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Isolation and Characterization of *Kappa*-Carrageenan from *Hypnea musciformis* (Red Alga) Collected from Karachi Coast, Pakistan

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Abstract. *Hypnea musciformis*, collected from Karachi coast of Pakistan, was used for the isolation of a thickening and emulsifying agent, carrageenan. Various extraction procedures were employed and the yield of carrageenan obtained was in the range of 34-44%. Total sugar contents were found to be 31.8-55.4%. 3,6-Anhydrogalactose, a component of the total sugar, was present in the range of 19.9-27.6%. Sulphate and ash contents were high, 14.8-41% and 15.4-53%, respectively. The positive rotation of these polysaccharides indicated a predominance of α-D-glycosidic linkages in their structure. IR spectral studies showed *kappa*-carrageenan as the major phycolloid, with a very small contamination of *iota*-type carrageenan, whereas *lambda*-type was not detected. Polysaccharides obtained showed a positive elicitor activity in garden peas (*Pisum sativum*). HPLC analysis indicated the presence of a single major component.

Keywords: carrageenan, Rhodophyta, polysaccharides, elicitor activity, *Hypnea musciformis*, phycolloid

Introduction

Commercially important polysaccharides from red seaweeds (Rhodophyta) belong to the group of polydisperse, long chain, water-soluble galactans. Their building block is made up of alternating 3-linked β-galactopyranose and 4-linked β-galactopyranose, which can be variably modified and/or substituted. In carrageenans, the 4-linked units are in the D-configuration, whereas in agars they are in the L-configuration (Usov, 1992). The gelling and thickening properties, and the protein reactivity of these phycolloids have led to their widespread commercial uses in the industry, including food and beverages, pharmaceuticals and cosmetics (Nishizawa, 2002). These are also used as biofertilizers in the agriculture sector. Because of the wide commercial applications of carrageenans, various extraction procedures to obtain this product from red algal plants have been reported in literature, while their structural determinations are done using spectroscopic techniques (Greer *et al.*, 1984). Karachi, Pakistan has a large coastal area yielding large quantities of marine algae. Unfortunately, these seaweeds are not utilized in Pakistan, either as marine vegetables or for extracting commercial compounds (Husain *et al.*, 2001), whereas huge amounts are spent on the import of seaweed products. The aim of the present study was to develop an effective procedure for the exploitation of these seaweeds so that carrageenans may be extracted in good quality and quantitiy from *Hypnea musciformis*. Another objective was to explore the nature of these polysaccharides as the inducers of hypersensitive response, characteristic of resistance mechanism in plants against diseases, especially in terms of induced browning and production of phytoalexins (Nicholson and Wood, 2001). Garden peas (*Pisum sativum*) were used as the test plant for the elicitor activity experiments.

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Materials and Methods

General method of extraction. *Hypnea musciformis* (red alga) was collected from Karachi coast, Pakistan in February and November 2003. The plant was cleaned of epiphytes, washed, dried, and ground to a fine powder. Representative material of the plant sample (25 g) was pretreated with HCl (0.1 N)/formaldehyde (20%), and extracted with water (tap/distilled) at 70-80 °C, with constant stirring for 6 h. Supernatant was collected and the residue was re-extracted twice under similar conditions. Experimental conditions were varied for optimizing the extraction procedures and carrageenans were obtained, as detailed below in different extraction protocols, by ethanol precipitation or by direct drying on a waterbath.

Extraction-1. Dry plant powder, pretreated with HCl (0.1 N), was stirred for 30 min in an icebath, washed extensively with tap water to remove traces of acid, followed by washing with distilled water. The supernatant was dried in a waterbath.

Extraction-2. Dry plant powder, pretreated with HCl (0.1 N), was washed, followed by extraction with distilled water. Supernatant was precipitated with 95% ethanol (three volumes of the extract), and the precipitate was dried.

Extraction-3. Dry plant material, pretreated with HCl (0.1 N), was washed and extracted with tapwater and dried in a waterbath.

Extraction-4. Dry plant material, pretreated with HCl (0.1 N), was washed and extracted with tapwater, precipitated with alcohol, and the precipitate was dried in a waterbath.

Extraction-5. Dry plant material, pretreated with HCl (0.1 N), was washed and extracted with distilled water. Supernatant was precipitated with 0.3 M KCl (solid) and the precipitate was dried in a waterbath.

Extraction-6. Dry plant material was mixed with formaldehyde (20%), left overnight, washed and extracted with distilled water. Supernatant was dried in a waterbath.

Analytical methods. Moisture, ash and total carbohydrate contents were determined. Acid hydrolysis was done for different time periods, 30, 90 and 180 min. Monosaccharide components were identified by paper chromatography, as described elsewhere (Bi and Iqbal, 1999). 3,6-Anhydrogalactose was determined colourimetrically using the modified resorcinol method (Yaphe and Arsenault, 1965). Sulphate content was determined in accordance with the method of Dodgson and Price (1962). Optical rotations of carrageenan solutions of known concentration (1% of **extracts-1, 2, 3, 4** and commercial carrageenans, and 0.1% of **extracts-5 and 6**) were determined with a digital polarimeter (Jas. Co. Dip. 360) using 50 mm tubes and sodium D line at 589 cm wavelength. Fourier transform infrared (FTIR) analyses were performed on a Nicolet Avatar 370 DTGS infrared fourier transform spectrometer.

Elicitor activity and extraction of induced secondary metabolites (ISM) from garden peas. A general method of elicitor application was employed (Whitehead *et al.*, 1982). Fresh garden peas (*Pisum sativum*), 100 g, were pealed and cotyledons were sterlized with 1% sodium hypochlorite solution, washed extensively with distilled water, followed by washing with sterilized water. The elicitor preparation (20 µl), made from **extract-5**, at a concentration of 100 µg glu eq/ml and sterilized water, were applied to the cut surface of cotyledons. The browning induced in the samples was recorded after 24 h of incubation, and the samples were dipped in distilled alcohol (95%) for the extraction of induced secondary metabolites.

Separation of ISM by HPLC. HPLC separation was accomplished on 4.6 mm x 25 cm reverse phase C_{18} column (Perkin Elmer), with a variable UV-detector (254 nm) and isocratic solvent system. A guard column of pellicular C_{18} hydrocarbon chemically bonded to glass beads was placed before the analytical column. Initially, 70 : 30 (acetonitrile : water), having 0.5% acetic acid, was run for 10 min and then acetonitrile (100%) was run for further 25 min.

Sample preparation for HPLC. Dry alcoholic extract of the treated and control samples were dissolved in 1 ml of the initial solvent (70 : 30; acetonitrile : H_2O). This solution (100 µl) was further diluted with 2 ml of the same solvent and filtered with 0.45 µm filters, and 20 µl of the clear solution was applied on the column.

Results and Discussion

Various extraction procedures were applied during the present study to isolate and characterize carrageenans from *H. musciformis* (red alga). The average total recovery of galactans from hot water extractions was about 39% of the dry alga (Table 1), which was similar in range as described earlier (Knutsen *et al*., 1995). Generally, yields were high in the extracts pretreated with mild acid (38-44%). Moisture and ash contents of the products were in the range of 3.1-7.7% and 15.4-18.4%, respectively, whereas **extract-5** had very low moisture (0.32%) and the highest ash content (53%). Total sugar contents were in the range of 31.8-55.4%. High sugar content in aqueous hot extract of *H. musciformis* has been also reported in a previous communication (Bi and Iqbal, 1999). It is documented that plants belonging to Rhodophyceae (red alga) commonly have sulphated galactan (Miller and Blunt, 2002). High sulphate contents (23.6-41.0%, except in **extract-5**) and 3,6-anhydrogalactose (20-27%), which were derived from the total sugar, confirm the earlier findings for these contents reported by Chiovitti *et al*. (1996).

Various characteristic properties of the phycolloids obtained using different extraction procedures are summarized in Table 2. After extraction and drying of samples, no odour was found in any extract. Most of the extracts were brown in colour. The alkali treatment of **extract-5** gave the sample a creamish white colour, close to the colour of commercial carrageenan.

Table 1. Yield and chemical composition of algal extracts of *Hypnea musciformis* (%; w/w)

Extracts*	Yield	Moisture	Ash	Sugar contents[a]	Sulphate
Extract-1	40.0	7.7	15.7	45.3 (20.9)	31.1
Extract-2	38.0	4.6	18.4	50.8 (21.8)	25.4
Extract-3	44.2	6.9	16.3	41.1 (23.1)	35.4
Extract-4	39.1	5.1	18.1	46.5 (27.6)	30.1
Extract-5	40.0	0.32	53.0	31.8 (25.9)	14.8
Extract-6	34.2	5.4	15.4	55.4 (22.3)	23.6
Carrageenan[c]	nd[b]	3.1	15.7	40.1 (19.9)	41.0

* = see Materials and Methods for the extraction procedures used for **extracts 1-6**; a = total sugar, within parenthesis is the value of 3,6-anhydrogalactose derived from the total sugar; b = not determined; c = commercial carrageenan used as the reference

Table 2. Characteristics of phycolloids of *Hypnea musciformis*

	Extract-1	Extract-2	Extract-3	Extract-4	Extract-5	Extract-6	Commercial carrageenan
Colour	reddish-brown	light-brown	dark-brown	light-brown	creamish-white	brown	pinkish-white
Solubility in water (2%; w/v)	dissolved at 60-70 °C	dissolved at 60-70 °C	dissolved at 60-70 °C	dissolved at room temperature	dissolved at 60-70 °C	dissolved at 60-70 °C	dissolved at room temperature
Methylene blue test	ppt formed	ppt formed	ppt formed	ppt formed	ppt formed	ppt formed	ppt formed
Milk reactivity	positive	positive	positive	positive	positive	positive	positive
pH of aqueous solution (1%; w/v)	4.4	4.2	4.7	4.8	5.2	4.8	7.7
Aqueous gel strength (2%; w/v)	non-gelling	gel formed at 4 °C	non-gelling	non-gelling	gel formed at room temperature	gel formed at 4 °C	viscous solution at 4 °C
Optical rotation ($[\alpha]_D^{25}$)	+ 58.2°	+ 48.1°	+ 52.0°	+ 52.0°	+ 30.0°	+ 64.0°	+ 53.8°

Except **extract-4** and the commercial carrageenan, all other samples were soluble in water (2%) at 60-70 °C within 30 min, only a very small amount of insoluble material remained in some samples. Formation of precipitate, with methylene blue, and positive test with milk are the characteristic tests of carrageenans. pH of aqueous solution (1%) of **extracts 1-6** showed acidic nature (pH = 4-5), whereas the commercial carrageenan had slightly alkaline nature (pH = 7.7). The gelling strength of the aqueous solutions (2%) was recorded at room temperature and at 4 °C. Some of the **extracts-1, 3, 4** were non-gelling, but **extract-2, 6** formed thick and viscous gels at 4 °C. It has been reported that the presence of 3,6-anhydro sugar causes gelling, but if it is replaced by the 6-sulphate sugar, the gelling power is considerably lessened and the 2,6-disulphate in place of 3,6-anhydro sugar results in the complete loss of gelling power (Percival and Mcdowell, 1990). This suggests that gelling property not only depended on the anhydro sugar, but was also dependent on the sulphate content of the phycolloids. It is also possible that acid treatment resulted in the hydrolysis of the linkage at 3,6-anhydro-galactose and made the samples totally non-gelling, whereas **extract-5** with low sulphate (14.8%) showed the highest gelling strength and thus formed thick and stable gel at room temperature. Some non-gelling polysaccharides obtained from marine algal plants have been also reported by Parekh *et al.* (1989). It was surprising that the commercial carrageenan only formed viscous solution at low temperature (4 °C), which may be due to its high sulphate content (41%). The marketed carrageenan products are blended materials, which are used for different purposes, rather than as a pure product (Thomas, 1997). The positive optical rotation of these polysaccharides indicates a predominance of α-D-glycosidic linkages in their structure.The monosaccharide composition, as determined by acid hydrolysis and paper chromatography, showed galactose as the major sugar component of each extract and released as early as 30 min of hydrolysis. Regular increases were observed, which maximized at 3 h of hydrolysis. Xylose/arabinose and glucose alongwith minor amounts of fucoses, were also observed in the chromatogram. The intensity of spots was high in the extracts pretreated with mild acid, which may be due to some addition of glucose from starch as a contaminant.

Infra-red technique has been commonly used in the characterization of carrageenans (Estever *et al.*, 2002; Falshaw *et al.*, 1996). The IR spectra of the crude **extracts 1-6**, alongwith the commercial carrageenan, were recorded (Fig. 1). All the spectra displayed a broad absorption band at 1210-1220 cm^{-1}, corresponding to sulphate ester, which is common to all the sulphated polysaccharides and increased in size with the sulphate content. In the commercial carrageenan spectra, this band was fairly sharp and strong, which is in accordance with the high sulphate content of the sample (Table 1). The diagnostic region (940 cm^{-1} - 800 cm^{-1}) of the IR spectra of polysaccharides resembled that of *kappa*-carrageenans elaborated by the red algal plant, *H. musciformis* (Knutsen *et al.*, 1995). The characteristic bands at 930 cm^{-1} and 840 cm^{-1} represent the anhydrogalactose and galactose-4-sulphate, respectively. The

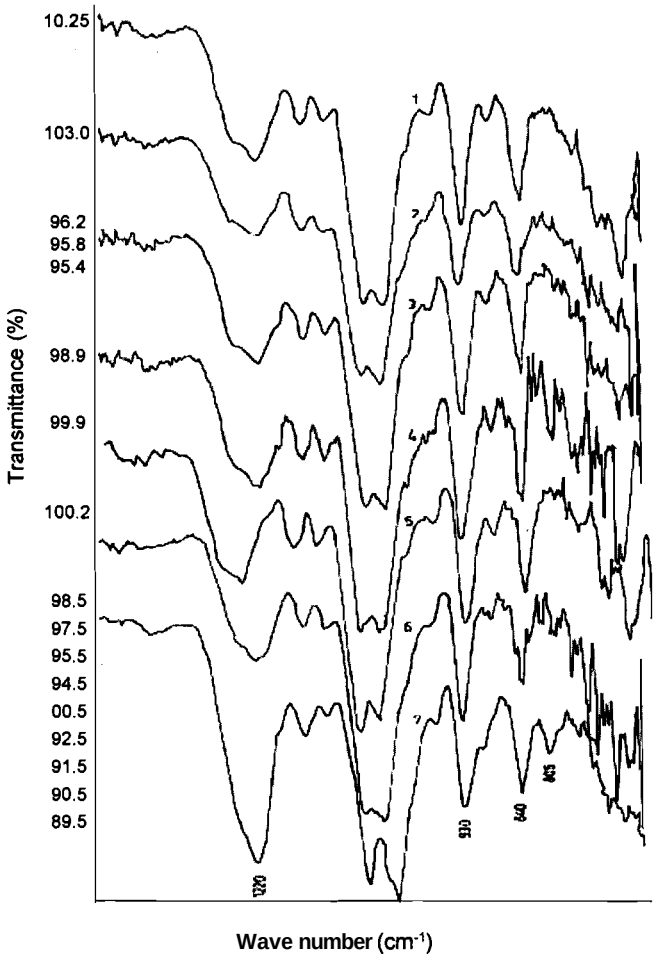

Fig. 1. Fourier transition infrared (FTIR) spectra of polysaccharide preparations from Hypnea musciformis (extracts 1-6) and a commercial sample of kappa-carrageenan (extracts-7); see Materials and Methods for the extraction procedures used for extracts 1-6.

lack of absorbencies at 820 and 830 cm^{-1} in all the spectra suggests that these samples were not galactans of only one common type. However, extracts-4, 5 and the commercial carrageenan had a small, but prominent, band at 805 cm^{-1} showing iota-carrageenan type structure present in these phycolloids (Chiovitti et al., 1996), while other spectra displayed complete elimination of this absorption band. Greer et al. (1984) found that polysaccharides obtained from H. *musciformis* comprised of 73% of kappa-carrageenanand 17% of iota-carrageenan. Analysis of carrageenan from different algal sources has revealed hybrid nature of these polymers (Bellion *et al.*, 1982). A careful examination of all the spectra leads to the conclusion that carrageenans derived from H. *musciformis* had features of both kappa- and very small portion of iota-carrageenan.

Seaweed polysaccharides have been reported as elicitors of plant defence mechanism in the tissues of chickpea in terms of induced browning and phytoalexin production (Bi and Iqbal, 2003). On the basis of chemical composition and IR spectral studies, Extract-5 was noted to be a close representative of kappa-carrageenan and was investigated for its elicitor activity in the tissues of garden peas (*Pisum* sativum). For this purpose, pea tissues were inoculated with 100 µg glu eq/ml preparation of elicitor **(Extract-5)**. After 24 h of incubation, high intensity of browning was produced in the treated samples as compared to the control. Typical chromatogram (Fig. 2) showed the resolution of alcoholic extract of elicited tissues of peas using HPLC analysis. The prominent and sharp peak-A represents induced secondary metabolites eluted in the organic phase (100% acetonitrile). It is reported that the phytoalexin 6α-hydroxy pterocarpan and phenolic contents, as well as the enzyme activity, in peas was increased on fungal infection and elicitor treatment (Katoch et al., 2002; Banks and Dewick, 1982).

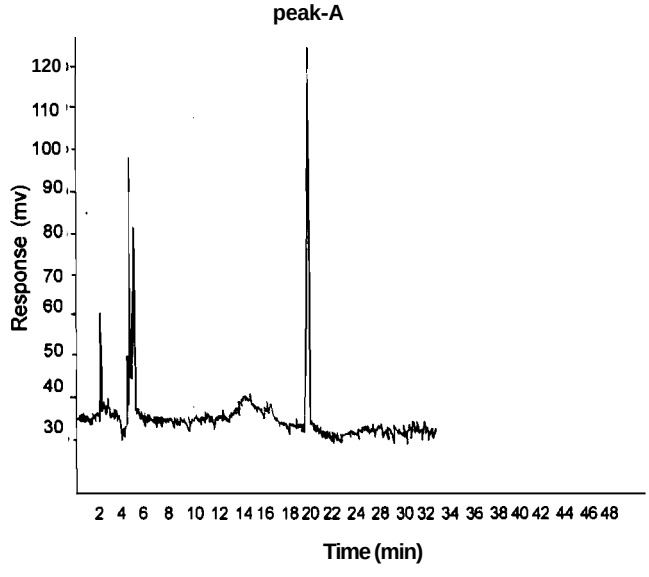

Fig. 2. HPLC separation of induced secondary metabolites (peak-A) in pea cotyledons treated with elicitor preparations (polysaccharide) of Hypnea musciformis.

The present studies indicated that seaweeds of Karachi coast can be utilised for the production of the commercially usable carrageenans, which have extensive application in various industrial sectors.

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