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Isolation, fractionation and modification of sulfated polysaccharides from *Spirulina platensis* and its antitumor activity

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ABSTRACT

The water-soluble polysaccharide obtained from a blue-green alga *Spirulina platensis* by boiling water extraction and ethanol precipitation (118.25g kg^{-1} on dry weight basis) was fractionated by DEAE-cellulose column chromatography, and purified by Sephacry S-300 giving two active fractions termed Ca-SPIIa and Ca-SPIIb. These fractions were mainly composed of glucose, galactose, rhamnose, fructose, glucouronic acid, mannose, xylose with the molar ratio (6.5: 3.3: 50.0: 38.5: 9.6: 1.5: 1.0) and (7.2: 3.5: 55.0: 40.2: 8.5: 2.0: 1.0) and sulfate percent (2.25% and 3.47%, respectively). Calcium ion binding with the anionic part of a molecule was replaced with various metal cation and their inhibitory effects on Ehrlich Ascites Carcinoma Cells (EACC) was determined. The Ca-SPIIb was able to kill 95% of the tumor cells when they were incubated with 200µg. While, replacement of Ca^{+2} with Na^{+}, Ag^{+}, Cu^{+2} and H^{+} reduced the activity.

KEYWORDS

Spirulina platensis;
Blue-green alga;
Antitumor activity;
Ehrlich ascites carcinoma cells;
Polysaccharide.

INTRODUCTION

Spirulina platensis is a blue-green alga growing in some African and central and South American lakes rich in salts. *Spirulina* contains much good-quality protein as well as carotenoids, vitamins and minerals; it has received attention as a most promising and nutrition food source[12]. *S. platensis*, which belongs to blue-green alga, regionally has been used as a source of nutrition food in some African areas[3]. It also exhibits a variety of biological properties such as reduction of hypercholesterolemia[21], enhancement of immune responses[4,13], prevention of oral cancer[27] and inhibits cancer cell proliferation and induces apoptosis[28]. Ca-SP is a novel sulfated polysaccharide isolated from a hot water extract of a blue-green alga *S. platensis*[12,14]. Calcium sulfated polysaccharide consists of rhamnose, 3-O-methylrhamnose, 2,3 di-o-methyl rhamnose, 3-o-methylxylose, uronic acid, sulfate and calcium ion[23], and includes two types of disaccharide repeating units, o-methyl-rhamnose and o-hexuronosyl rhamnose[16,23,24]. In vitro it has been shown that Ca-SP stimulates heparin cofactor II, a physiological inhibitor of thrombin, and exhibits antithrombin activity[15], by a unique mechanism different from that of heparin[16,17]. Furthermore, Ca-SP acts on cultured human fetal lung

fibroblasts and promotes the synthesis of tissue plasmingen activator but not that of plasmingen activator inhibitor type[18]. These results suggest that Ca-SP can be an origin of antitheromnogenic, fibrinolytic and anti-atherogenic medicines. Previous studies have shown that Ca-SP inhibited the replication of several enveloped viruses, including herpes simplex virus type I (HSV-1), human cytomegalovirus, measles virus, mumps virus, influenza virus and human immunodeficiency virus-1 (HIV-1)[12,19]. However, other biological properties of Ca-SP, such as its effect on tumor invasion and metastasis, are still unknown. In the present study, isolation and purification of a new sulfated polysaccharide from *S. platensis* and the investigation of the effect of fractions and modified polysaccharides on tumor cells.

MATERIALS AND METHODS

Algal source

The biological material used in this investigation was the cyanobacterium *S. platensis*. This organism was obtained from algal collection, at the University of Mansoura, Egypt. The axenic culture of *S. platensis* was maintained in 2 L Erlenmeyer flasks containing the nutrient medium described by Abida and Gawa[1] 6-7 days-old culture was used as inocula. The culture flasks were incubated for 15 days at 25 ± 2ºC under continuous illumination (using florescent lamps) at 4000 lux. At the expiry of the experimental period (15 days), the culture biomass was separated from its medium by centrifugation at 10000rpm for 20 min (Sigma-Laborzentrifugen, 2K 215), washed several times with bidstilled water to dissolve the salts and frozen at -20ºC.

Isolation and purification of sulfated polysaccharide

The freezed-dried powder of *spirulina* was extracted with boiling water for 1h. After centrifugation at 5000 rpm for 10min, the precipitate was treated with the same way. The extracts were treated with 10% trichloroacetic acid (TCA) (Hayashi et al. 1996b). The soluble fraction polysaccharide obtained from the (TCA) was dialyzed against distilled water for 24 h. The supernatant concentrated and precipitated with 4-volumes of alcohol, the precipitate was collected by centrifugation and freeze dried. The crude polysaccharide was dissolved in 0.01M Citrate buffer pH (7.0, containing 0.1 M NaCl) was applied to a column (2.4×40 Cm) of Sephacryl S-300. The column was eluted with the same buffer[12] and fractions of (5 ml) were collected and analyzed with phenol-suforic acid reagent at 490 nm[5] and protein at 280 nm[29] using a Shimadzu UV-vis spectrophotometer, model 2401CP. The second fraction was concentrated, dialyzed against distilled water to remove NaCl and then again freeze dried to a colorless powder (Ca-SPII). Ca-SPII and was dissolved in water and then applied to a column (2.0×40 Cm) of DEAE-cellulose. After elution with water, the column was eluted with continuous gradient from (0.0-3.0 M NaCl and fractions of (5 ml) were collected and tested for carbohydrates and proteins as above. The fractions (Ca-SPIIa and Ca-SPIIb) were re-chromatography on a Sephacryl S-300 column (2.0×40 Cm) by 0.01M Citrate buffer pH 7.0 containing 0.1M NaCl. Fractions were combined, concentrated, dialyzed and freeze dried.

Analysis of component sugars

Sample (5 mg) was hydrolyszed with 90 % HCOOH (1 ml) for 5 h at 100ºC in a sealed glass tube[8]. After evaporation to dryness, the hydrolyzate dissolved in 1ml water and the purified hydrolyzates (20 µl) were applied to a Simadzu LC10A HPLC[7]. Determination of sulfate in the hydrolyzate solution by Barium Chloride-Tween 20 reagent[9].

Determination of molecular weight

The average molecular weight of the polysaccharide was estimated by gel filtration using standard dextran 2000000, 700000 and 40000 (Fluka)[11].

Desulfation of polysaccharides

The polysaccharide (100 mg) was treated with 2% MeOH/HCl (20 ml) for 12h at 25ºC and then dialyzed against deionized water followed by freeze drying[30].

Removal of calcium

Ca-SPIIb dissolved in water was applied to a cation exchange column (1.5×15cm) on Dewex 50w×-8 (H^+ form) and eluted with water. The polysaccharide containing fractions were combined and freeze dried to give a white powder H-SPIIb[12].

Regular Paper

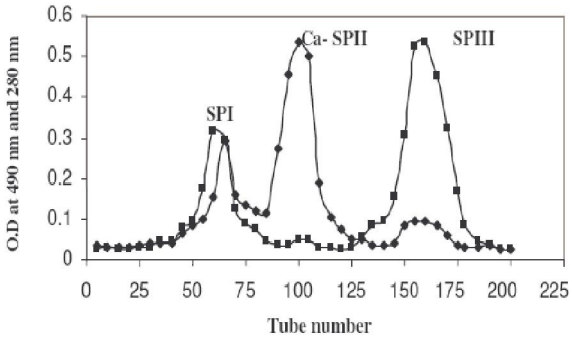

Figure 1 : Elution profile of SP by Sephacryl S-300 column chromatography (2.4×40Ccm), (-■-) absorbance at 280 nm; (-◆-) absorbance at 490 nm

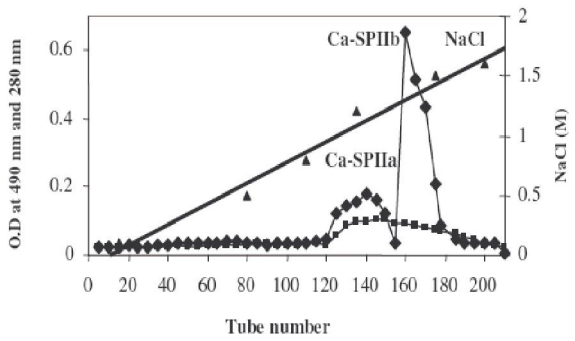

Figure 2 : Elution profile of Ca-SPII by DEAE-cellulose. (-■-) absorbance at 280nm, (-◆-) absorbance at 490 nm

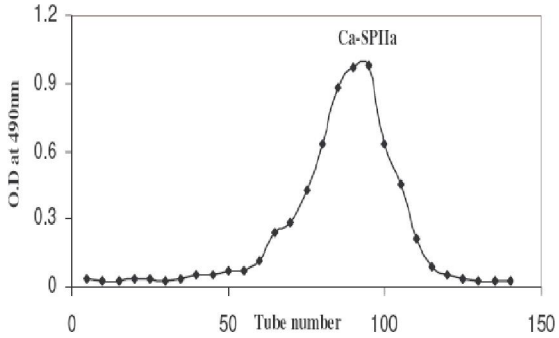

Figure 3 : Gel filtration for the chromatography of Ca-SPIIa on Sephacryl S-300 (2.0×40Cm).The column was equilibrated with 0.01 M Citrate buffer, pH 7.0 containing 0.1 M NaCl at flow rate 0.5 ml/min and 5 ml fraction

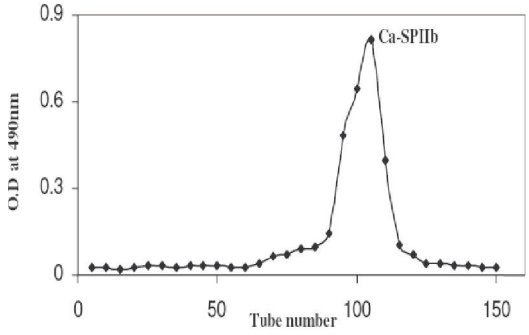

Figure 4 : Gel filtration for the chromatography of Ca-SPIIb on Sephacryl S-300 (2.0×40Cm), the column was equilibrated with 0.01 M Citrate buffers, pH 7.0 containing 0.1 M NaCl at flow rate 0.5 ml/min and 5 ml fraction

Exchange of metal ions

A Dowex 50wx 8 column (1.5×15 cm) was pre-equilibrated with each metal salt solution and the solution of Ca-SPIIb in water was passed through this column. The eluted solutions were freeze dried to give metal-exchanged sulfated polysaccharides[24]. The products were hydrolysis by 65% nitric acid and metal elements were analyzed by atomic absorption spectrometer model Spectrometer AA, Varian.

Viability of ehrlich ascites carcinoma cells (EACC)

The tumor cell line

The original tumor cells were obtained from National Cancer Institute, Cairo University.

Viability of tumor cells

This was performed using an *in-vitro*. Viability of tumor cells was measured by a modified cytotoxic trypan blue exclusion technique[2].

Statistical analysis

The obtained data were subjected to One-way ANOVA and the differences between means were at the 5% probability level using Duncan's new multiple range tests. The software SPSS, version 10 (SPSS, Richmond, USA) was used[6].

RESULTS AND DISSCUTION

Isolation and purification of sulfated polysaccharide

Freeze-dried powder of *S. platensis* was extracted with boiling water, and the hot water extract was treated with 10% TCA. The crude polysaccharide (SP) was obtained from the TCA-soluble fraction by dialysis three times against bi-distilled water followed by lyophilization (118.25g kg^{-1}). Gel filtration of crude polysaccharide on Sphacryl S-300 gave three fractions as shown in figure 1. Among these fractions, only Ca-SPII had

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TABLE 1 : Monosugars molar ratios of Ca-SPIIa and Ca-SPIIb obtained from columns chromatograph

Fraction	Molar ratio						
	Glucose	Galactose	Rhamnose	Fructose	Glcouronic acid	Mannose	xylose
Ca-SPIIa	6.5	3.3	50.0	38.5	9.6	1.5	1.0
Ca-SPIIb	7.2	3.5	55.0	40.2	8.5	2.0	1.0

antitumor activity against Ehrlich ascites carcinoma cells (EACC). Ca-SPII was then subjected to an ion-exchange column chromatography on DEAE-cellulose (Figure 2). After elution with water, the two fractions Ca-SPIIa and Ca-SPIIb were eluted with NaCl ranging (1.0 to 1.3 M) and (1.4 to 1.5), respectively. Then further purified by a column chromatography on Sephacryl S-300 to give a colorless polysaccharide Ca-SPIIa and Ca-SPIIb figure 3 & 4.

Chemical composition and molecular weight of sulfated polysaccharide

Acid hydrolysates of the two fractions SPIIa and SPIIb by HCOOH and analysis by HPLC were suggested to contain glucose, galactose, rhamnose, fructose, glucouronic acid, mannose, xylose. The molar ratios of these components were (6.5: 3.3: 50.0: 38.5: 9.6: 1.5: 1.0) and (7.2: 3.5: 55.0: 40.2: 8.5: 2.0: 1.0), respectively (TABLE 1). The molecular weights of Ca-SPIIa and Ca-SPIIb were determined using a calibration curve of standard dextrans and found to be 10.5×10^5 and 9.1×10^5, respectively. The sulfate percent from Ca-SPIIa and Ca-SPIIb was found to be 2.25% and 3.45% respectively. Our results are disagreement with the results of Hayashi et al. and Mishima et al.[12,26], who founds that the sulfated polysaccharide from *S. platensis* was composed of rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, glucouronic acid, galactouronic aid and sulfate. While[22] cited that a novel sulfated polysaccharide isolated from a hot water extraction of *S. platensis* consisted of rhamnose, xylose, uronic acid and sulfate. On the other hand[26], found that the molecular weight of sulfated polysaccharides obtained from *S. platensis* was 2.6×10^5, and 3.1×10^5 by gel filtration on Sepharose 6B and light scattering experiments respectively.

Modification of sulfated polysaccharide

When Ca-SPIIb was eluted with distilled water through an ion exchange column of Dowex (50w×-8) followed by lyphilization, a colorless powdered polysaccharide H-SPIIb free from calcium was obtained. On the other hand, Ca-SPIIb solution was passed through

TABLE 2 : Inhibitory effect of Ca-SPIIb, H-SPIIb, Desulfated SPIIb and modified polysaccharide on the viability of (EACC)

Modified polysaccharide	Concentration µg/2ml	Mean percentage of viable cells	Mean percentage of dead cells
Ca-SPIIb	0	95.33±1.453 [a]	4.66±1.453 [a]
	10	88.33±1.763 [b]	11.66±1.763 [b]
	50	73.00±4.163 [c]	27.00±4.163 [c]
	100	52.33±1.763 [d]	47.66±1.763 [d]
	150	5.33±0.333 [e]	94.66±0.333 [e]
	200	4.66±0.667 [e]	95.33±0.667 [e]
H-SPIIb	10	90.33±0.882 [b]	9.66±0.882 [b]
	50	85.66±0.667 [c]	14.33±0.667 [c]
	100	81.66±1.763 [d]	18.33±1.763 [d]
	150	64.66±0.882 [e]	35.33±0.882 [e]
	200	38.66±0.882 [f]	61.33±0.882 [f]
Desulfated-SPIIb	10	89.66±1.459 [b]	10.33±1.459 [b]
	50	81.00±1.527 [c]	19.00±1.527 [c]
	100	58.66±0.882 [d]	41.33±0.882 [d]
	150	41.00±1.527 [e]	59.00±1.527 [e]
	200	39.00±1.154 [e]	61.00±1.154 [e]
Na-SPIIb	10	88.00±1.527 [b]	12.00±1.527 [b]
	50	78.33±1.763 [c]	21.66±1.763 [c]
	100	57.33±1.763 [d]	42.66±1.202 [d]
	150	42.66±1.453 [e]	57.33±1.453 [e]
	200	36.66±0.882 [e]	63.33±0.882 [e]
Ag-SPIIb	10	86.33±1.764 [b]	13.66±1.764 [b]
	50	81.33±1.856 [c]	18.66±1.856 [c]
	100	66.66±1.453 [d]	33.33±1.453 [d]
	150	48.00±1.527 [e]	52.00±1.527 [e]
	200	35.33±0.882 [e]	65.66±0.882 [e]
Cu-SPIIb	10	88.33±1.202 [b]	11.66±1.202 [b]
	50	57.00±1.155 [c]	43.00±1.155 [c]
	100	31.33±0.882 [d]	68.66±0.882 [d]
	150	19.66±0.667 [e]	80.33±0.667 [e]
	200	19.33±0.667 [e]	80.66±0.667 [e]
Ca-SPIIa	10	88.00±1.155 [b]	12.00±1.155 [b]
	50	83.66±0.882 [b]	16.33±0.882 [b]
	100	69.66±0.667 [c]	30.33±0.667 [c]
	150	31.33±0.882 [d]	68.66±0.882 [d]
	200	23.33±0.882 [e]	76.66±0.882 [e]

***Means ± S.E for *n*=3. The mean having the same litter in the same column for modified polysaccharide are not significantly different**

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on the Dowex column pre-equilibrated with metal (Na^+, Ag^+ and Cu^{2+}) solution was obtained derivatives Na-SPIIb, Ag-SPIIb and Cu-SPIIb. Furthermore, treatment of H-SPIIb with 2 % methanol hydrogen Chloride at 25 ºC gave a desulfated polysaccharide. These results suggested that Ca-SPIIb is a sulfated polysaccharide chelating calcium ion and mainly composed of glucose, galactose, rhamnose, fructose, glucouronic acid, mannose and xylose.

Effect of metal cation on antitumor activity

The previous finding[26] prompted use to examine other effects of exchanging Ca^{2+} with other metal cations on antitumor activity. Exchange of metal cations was preformed by passing through a cation exchange column on Dowex (50w×-8) resin equilibrated performed with a salt of corresponding metal cation. Each derivative thus obtained was assayed for antitumor against EACC as well as inhibitory activity against EACC. As shown in TABLE 2 Ca-SPIIb and Cu-SPIIb exhibited potent antitumor activity, while the potency of other derivatives remarkably decreased in antitumor activity.

Particularly, replacement of Ca^{2+} with H^+ or Ag^+ almost completely eliminated the antitumor activity as a result of a marked decrease in activity. It is noteworthy that Cu-SPIIb showed relatively stronger antitumor activity than H-SPIIb, although Cu^{2+} was assumed to be more toxic than H^+ in cells. Our previous experiments comparing inhibitory effects of Na-SPIIb on antitumor activity formation with those of Ca-SPIIb suggested that a divalent metal cation such as Ca^{2+} could build up a certain molecular form which is essential for exerting an antitumor effect even at lower concentration. However, present data indicate that Ca^{2+} might be a specific metal cation which can form a pot antitumor molecule[26]. One plausible explanation includes the possibility that Ca-SPIIb may bind to laminin receptors such integrins and 67kDa protein on the tumor cell surface and consequently lead to the inhibition of tumor cells. On the other hand, Hans and Klaus[10] have demonstrated that specific receptors for rhamnose were present types of primary tumor sits, such as sarcoma, melanoma and Delano carcinoma in tissue. Since a major component in Ca-SPIIb is rhamnose (TABLE 1), it is also conceivable that Ca-SP may be recognized by such receptors for rhamnose on the surface. In general, the polysaccharide isolated from different strains seemed to have different *in-vitro* antiumor activity depending on their monosugars composition, protein content, molecular weight and chain conformation[20]. The antitumor activity of sulfated polysaccharides may be due to the following reasons: (i) interaction between the polysaccharides and proteins located on the cell membrane of tumor cells, (ii) interaction between the polysaccharides and carbohydrate moieties located on the cell surface, (iii) polysaccharides may attach to cell surface and cause metaphase block[25].

CONCLUSION

Improved knowledge of the composition and analysis of polysaccharide isolated from a hot water extraction of *S. platensis* would assist in medicinal application of this edible alga. It could be said that the *S. platensis* give considerable yield polysaccharide which are rich in sulfate and calcium. Moreover, recovered sulfated polysaccharide could be antitumor against EACC.

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