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CHEMICAL INVESTIGATION AND ELICITOR ACTIVITY OF POLYSACCHARIDE OF RED ALGAE *HYPNEA MUSCIFORMIS* AND *BOTRYOCLADIA LEPTOPODA*

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Hypnea musciformis and *Botryocladia leptopoda* (red algae), collected from Karachi coast were studied for chemical investigation and elicitor activity. Ash content of *H.musciformis* was bit high (40%). Yields of High Molecular Weight Crude Elicitor Preparations HM.WCEP "Polysaccharides" of two algal genus were high (14-49%) in NaOH extracts. These HMWCEP were chemically analysed for total sugar protein, SO_4 group and uronic acid contents. Simple profile of monosaccharide consisting of galactose, glucose, fucose, mannose and galactoronic acid were detected in acid and aqueous extracts of *H. musciformis* and *B. leptopoda* respectively as compared to its alkaline extracts. Elicitor activity of HMWCEP was determined in terms of induced browning in *Cicer arietinum* (chick pea) tissues. A pronounced browning was produced by the samples treated with various extracts of *H. musciformis*. The response was low in the samples treated with various preparations of *B. leptopoda*.

Key words: Seaweed, Elicitor, Phytoalexin, Browning.

Introduction

Natural or synthetic molecules which are able to elicit induced resistance against diseases in plants (Bailey 1982; Darvill and Albersheim 1984) are grouped together and known as "Elicitors" (Keen *et al* 1972). Substances of microbial origin including polysaccharides, proteins and fatty acids (Clarence and Edward 1991; Vogelsang *et al* 1994; Castoria *et al* 1995) induce hypersensitive responses in terms of induced browning (Theodorou and Smith 1979) and phytoalexin production (Iqbal and Paxton 1991; Koga *et al* 1996) along with some other responses. It is previously reported that in most of the cases elicitor activity was associated with the polysaccharide fractions of various preparations. Seaweeds are generally comprised of 40 - 69% of carbohydrate. The major interest of this study was to exploit these polysaccharides as an inducer of hypersensitive responses. Karachi has a large coastal area and produces a huge amount of marine algae. The purpose of the present work is to follow the extraction process quantitatively. High Molecular Weight Crude Elicitor Preparations "HMWCEP" were obtained by ethanol precipitation and lyophilization of polysaccharides of various seaweed extracts. Chemical composition in terms of total sugar, protein sulphate group and uronic acid contents was determined Initially these HMWCEP were evaluated for their elicitor activity in terms of induced browning.

Materials and Methods

Collection and pretreatment of algae. Two algal varieties, *Hypnea musciformis* and *Botryocladia leptopoda* of class Rhodophycea were collected from Hawksbay, coastal area of Karachi in the month of November 1993 and February 1994. Fresh seaweeds were collected, washed with running tap water and air dried under shade.

Water moisture and ash content of seaweed. 100 gram of each fresh sample was thoroughly washed and dried at room temperature under shade, till constant weight. One gram of air dried sample was heated at 110 °C in the oven till constant weight and moisture content was determined. 0.5 gram of each sample was heated on burner to remove soot and then burnt in furnace at about 600-800 °C for about 2 h then another 2h heating was carried out for complete ashing.

Sequential extraction of red algae. Aqueous extraction cold/hot). One gram of dried sample was finely chopped and dipped into 100 ml distilled water, magnetically stirred at room temperature for 16-18h for an exhaustive extraction filtered through Whatman filter paper No. 1. The residue was re-extracted with 100ml water at 60-65 °C for another 12-16 h filtered and stored at -20 °C.

Alkali and acid (cold/hot) extraction. Another 1 g of the substance was subjected to cold/hot extraction under similar conditions outlined above using 100ml of 0.1N NaOH and 0.1N HCl, extracts were stored frozen. In this way six extracts of each sample and a total of 12 extracts were prepared.

Crude elicitor preparations and chemical analysis of HMWCEP. Acid alkali and aqueous (cold/hot) extracts of each algal variety were treated with 1:3 volume of distilled ethanol and stored at 4-6 °C for 3-4 days to precipitate out polysaccharides. The supernatant was discarded and the pre-

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cipitate, High Molecular Weight Crude Elicitor preparation "HMWCEP" was collected by centrifugation at 3000-4000 rpm for 20-30 min dissolved in minimum quantity of water and lyophilised to give the dry weight of the material (Table 1).

Total carbohydrate content of these HMWCEP was determined by phenol sulfuric acid (Dubois *et al* 1956). Total protein was determined by Bradford method (1976) and bovine serum albumin was used as standard. Assay for sulphate group was done by the Dodgson's method (1961). Total acidic sugar was determined by the method employed by Bitter and Muir (1962).

Acid hydrolysis and monosaccharide composition of HMWCEP. 100µl of stock (1mg ml^{-1}) solution of HMWCEP of each algal variety of Rhodophycae were taken into air tight sealed tubes and dried over KOH pellets in a desiccator maintained under vacuum. 100µl of 90% formic acid was added, flushed with nitrogen and heated at 100°C for 8h. After hydrolysis samples were left overnight in vacuum desiccator over P_2O_5. Dried hydrolysate resuspended into 50µl of distilled water Paper chromatography was carried out on Whatman No.1 filter paper with the following solvent system, Butanol: Acetic acid: Water (4:1:5). Chromatograms were developed with silver nitrate reagent (Georg and William 1974).

Germination of seeds and elicitor activity. A general method of elicitor application was employed in all experiments as previously described by Whithead *et al* (1982). Chickpea *(Cicer arietinum)* were germinated on filter paper placed on cotton hed in a tray, incubated at 25 °C in the dark. The excised cotyledons (2-3 days) were surface sterilised by immersion in 1% Sodium hypochlorite for 2 min and then washed extensively with distilled water, finally rinsed with sterile water elicitor preparation at a concentration of 100µg glc eq ml^{-1} were used. Treated and control samples were prepared by application of 20µl of test solution and sterile H_2O (control) to the cut surface of cotyledons placed in a petri dish (10-15 cotyledons) on a moistened filter paper and incubated at 25 °C in the dark for a specified period of time (24h).

Table 1

Sequential extraction and yield of HMWCEP ($g\%\ w\ w^{-1}$)

Name of Algae	Water ext		NaOH ext		HCl ext	
	Cold	Hot	Cold	Hot	Cold	Hot
Hypnea musciformis	1	17	32	49	20.1	1.3
Botryocladia leptopoda	6	1	14.2	8	10	-

Results and Discussion

Two algal varieties *Hypnea musciformis* and *Botryocladia leptopoda* family Rhodophyceae were collected from Karachi coast, however *B.leptopoda* was found in small quantity. It was observed that generally collected samples were mixed with other varieties of algae, dominated by one variety. Water content of *B.leptopoda* and *H.musciformis* was high, 95.2% and 91.2% respectively, probably due to its beaded and capsulated structure. Similarly the moisture content of these two varieties was also high i.e. 11.6 and 10.8%. It is likely that the sea salts retained and absorbed water from the atmosphere at the later stages on storage. Ash content of the samples were determined and surprisingly *H. musciformis* showed a higher ash content (40%) as compared with the values reported earlier (Qari 1988).

Separation of polysaccharides in algae is often possible by sequential extraction and it also provides information on the range of polysaccharide present (Percival and Mcdowell 1990). Higher yields of HMWCEP were observed in (cold/hot) NaOH extracts of both the varieties ranging from 14-49%. Hot aqueous and cold acidic extracts of *H.musciformis* showed an increased amount of HMWCEP i.e. 17% and 20% respectively. Yields were low (1-10%) in other extracts of *B.leptopoda* and *H. musciformis* as mentioned in Table 1.

Chemical composition of HMWCEP showed that higher sugar contents 24-33% and 23-45% were observed in NaOH extracts of both the algal plants Major difference of sugar content (8-64%) was observed in cold/hot fractions of aqueous extracts of *H.musciformis.* Results in Table 2 suggested that total sugar content (cold & hot) of acidic and aqueous fraction was high but overall yield of HMWCEP of these extracts was low (Table 1) whereas yields of NaOH ware high along with the higher sugar content. It appeared that under specified conditions NaOH could be the best extracting media, not necessarily with the best elicitor activity.

Protein was found in all the cold/hot fraction of *H.musciformis* ranging from 0.9-5.5%. Only one fraction aqueous (cold) extract of *B. leptopoda* showed a protein content of 4%. Likewise plants, the nature of carbohydrate protein linkages in seaweeds is not deduced yet (Thompson and Preston 1967).

It is reported in literature that plants belonging to Rhodophycea are commonly sulphated galactan (Percival 1978). Analysis showed that a higher (20-80%) SO_4 content was found in all extracts of *H. musciformis* and *B.leptopoda.* Uronic acid was found in each extract of the two algae especially high contents (3-6.4%) were observed in acidic extracts of *H. musciformis.*

Table 2
Chemical composition of HMWCEP of red algae

Name of algae	Extracts	Expressed as g%							
		Sugar		Protein		Uronic acid		Sulphate	
		C	H	C	H	C	H	C	H
Hypnea musciformis	H_2O	8	64	1.2	1.5	1.6	2.8	79	29
	0.1N NaOH	24	33	0.9	2.3	1.0	1.4	17	46
	0.1N HCl	12	40	0.9	5.5	6.2	6.4	68	43
Botryocladia leptopoda	H_2O	32	30	4.0	–	2	0.6	6	22
	0.1N NaOH	23	45	NF	NF	2.2	2	70	47
	0.1N HCl	64	–	NF	NF	3.3	–	30	–

C, Cold; H, Hot,; NF, Not found

Table 3
Elicitor activity in terms of induced browning

Name of algae	Cotlyledons treated extracts	Elicitor activity	
		Cold	Hot
Hypnea musciformis	H_20	++++	+++
	0.1N NaOH	++	++
	0.1N HCl	++++	+++
	Control Wound	0	
	Control H_20	0/+	
Botryocladia leptopoda	H_20	+	+
	0.1N NaOH	++	+
	0.1N HCl	+	+
	Control Wound	0/+	
	Control H_20	0/+	

Data are mean of two replicates and findings of two determinations
Degree of browning = 0/+ for least browning.

The principal component sugar in the red algae previously reported as galactose, present as galactan or galactan sulphate (Percival 1978). These findings were found correct as after 6 h of hydrolysis galactose appeared as major component of the degraded products of various extracts of two algal varieties. A simple profile of monosaccharide was observed in aqueous and acidic extracts of these algae , whereas a complex mixture comprising of mannose, rhamnose, xylose and fucose were found in NaOH extracts of these preparations.

Induction of browning as a hypersensitive response in chickpea cell culture was previously reported in literature (Vogelsang *et al* 1994) In our experiment cut surfaces of etiolated cotyledons of chickpea were inoculated with the 100 µg glc eq ml^{-1} preparations of seaweed. After 24 h of incubation, browning was produced by the samples treated with various extracts of red algae, although the extent of browning was different for each sample (Table 3). A significant browning was produced by the samples treated with aqueous and acidic extracts of *H. musciformis* whereas a little browning was produced by the alkaline extracts. The alkaline extracts of *B.leptopoda* showed either none or a small activity. As we observed that sugar with greater content of SO_4 i.e. aqueous and acidic fractions of *H.musciformis* and the alkaline extract of *B.leptopoda* exhibited higher activity which suggested that some structural arrangements were more active but repeated experiments showed a positive and definite elevated browning in the samples treated with the HMWCEP of *H.musciformis* which indicated that overall browning induced by *H.musciformis* was many folds higher as compared to various preparations of *B.leptopoda.*

Conclusion

It is concluded that treated tissues of *Cicer arietinum* responded differentially to various preparations of algae. Acidic and aqueous extracts of *H.musciformis* showed higher elicitor acitivity. The extent of browning produced was different for various samples which could be due to the compositional differences of these preparations. More active fractions may have some unique structures which are more likely to interact with the receptor molecule.

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