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Antiviral Sulphated Polysaccharide from Brown Algae Padina pavonia Characterization and Structure Elucidation

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Abstract : The hot water extract of brown alga *Padina pavonia* was fractionated into a neutral fraction P1 and a sulphated heteropolysaccharaide P2. Based on the antiviral activity P2 was fractionated by size exclusion chromatography into EPS-1 and EPS-2 with molecular weight of 160 and 85 kDa, respectively. Fucose and galactose were major sugars in EPS-1. The chemical structure of EPS-1 was elucidate by using periodate and Smith's degradation methods. Xylose was found to be a branched point of main chain at C5 of xylofuranose linked by (1---3)-glycosidic linkages. While glucose residues are located in the terminal units of side chains and main chain linked mainly by (1---4)-glycosidic linkages. Also the results showed that mannose and fucose are present in the main and/or side chains and linked mainly by (1---4) and (1---3)-glycosidic linkages, respectively. EPS-1 showed a marked antiviral activity against both HSV and HAV. Its inhibition effect against HSV was 72.3 % where against HAV was 73.3% at concentration of 20 µg/ml.
Key words: *Padina pavonia*, polysaccharides, antiviral, HSV, HAV.

Introduction

Recently, macroalgae have been used as a novel food with potential nutritional benefits in industry and medicine for various purposes. Furthermore, macroalgae have shown to provide a rich source of natural bioactive compounds with antiviral, antifungal, antibacterial, antioxidant, anti-inflammatory, hyper-cholesterolemia and hypolipidemic and antineoplasteic properties (1).Thus, there is a growing interest in the area of research on the positive effect of macroalgae on human health and other benefits. It was found that carrageenan, an algal polysaccharide, had no effect on virus attachment or penetration into host cells, but the synthesis of viral proteins inside the cells was inhibited (2). Similarly other sulfated algal polysaccharides selectively inhibited reverse transcriptase (RT) enzyme of human immunodeficiency virus (HIV) and its replication in vitro. The extracts of air dried marine algae *(Ulva lactuca),* collected from beside the Bulgarian Black Sea, were tested for their antiviral activity (3). They studied their effect on the reproduction of influenza virus (H1N1) in cultures of chorioallantoic membranes from 12- to 14-day-old chick embryos. The results showed an inhibitory effect on the reproduction of the virus, with reduction of the viral infectious titer. A number of biological and synthetic sulfated polyanions were tested, such as heparin; inhibit the replication of various mammalian viruses (4). They suggested that these negatively charged molecules including the sulfated algal polysaccharides, exert their inhibitory effect by interacting with the positive charges on the virus or on the cell surface and thereby prevent the penetration of the virus into the host cells. It was reported that a sulfated galactan isolated from extracts of red algae was a selective inhibitor of herpes simplex virus (HSV-1 and HSV-2). The mode of action of sulfated galactan could be ascribed to an inhibitory action on virus adsorption (5).

It was reviewed that the antiviral mode of action of sulphated polysaccharide (SP2) isolated from the brown alga *Sargassum patens* on herpes simplex virus type 2 (HSV-2) could be ascribed to the inhibition of virus adsorption, which is different from that of the current drug of choice acyclovir (6). Marine algae are the most important source of non-animal sulfated polysaccharides. Furthermore, the structure of algal sulfated polysaccharides varies according to the species of algae (7). Thus, each new sulfated polysaccharide purified from a marine alga is a new compound with unique structures and, consequently, with potential novel biological activities. Depending on this fact, this work aimed to investigate the water soluble sulphated polysaccharides extracted from marine brown alga *Padina pavonia* growing on the shores of Jazan, KSA and study its antiviral activity.

Materials And Methods

Materials source

The brown alga, *Padina pavonia*, collected periodically, during (2010) from red sea, Jazan, KSA. After collection, the seaweeds were thoroughly washed with running water to remove foreign substances, air dried and finally ground (8). Algal powder was depigmented using sequential extraction with petroleum ether and acetone in a Soxhlet apparatus. The residual material was air dried to yield depigmented algal powder (DAP). Extraction of DAP (5 g) with 0.1 M HCl (1:100, w: v) was conducted at room temperature for 24 h under constant stirring (twice). The acid insoluble residue was extracted with 2% Na_2CO_3 (1:100 w: v) at 45–50 ºC for 5 h under constant stirring (9). .

Extraction with hot water

The alkali insoluble residual material was again extracted with water at 80 ºC for 2 h (twice) and the combined extract was dialyzed, concentrated in vacuum, and treated with 10% cetyl trimethyl ammonium bromide (CTAB) solution. The precipitate formed was centrifuged (30 min, 5000 rpm), washed with water and stirred with 20% ethanolic KI solution (3 × 50 ml). After washing with ethanol the precipitate was dissolved in water, dialyzed exhaustively and lyophilized to give P1. The superannuated was dialyzed, concentrated, and diluted with 4 volumes of ethanol. The precipitate formed was then dissolved in water and lyophilized to give a neutral polysaccharide P-2. Each fraction was subjected to antiviral assay.

Size exclusion chromatography (SEC)

The active fraction **P-2** was subjected to size exclusion chromatography (SEC) on a column (2.5 x 60 cm) of Sephacryl S-200. The void volume was determined using dextran blue. The column was equilibrated and later eluted with 0.1 M NaCl. An aqueous solution of sample (30 mg) was dissolved in 2.0 ml of 0.1 M NaCl and loaded on to the column bed. Fractions (5.0 ml) were collected and tested for the total carbohydrates by the phenol-H_2SO_4 reagent (10). Appropriate fractions EPS-1 and EPS-2 were pooled, dialyzed and lyophilized. The two fractions (EPS-1 and EPS-2) were subjected to antiviral assay.

Antiviral Activity

The polysaccharide was dissolved as 10 mg in 1mL of 10% DMSO in water. The final concentration was 10µg/µL (Stock Solution). The dissolved stock solution was sterilized by the addition of 10 µg/mL antibiotic antimycotic mixture (10 U penicillin G sodium, 10µg streptomycin sulfate and 250 µg amphotericin B) (11). A 6-well plate was cultivated with Vero cell culture (105cell/mL) and incubated for 2 days at 37°C. HAV and HSV were diluted to give 104 PFU/mL final concentrations and were mixed with the tested polysaccharide at two concentrations and incubated 1 h at 40 °C. Growth medium was removed from the multi well plate and virus- polysaccharide mixture was inoculated (100 µL/well). After 1 h of contact time, the inocula were aspirated and 3 mL of DMEM with 1% agarose was overlaid on the cell sheets. The plates were left to solidify and incubated at 37 °C until the development of virus plaques occurred. Cell sheets were fixed in 10% formalin solution for 2 h, and stained with crystal violet stain. Control virus and cells were treated identically without polysaccharide. Virus plaques were counted and the percentage of reduction was calculated.

Molecular weight determination of EPS1 and EPS-2

The average molecular weight of the fractions EPS-1 and EPS-2 were determined by a size exclusion chromatography (SEC). Standard dextrans (40, 500, 700 and 2000 KDa, Fluka Chemical Co., Bush, Switzerland) were passed through a (2.5 × 60 cm) Sephacryl S-200 column, and then the elution volumes were plotted against the logarithm of their respective molecular weights (12).

Sugar analysis of EPS-1

Total sugars and uronic acids were determined by the phenol-H_2SO_4 reagent (9) and *m*-hydroxydiphenyl (13), respectively. The polysaccharides EPS-1 was hydrolyzed with 2 M trifluoroacetic acid in a sealed tube. Hydrolysis was carried out at 105 ºC for 2 h. After the hydrolysis, the acid was removed by flash evaporation on a water bath at a temperature of 40 ºC and co-distilled with water (5 ml × 3) (14). The purified hydrolyzates (20 µl) were analysis by HLPC as described before (15).

Desulphation of polysaccharide

The polysaccharide (150 mg) was treated with 2% MeOH/HCl (20 ml) for 72 h at 25ºC and then dialyzed against deionized water followed by freeze drying (EPS-ID) (16).

Sulphate estimation

The polysaccharide (100 mg) was hydrolyzed in a sealed tube with 1 ml HCl (1 M) at 85°C for 24 h. Then it was neutralized to pH 7.0 with NaOH solution and diluted to 10 ml with distilled water. The sulphate content was determined by modified barium chloride method (17).

Infra red spectroscopy

The polysaccharide fractions EPS-1 and the other desulphated EPS-ID fractions were also characterized using a Fourier transform infrared in Burcker Vector 22-spectrophotometer. The dried polysaccharides were ground with KBr powder and pressed into pellets for FT-IR spectra measurement in the frequency range of 100-4000 cm^{-1} (17).

Periodate oxidation and Smith degradation of EPS-1

Samples of EPS-1 in addition to desulphated fractions EPS-ID (20 mg) were added separately to 30 ml $NaIO_4$ (0.1 M) solution in round bottom flasks, and the mixtures were kept at 4ºC in the dark (18). Aliquots (2 ml) were removed after different times and the consumption of periodate and formic acid produced was determined (19). After complete oxidation, the oxidation process was stopped by the addition of ethylene glycol and the solutions were dialyzed. The polyaldehyde was reduced with excess of $NaBH_4$ at room temperature overnight. The reaction was terminated by addition of ice cold of 4N acetic acid; the solutions were dialyzed again and lyophilized (20). A portion of the resulting polyalcohol-polysaccharide was hydrolyzed with formic acid 90% for 5 h. The sugars and sugar alcohols were determined by HPLC (15).

Methylation analysis of EPS-ID

The polysaccharide (EPS-ID) was methylated separately (21). The methylated products were isolated by partition between $CHCl_3$ and water (5:1, v/v). The products were then hydrolyzed with 90% HCOOH (5 ml) for 5 h, and excess HCOOH was evaporated by co-distillation with distilled water. The hydrolyzed products were then reduced with $NaBH_4$ (24 mg) and acetylated with pyridine-acetic acid (22). The alditol acetate of the methylated sugars was analyzed by GC-MS Finnegan SSQ-7000 instrument using column (DB-5, 0.25 mm ID, 30 m) (23). Qualitative and quantitative identification of the methylated sugars by comparing retention time and mass fragmentation patterns with those of the available authentic data base were performed.

Results And Discussion

Isolation and purification of polysaccharide from DAP

The central goal of this study was determination of the polysaccharides classes present in *P. pavonia*. Sugar composition of depigmented algal powder (DAP) was determined. Algal glucans are water soluble, but their solubility depends on the temperature of the medium. The less branched glucans are soluble in warm water (80 ºC), but are usually neutral. On the other hand, the anionic polysaccharides form insoluble salt with detergents such as CTAB. Therefore, attempts have been made to separate glucan from other anionic polysaccharides present in the hot water by taking advantage of their differential solubility. Indeed, CTAB separates the crude hot water extracted polymers into two fractions **P-1**and **P-2**. The neutral fraction **P-1** consisted of glucose as the only neutral sugar. Based on calibration with standard dextrans, the molecular weight of this glucan would be 60 kDa. This glucan containing fraction (P-1) does not produce blue coloration with iodine. On the other hand, P-2 consisted of fucose, xylose, galactose and glucose. The crude polysaccharide (P-2) was separated by size exclusion chromatography on Sephacryl S-200 column, into two fractions, EPS-1 and EPS-2 was eluted with NaCl solution **(Figure 1).** Moreover, the average molecular weights of EPS-1 and EPS-2 were around 160 and 85 kDa, respectively. The EPS-1 found more active as antiviral against HAV and HSV.

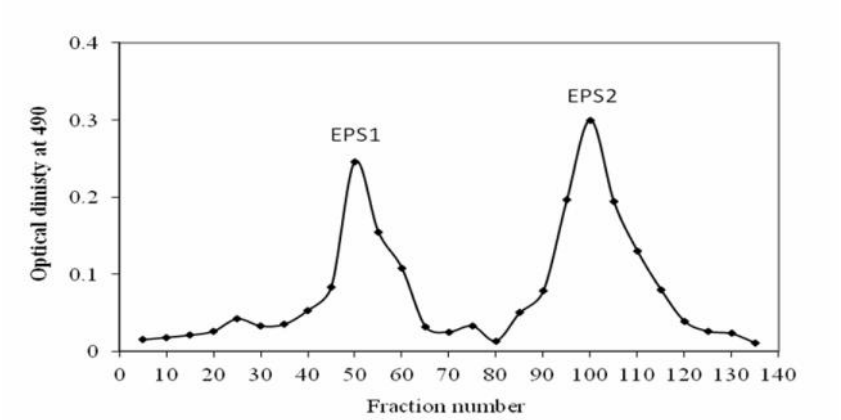

Figure 1: Size exclusion chromatography of P-2 on Sephacryl S-200 column.

Compositional analysis

The monosugars content of EPS-1 and EPS-2 were obtained by formic acid hydrolysis followed by HPLC analysis. fucose, xylose, galactose and glucose were detected with molar ratios of (6.1: 1.0: 2.0: 1.1) and (3.7: 1.0: 0.8: 4.7) in EPS-1 and EPS-2, respectively. Fucose and galactose were the major sugar in the fraction EPS-1, glucose was the major sugar in the fraction EPS-2 (**Table 1).**

Desulphation and reduction

After desulfation of EPS-1 the yield was about (55%). The desulphated polysaccharides coded EPS-ID. The native and desulphated polysaccharide fractions indicated that the backbone of the former was unaffected by desulphation (**Figure 2).**

Table 1: Sulphate percentages, monosugar molar ratios of sulfate polysaccharide fractions obtained from column chromatography

Fraction	$SO^{=}_{4}$(%)	Molar ratio			
		Fucose	Xylose	Galactose	Glucose
P-2	18..58	7.4	1.0	2.3	4.9
EPS-1	25.10	6.10	1.0	2.0	1.1
EPS-2	7.48	3.7	1.0	0.8	4.7

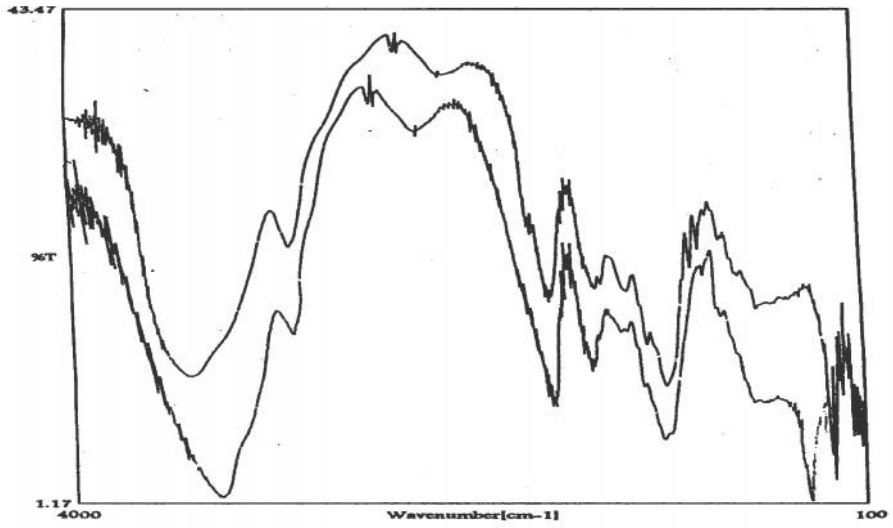

Figure 2: Infrared spectra of EPS-1 and EPS-1D from *P. pavonia*

Periodate oxidation and smith's degradation

The EPS-1 and EPS-1D showed abundance $NaIO_4$ uptake, during oxidation. The consumption of periodate by (EPS-1) and (EPS-1D) were (0.47) and (0.74) respectively. The low periodate consumption by (EPS-1) refers to some resistance of the polysaccharide molecules, towards oxidation. This resistance could be attributed to branching of the polysaccharide molecules, and due to presence of (1---3) linked sugar units, and the presence of sulfate groups masking one or more of the vicinal hydroxyl groups. The periodate consumption of desulphated fraction may be less than one mole periodate per one mole anhydrosugar unit, because some of these units may not be affected. These results agreed with the immune sugars during the smith's degradation experiment. Reduction of the resulting polyaldehyde with $NaBH_4$ followed by hydrolysis and HPLC analysis, revealed the presence of erythritol, thritol, and glycerol in addition to free fucose and xylose as shown in **Table (2).** The formation of theritol demonstrated the presence of (1---4)-linked galactose residues, whereas the detection of theritol indicated the presence of glucose as non-reducing end of the chains. The formation of erythritol demonstrated the presence of (1---4)-linked glucose residues. The detection of unreduced xylose and fucose indicated that some of these sugars residues are resistant towards periodate oxidation. The previous detection of sulfate-free xylose in the exterior portion and the resistant of a high proportion of xylose residues towards periodate oxidation, suggest the presence of (1---3)-linked xylose residues. On the other hand, the detection of relatively small quantities of glycerol in the polyalcohol hdrolyzate confirmed the presence of glucose as the (1---4)-linked at non-reducing terminus **(Table 2)**. The appearance of an immune portion of fucose accords with the previous demonstration which showed the presence of (1---3)-linked fucose residues (24- 27).

Table 2 HPLC results of Smith's degradation of EPS-1 and EPS-ID

Fraction	**Molar ratios**						
	Fucose	Xylose	Glucose	Galactose	Thritol	Erythritol	Glycerol
EPS-1	6.2	1.0	1.7	0.8	0.3	0.2	0.8
EPS-1D	6.1	1.0	0.2	0.1	1.5	1.7	1.3

Fourier transforms infra red spectroscopy (FTIR)

FTIR spectra of (EPS-1) and (EPS-1D) were similar and showed an intense band of absorption at 1240-1260 Cm^{-1} indicating the presence of sulfate ester (28). Two other bands 791 and 852 Cm^{-1} arising from the sulfate group of polysaccharides were also observed. On the other hand, IR spectra of the desulfated polysaccharide fractions showed reduction of these bands (Figure 2). The absorption at 890cm^{-1} indicated the β-glycosidic linkages of polysaccharide fractions (29)

Glycosidic Linkages

The EPS-1D was methylated as describe before (21), and then hydrolyzed with acid. The alditole of methylated products were analyzed by GC-MS using a DB-5 capillary column. The EPS-ID gave five components namely 2,3,6-tri-O-methyl-glucose; 2,3,6-tri-O-methyl-galactose, 2-O-methyl-xylose; 2,3,4,6-tetera-O-methyl-glucose and 2,4-dio-O-methyl-fucose in molar ratio of 1.57: 0.95: 1.00: 0.47: 7.85, respectively (**Table 3).** The results of methylation analysis of the derivatives EPS-1D suggest that the xyloses are in furanose form and are (1--3,5)-linked, due to presence of 2-O-methyl xylose, in addition to the presence of xylose as immune units in smith's degradation results (30). It could be said that xylose is a branched point of main chain at C5 of xylofuranose linked by (1---3)-glycosidic linkages. In addition to the appearance of 2,3,4,6 tetra-O-methyl glucose and 2,3,6-tri-O-methyl glucose indicating that glucose residues are located in the terminal units of side chains and main chain linked mainly by (1---4)-glycosidic linkages. These results agreed with appearance of glycerol and erythritol in the Smith's-degradation results. The presence of 2,3,6-tri-O-methyl galactose and 2,4-di-O-methyl fucose suggests that mannose and fucose are present in the main and/or side chains and linked mainly by (1---4) and (1---3)-glycosidic linkages, respectively. These results agreed with the appearance of fucose as immune unit and erythritol in the Smith's results (24, 31, 30, and 32).

Table 3: Linkage analysis of the constituent sugars of EPS-ID

Methylated sugars	Mode of linkages	Mass fragments *(m/z)*	Molar ratio
2,3,6-tri-*O*-methyl-glucose	--1) Glc (4---	43,45,59,71, 87, 101, 117, 129, 145, 161, 205	1.57
2,3,6-tri-*O*-methyl-galactose	--1) Gal (4---	43,45,59,71, 87, 99, 113, 129, 143, 161, 201	0.95
2-O-methyl-xylose	--1) Xyl (3,5--	43,45,59,71, 87, 101, 117, 129, 145, 161, 205, 173, 233	1.0
2,3,4,6-tetra-*O*-methyl-glucose	Glc (1-----	43,45,59,71, 87, 101, 117, 142, 161, 201, 261	0.47
2,4-di-*O*-methyl-fucose	--1) Fuc (3---	43,45,59,71, 87, 101, 117, 129, 143, 159, 201, 261	7.85

Table 4: *In-vitro* antiviral activity of polysaccharides isolated from *P. pavonia* against HSV and HAV

Fraction	Conc. µg/ml	Anti-viral activity					
		HSV			HAV		
		Initial virus (PFU/ml)	Final virus (PFU/ml)	Inhibition (%)	Initial virus (PFU/ml)	Final virus (PFU/ml)	Inhibition (%)
P-1	10	0.94×10^6	0.91×10^6	3.2	1.2×10^6	1.16×10^6	4.2
	20	0.94×10^6	0.87×10^6	7.5	1.2×10^6	1.1×10^6	8.3
P-2	10	0.94×10^6	0.68×10^6	27.0	1.2×10^6	0.9×10^6	25.0
	20	0.94×10^6	0.61×10^6	35.9	1.2×10^6	0.81×10^6	32.5
EPS-1	10	0.94×10^6	0.32×10^6	65.0	1.2×10^6	0.52×10^6	56.7
	20	$0.9\ 4 \times 10^6$	0.26×10^6	72.3	1.2×10^6	0.32×10^6	73.3
EPS-2	10	0.94×10^6	0.75×10^6	20.2	1.2×10^6	1.0×10^6	16.6
	20	0.94×10^6	0.69×10^6	26.6	1.2×10^6	0.98×10^6	18.3

***In-vitro* antiviral activity**

The crude polysaccharides (P-1 and P-2) as well as fractions (EPS-1 and EPS-2) were evaluated for their antiviral activity against HSV and HAV by a virus plaque reduction assay as shown in **Table (4).** The fraction EPS-1 showed a marked antiviral activity; this fraction was more active than others. An explanation of these results may be indicating that the fraction EPS-1 has a higher amount of sulfate ester groups and high molecular weight than the other fraction. In general, the antiviral activity of sulfated polysaccharides increases with the degree of sulfation and molecular weight (33). A next point of interest is the influence of the distribution of sulfate groups along polymer chain and the conformational flexibility of this chain for adopting a definite shape that might be required during the formation of polysaccharide-virus complex (34). It is likely that post-infection involved inhibition of early post absorption steps such as virus internalization and inhibition of cell-to-cell transmission in successive cycles of replication (35).

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