5	24.1	89.6

chromatographic media such as DEAE-Cellulose or DEAE-Sepharose (Mou et al., 2004). Dialyzed soluble fraction of aqueous extract was further purified on DEAE-Cellulose. No sugar was detected up to 20–30 ml. The bound fraction was eluted with 1 M NaCl solution. Applied Polysaccharides were resolved into a sharp symmetrical peak, peak-A known as purified fraction-A (HMWPF-A) where as peak-B is polydisperse in nature and indicate heterogeneity in composition (see supplementary data Fig. S1).

Total column recovery is low (75%) this suggest that some charged sugars were irreversibly bound to the column and not eluted under these elution conditions (Table 3). After purification by Anion-Exchange Chromatography, fraction eluted as peak-A was evaluated for its elicitor activity and enhance elicitor activity was observed. The principal component sugar in red algae from previous reports was galactose present as galactan or galactan sulfate (Chiovitti et al., 1996). Mixture of standard monosaccharides was well separated (supplementary Fig. S2) during chromotography. Major monosaccharide residues released were galactose. Spots found in the developed chromatogram with lower RF values were not identified and could be some oligosaccharides or protein/pigment substances.

Range of molecular weight and homogeneity of purified fraction-A obtained by the Anion-Exchange Chromatography was determined. Initially sample was applied on sephadex G-25, column-I. A typical chromatogram in (see supplementary data Fig. S3) showed two peaks A-I and B-I. Peak A-I eluted in the void volume and separation achieved under peak B-I indicated a broad distribution of components suggesting polydisperse nature of applied material. Fraction eluted in the void volume A-I were pooled together, concentrated and rechromatographed on sephadex G-50, column-II. Major portion of applied sample was eluted in or near the void volume under peak A-II giving an apparent molecular range >20,000 (see supplementary data Fig. S4). Fractions 10–20 ml were collected and further applied on sephadex G-75, column-III, separation showed two distinct and major peaks (see supplementary data Fig. S5). Peak A-III corresponds to void volume with an apparent molecular range ≥70,000 and peak B-III elaborated to low molecular weight region and was quite heterogeneous in its construction. Column recoveries were given in Table 4.

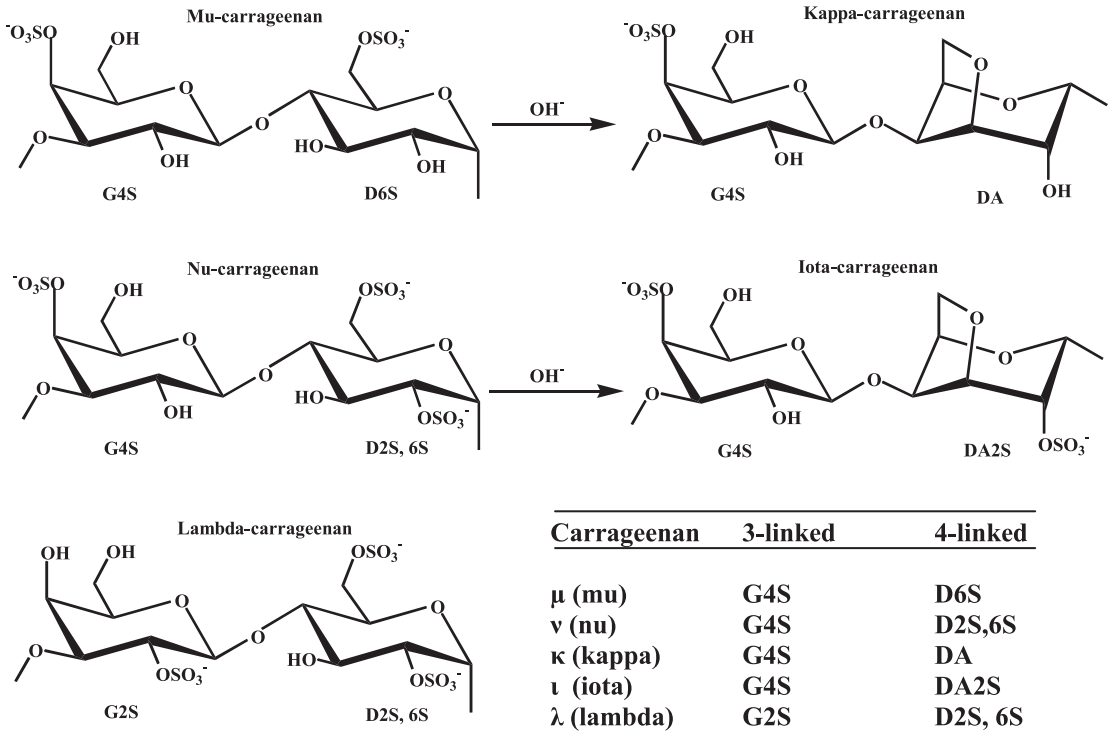
The characteristic bands observed in the spectra of aqueous crude extract (Fig. 4) correspond to characteristic bands of DA2S, 3, 6-anhydrogalactose-2-sulfate at 803.99 cm^{-1} for iota carrageenan, G2S galactose-2-sulfate or D2S, 6S, galactose-6-sulfate at 822.27 cm^{-1} for lambda carrageenan and G4S galactose-4-sulfate at 840.91 cm^{-1} for kappa carrageenan. A careful examination of the spectra of aqueous extract (crude) leads to the conclusion that carrageenan derived from *H. musciformis* had a major features of kappa carrageenan with very small portion of iota and lambda carrageenan (Fig. 5). Spectrum of HMWPF-A in Fig. 5 showed strong characteristic band of G4S, galactose-4-sulfate at 846.95 cm^{-1}; DA, 3, 6-anhydrogalactose at 928.92 cm^{-1} and S, sulfate ester at 1221.25 cm^{-1} that confirms 100% kappa carrageenan presence while the characteristics bands for iota carrageenan and lambda carrageenan were not found in the spectrum. This suggests that small amount of iota and lambda carrageenans were eliminated from the native polysaccharide during the process of purification. Values of absorption bands are summarized in Table 5 for crude and purified fraction-A and also it is in agreement with the previous finding (Villanueva & Montano, 2003).

The purified fraction (HMWPF-A) Chemical shifts (ppm) in the ^{1}H NMR spectrum in Table 6 were tentatively characterized referring the reported values (Villanueva & Montano, 2003). H-1 signal at δ 4.91040 ppm has a close proximity of δ 5.10 ppm for DA anhydrogalactose 4-linked residue in k-carrageenan and H-1 signal at δ 4.65614 ppm assign to G4S in k-carrageenan. These anomeric signals at δ 4.63 and 5.10 ppm are characteristic of k-carrageenan (Villanueva & Montano, 2003). Slight changes in the chemical shifts as compare to reported values were attributed to the substituents and the substitution pattern of typical carrageenan back bone. Data for ^{13}C NMR spectroscopy provided evidences that k-carrageenan is a dominating sugar of purified fraction-A. The observed values for C-1 to C-6 are given in Table 6.

Main purpose of this study is to evaluate seaweed polysaccharides as an elicitor of disease resistance responses in plants. A common indication of pathological metabolism is some discoloration/browning of the affected tissues. Isoflavones and related pterocarpans constitute a group of secondary metabolites primarily in leguminous plants and are known for their role as phytoalexins (Barz & Mackenbrock, 1994). Homogenous pure fractions of active elicitor molecules with known structure are highly desirable for targeting receptor molecules and to understand the recognition mechanism of plant pathogen or plant elicitor defense interaction.

Research work was designed to evaluate these polysaccharides as an inducer of hypersensitive responses characteristic to resistance mechanism against plant disease. It is previously reported that isolation and exhaustive purification of elicitors from microbial source generally end up with very small quantity and one of the purpose of present work is the exploitation and utilization of marine resources for the production of potentially active elicitors and of course will be produced in large quantity. This research can also be of much importance with respect to the plant disease control and hence of practical importance by developing various resistant varieties of plants, especially of crop plants for future agriculture industry. In another work we have conducted field trials of k-carrageenan elicitor preparation to examine the effect of elicitor on the production of induced secondary metabolites, ISMs (the disease resistance compounds) and on various growth characters of Chickpea and maize plants grown in the field of PCSIR Laboratories Complex, Karachi. The treated plants responded well to the applied elicitor in terms of average plant height, number of leaves, branches, flowers, fruits and ISMs production (Bi, Iqbal, Arman, Ali, & Hassan, 2010). This suggested that carrageenan elicitor can be used as a potent plant protectant as well as growth promoting agent for crop plants.

Initial results for elicitor activity of Peas tissues were not found very promising, induction of browning and estimation of induced metabolites (phytoalexins) in treated tissues of Peas were insignificant as compared to controls (Bi, Iqbal, Ali, Arman, & Hassan, 2008). Cotyledons of Peas are sweeter than Chickpea and sugars from *H. musciformis* may found some resistance from chemical composition and structure of the cell surface barrier from Peas tissues but this need to be studied. The induction pattern of secondary metabolites in respect of new source of elicitor (polysaccharides from seaweeds) which is non pathogenic origin and non specific is in progress. LC–ESI $(MS)^{n}$ characterization of induced components allowed detailed investigation of metabolic responses. The main advantage of this technique is that a very small amount of sample is required for complete analysis. Under the LC–ESI-MS conditions employed, nine compounds Naringin, Naringin malonate, Liquiritigenin, Naringenin, Biochanin A, Daidzein, Formononetin, Maackiain and Medicarpin were identified from elicited tissues of Chickpea. End product phytoalexins, Maackiain and Medicarpin



Carrageenan	3-linked	4-linked
μ (mu)	G4S	D6S
ν (nu)	G4S	D2S,6S
κ (kappa)	G4S	DA
ι (iota)	G4S	DA2S
λ (lambda)	G2S	D2S, 6S

Fig. 4. Schematic representation of the different repeating units of carrageenans and the letter codes nomenclature, as developed by Knutsen, Myslabodski, Larsen, and Usov (1999).

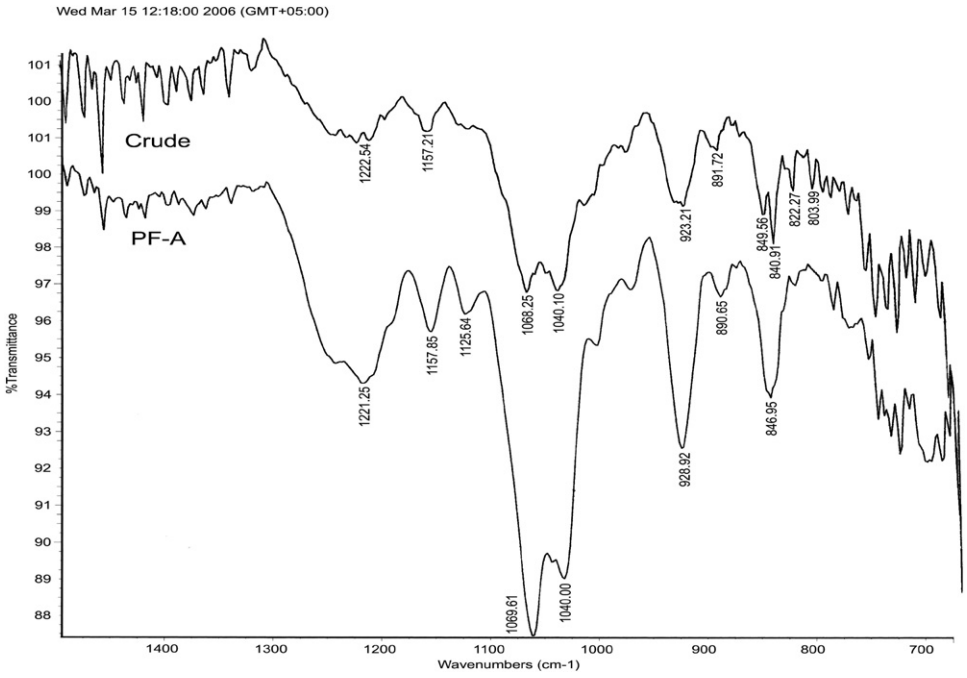


Fig. 5. FTIR spectra of k-carrageenan, crude and PF-A of *H. musciformis*.

Table 5
Characteristic bands of crude and purified HMWPF-A fractions of *H. musciformis* using FTIR.

Extract/fraction	Absorption bands (cm^{-1})	Tentative assignments	
Crude	803.99	DA2S	3, 6-anhydrogalactose-2-sulfate
	822.27	G2S galactose-2-sulfate/D2S, 6S galactose-6-sulfate	
	840.91	G4S	Galactose-4-sulfate
	1222.54	S	Sulfate ester
HMWPF-A	846.95	G4S	Galactose-4-sulfate
	928.92	DA	3, 6-anhydrogalactose
	1221.25	S	Sulfate ester

Table 6
Chemical shifts observed in the ^{1}H NMR and ^{13}C NMR spectrum of the HMWPF-A of *H. musciformis*.

Fractions	Residue	H-1	H-2	H-3	H-4	H-5	H-6_{endo}	H-6_{exo}
	G4S	4.65614	3.62037	3.95706	4.75137	3.80448	3.84173	3.80784
	DA	4.91040	4.33766	4.47883	4.52368	4.67547	4.45754	4.41322
HMWPF-A	–	C-1	C-2	C-3	C-4	C-5	C-6	–
	G4S	104.569	71.5699	80.4816	75.9639	76.8665	63.3753	–
	DA	96.986	74.2000	81.2853	78.8058	78.8880	71.8058	–

were induced two to three folds or even more than the control levels. Due to low profile of elicitor activity in treated tissues of Peas, induced components were not quantified but a successful HPLC method was developed and five compounds were identified as afrormosin, anhydropisatin, Pisatin, pseudobaptigenin and Maackiain (Arman, 2011).

Question arises why polysaccharides from *H. musciformis* are effective in Chickpea tissues and not for Peas tissues. To resolve this question it is proposed that in future experiments seaweed polysaccharides shall be used against various cash crops i.e. Tomato, Potato, Beans, Cotton seeds, etc. to determine and establish the activity spectrum of these polysaccharides. It is very much desirable to study the systemic induction of phytoalexins (isoflavonoids and their glyco-conjugates) in cell cultures of selected host inoculating with respective fungal pathogen, pathogenic derived elicitors and elicitor preparation from seaweed under identical conditions. The progress and induction pattern and quantification of induced secondary metabolites in each case shall be monitored with time and doses. This might explain what types of isoflavonoids glyco-conjugates are induced at what time and at what concentration.

4. Conclusions

Crude and purified fractions were isolated and chemically analyzed, maximum sugar was found as 64.3–67.6% (containing 27.6–30.2% of anhydrogalactose), very small amount of protein and uronic acids were found, sulfate content was high ranging between 14.1% and 17.4%. Acid hydrolysis and Paper Chromatography revealed galactose as a major component of these polysaccharides. Purified fraction was obtained by Anion-Exchange Chromatography and provided a homogenous fraction having mass range molecular range $\geq$70,000 determined by Gel Permeation Chromatography. Polysaccharides of red algal plants (*H. musciformis*) are charged sugars, for their identification and determination of monosaccharide composition, it is more advisable to hydrolyze polysaccharides under control condition using various strength of acid, at a different range of temperature and for various length of time to provide useful characterizable fragments and absolute composition. On the basis of IR, ^{1}H and ^{13}C NMR studies, purified fraction was safely characterized as k-carrageenan. Purified fraction-A produced intense browning as compare to the Purified fraction (PF-B) of *H. musciformis*. Overall browning produced by Chickpea was comparatively higher where as browning produced by Peas was low. On the basis of present results, It is concluded that seaweed polysaccharide elicitor k-carrageenan of *H. musciformis* can be used as a potent plant protectant as well as the good growth promoting agent especially for the Chickpea plant.

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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.2012.02.003.

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