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Anti HSV-1 Activity of *Spirulina platensis* Polysaccharide

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ABSTRACT

Aqueous extracts of *Spirulina platensis* were precipitated by cetyltrimethylammonium bromide (CTAB). The hot water extract was found anti Herpes simplex virus type 1 (HSV-1) activity at 50% inhibitory concentration (IC_{50}) values of 21.32 µg/ml. Partial purification by gel filtration of the crude extract on Sepharose 6B column gave two fractions, SHP-F1 and SHP-F2, which revealed about 4 and 2 times higher activity than that of the crude hot water extract, respectively. The crude hot water extract was a polysaccharide with rhamnose as the main sugar component. Calcium ion and sulfate groups in this polysaccharide had major roles in antiviral activity. However, the crude hot water extract polysaccharide contained approximately 42% carbohydrate and 31% protein. Decreasing the amount of protein by precipitation with trichloroacetic acid (TCA) resulted in higher purity of the crude hot water extract polysaccharide.

Key words: *Spirulina platensis*, Herpes simplex virus type 1 (HSV-1), polysaccharide

INTRODUCTION

Spirulina platensis is one of the edible microalgae that has been used as health food and feed for a long time. There is an increased interest in components of *S. platensis* because of their potential properties such as anti thrombin activity (Hayakawa *et al.*, 1996), lowering cholesterol level and blood pressure (Kato *et al.*, 1984; Nakaya *et al.*, 1988). Herpes simplex virus type1 is a common human pathogen causing infections of the orofacial mucosal region (Whitley and Roizman, 2001). Over the past decade, the incidence and severity of HSV infection have increased due to the increase in number of immuno-compromised patients produced by aggressive chemotherapy treatments, organ transplant and human immunodeficiency infections. Acyclovir, a synthetic drug which has remarkable effect against HSV-1 infection, inhibits virus replication by acting on viral DNA synthesis (Elion *et al.*, 1977; Schaeffer *et al.*, 1978). Acyclovir-resistant HSV infections have emerged due to the increase in drug use frequency (Field and Biron, 1994). Therefore, many researchers have attempted to search for effective and

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inexpensive anti-viral agents from natural sources. The inhibitory effects of polysaccharides from marine algae on virus replication were first reported almost four decades ago.

Gerber *et al.* (1958) reported that algal polysaccharides exhibited antiviral activity toward mumps and influenza B virus. Further, Hayashi *et al*. (1993) reported the anti HSV-1 activity of aqueous extracts from *S. platensis*. Our preliminary study revealed that both water soluble and non-polar extracts of *S. platensis* exhibited antiviral activity (HSV-1). This study investigated the anti HSV-1 activity of polysaccharides (water soluble compound) extracted from *S .platensis*. The isolation, partial purification, and composition determination of the anti HSV-1 activity extracts are described.

MATERIALS AND METHODS

Extraction of polysaccharides

The lipid component was extracted from freeze-dried powder of *S. platensis* with $CHCl_3$:MeOH (2:1). Then, the residue was extracted with distilled H_2O. After fillration, the filtrate was precipitated by 1% CTAB (cetyltrimethylammonium bromide). After centrifuging, the precipitant was washed stepwise with saturated sodium acetate in 95% EtOH, 95% EtOH, absolute EtOH and diethyl ether, respectively. The resulting in cold water extract polysaccharide was obtained. For the extraction of hot water extract polysaccharide, the same method was performed except using boiling H_2O.

Partial purification of polysaccharide

The hot water extract polysaccharide was dissolved in 0.01 M citrate buffer, pH 7.0 containing 0.1 M NaCl. The soluble portion was applied on to a Sepharose 6B (Pharmacia) column (3×30 cm) and eluted with the same citrate buffer. Fractions of 5 ml were collected and monitored using the phenol-sulfuric method with detection by spectrophotometer (Bausch&Lomb, Spectronic 21) at an absorbance of 485 nm (Hayashi *et al*., 1996a)**.** The collected fraction was concentrated using an evaporator (below 40°C under reduced pressure), dialyzed with deionized water and lyophilized.

Preparation of sugar derivatives for GC analysis

One milligram of sugar was treated with 1 ml of 20 g/l sodium tetraborohydride and cooled down to nearly 0°C. After standing over night, amberite IR-120 (H^+) was slowly added until no bubble. The solution was filtered through filter paper (Whatman #541). After filtration, the solution was evaporated under reduced pressure to thick syrup. The syrup was repeatedly dissolved in methanol and evaporated to remove boric acid. The syrup was further treated with 0.5 ml of acetic anhydride and 0.5 ml of pyridine at 80°C for 2 h. The solution was then immersed in an ice bath and 1 ml of methanol was added to the solution. The solution was then evaporated to remove methyl acetate. Then, 1 ml of heptane was added and evaporated to remove the remaining pyridine. Dried sample was dissolved in 200 µl of dichloromethane and analyzed by GC (Shimadzu, 17A) using Rtx-2330 capillary column (Blakeney *et al.*, 1983)**.**

Hydrolysis of hot polysaccharide solution

A 5 mg sample from partial purification of polysaccharide and 1 mg of internal standard (inositol) were mixed and treated with 2 N H_2SO_4 at 100°C for 8 h. The hot solution was neutralized with barium carbonate to pH 5 and filtered through filter paper (Whatman #541). Barium was eliminated from the supernatant using Amberite IR-120 (H^+) acidic cation-exchange resin. The solution was then applied to a Dowex 1-X8 anion-exchange column and eluted with distilled H_2O. The fraction was evaporated and converted to an alditol acetate derivatives form and analyzed by

GC.

Analytical methods Total carbohydrate content was estimated by phenol sulfuric acid assay (Dubois *et al*., 1956). Total protein content and lipid content were determined according to the methods of Lowry *et al*. (1951) and Folch Folch *et al*. (1957), respectively. Calcium content was carried out by Inductive Couple Plasma Spectroscopy (ICP, Model JY 124) and quantitative analysis of sulfate was performed by precipitation with 10% $BaCl_2$ (Burns, 1995).

Removal of calcium was achieved using a cation exchange column on Dowex 50W (X-8, H^+ form) (Hayashi *et al*., 1993).

Desulfation pH of the polysaccharide solution was adjusted to pH 7.6 with pyridine and the pyridinium salt was eliminated with dimethyl sulfoxide (containing 10% of MeOH) at 80-100° C (Nagasawa *et al*., 1977).

Antiviral activity was detected by using a colorimetric method modified from Skehan *et al*. (1990). Herpes simplex virus type 1 (HSV-1) was maintained in a Vero cell line (kidney fibroblasts of an African green monkey), which was cultured in Eagle's minimum essential medium (MEM) with the addition of 10% heat inactivated fetal bovine serum (FBS) and antibiotics. The test samples were put into wells of a microtiter plate at final concentrations ranging from 20-50 µg/ml. The viral HSV-1 (30 PFU) was added into a 96-well microplate, followed by plating of Vero cells (1×10^5 cells/ml); the final volume was 200 µl. After incubation at 37°C for 72 h, under 5% of CO_2 atmosphere, cells were fixed with 50% trichloroacetic acid (TCA) and stained with 0.05% sulforhodamine B in 1% acetic acid and optical density was measured at 510 nm using a microplate reader. Acyclovir was used as the reference compound.

Determination of cytotoxicity assay

Compounds were tested for their cytotoxicity against Vero cells (African green monkey kidney fibroblasts in 96-well tissue culture plates). One hundred and ninety µl of Vero cell suspension containing 1×10^5 cells/ml and 10 µl of tested compound were added to each well in triplicate. Elliptine and 10%DMSO were used as positive and negative control, respectively. The cells were incubated at 37°C for 72 h in 5%CO_2. After incubation, the cytotoxicity was determined by the colorimetric method as described by Skehan *et al*. (1990). The cytotoxicity was expressed as IC_{50}, i.e., the concentration of the compound which inhibits cell growth by 50%, compared with untreated cell.

RESULTS

Crude cold and hot water polysaccharides were obtained from extraction of dried *S. platensis* by distilled water (room temperature) and boiling water, respectively. The extracts were precipitated by CTAB solution. The freeze-dried extracts as fine creamy powder were shown in Figure 1. The yields of the cold and hot water extracts were 1.2 and 0.3 % (W/W), respectively. The hot water extract polysaccharide showed an IC_{50} value against HSV-1 at 21.32 µg/ml whereas no activity was detected in the cold water extract polysaccharide.

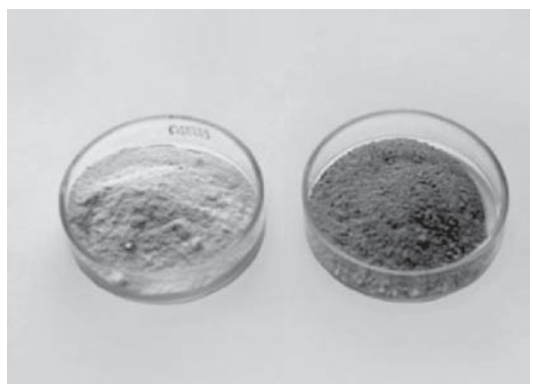

Figure 1 Cold water polysaccharide (pale color) and hot water polysaccharide (dark color).

The crude hot water polysaccharide was partially purified by gel-filtration on Sepharose 6B column. Two fractions, SHP-F1 and SHP-F2, were collected (Figure 2). Attempts to completely separate the two fractions by decreasing flow rate from 1.2 to 0.8 ml/min was not successful. The curde hot water polysaccharide comprised of approximately 40% of fraction 1 (SHP-F1) and 60% of fraction 2 (SHP-F2). The fractions of SHP-F1 and SHP-F2 were repeatedly run using the same method at a lower flow rate of 0.5 ml/min.

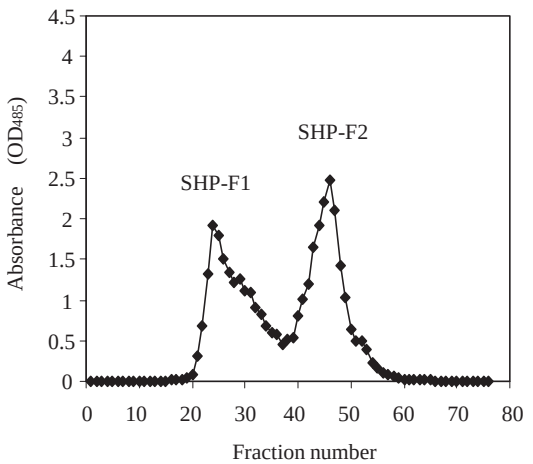

Figure 2 Elution profile of hot water polysaccharide by Sepharose 6B column chromatography.

After the pool fraction of SHP-F1 was repeatedly applied on Sepharose 6B column, the purified SHP-F1 was obtained (Figure 3A). However, SHP-F2 still exhibited 2 peaks of absorbances, a small peak and a bigger one, designated as SHP-F2/1 and SHP-F2/2, respectively (Figure 3B). When the partially purified fractions of the hot water polysaccharide (SHP-F1 and SHP-F2) were subjected to cytotoxicity and anti HSV-1 assays, both fractions exerted non-toxicity on the growth of Vero cells at the maximum concentrations tested and had significantly higher anti HSV-1 activity than the crude hot water polysaccharide (about 4 and 2 times, respectively) (Table 1).

The analysis of monosaccharide was performed by GC. It was found that SHP-F1 fraction contained only three sugars; rhamnose, ribose and arabinose, whereas, the SHP-F2 fraction contained rhamnose, ribose, arabinose, glucose, mannose, galactose and xylose. Both fractions contained rhamnose as the main sugar component (Table 2).

Table 3 showed the comparison of proximate analysis of dried cells of *Spirulina* and the crude hot water extract polysaccharide. Results showed that dried cells consisted of 21.9% carbohydrate, 61.4% protein, 7.2% lipid and 7.2%

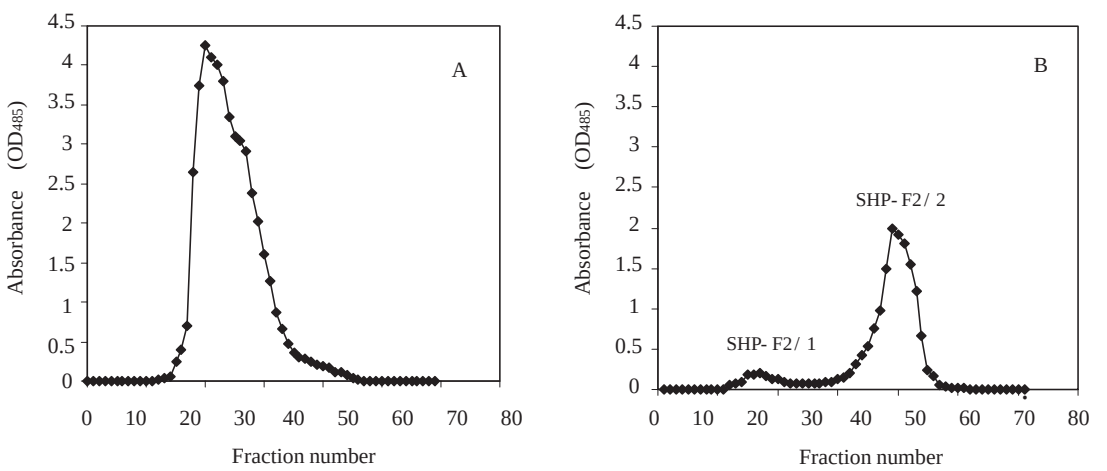

Figure 3 Elution profile of SHP-F1 (A) and SHP-F2 (B) by Sepharose 6B column chromatography.

ash. The crude hot water polysaccharide contained 42.5% carbohydrate, 31.0% protein, 12.9% ash and trace of calcium and sulfate.

Table 4 shows the remaining carbohydrate and protein precipitated by various concentrations of trichloroacetic acid (TCA).

These data suggested that at the highest concentration of TCA (50% TCA), 41% of the protein in dry cells was eradicated (a decrease from 31% to 18%), while the percentage of carbohydrate increased about 25% (from 42.5% to 53.1%). After the treated crude hot water polysaccharide (precipitated with 50% TCA) was tested for anti HSV-1 activity, results showed that the activity was not significantly different from the untreated crude hot water polysaccharide (data not shown).

To determine the role of calcium ion and sulfate groups of the hot water polysaccharide in antiviral activity, calcium ion and sulfate groups in the polysaccharide were eliminated before testing for cytoxicity and anti HSV-1 activity. The results indicated that all of the calcium-free

Table 1 Cytotoxicity and Anti HSV-1 activity of the hot water polysaccharide fractions from *S. platensis.*

Fractions	Cytotoxicity[a] (IC_{50}: µg/ml)	Anti HSV-1[b] (IC_{50}: µg/ml)
SHP-F1	> 50	5.25
SHP-F2	> 50	9.61

[a] maximum concentration of compound for cytotoxicity test was 50 µg/ml
compound was non-toxic to the growth of Vero cells when IC_{50} >50 µg/ml
(if compound was toxic on the growth of Vero cells, the compound will be subjected to the serial dilution for determination of IC_{50} value)

[b] % inhibition of HSV-1; < 25%= inactive, 25-35%= weakly active, >35-50% = moderately active, > 50%= active (the compound will be subjected to the serial dilution for determination of IC_{50} value)

Table 2 Sugar composition of fractions of the hot water extract polysaccharide.

Fractions	% Sugar composition						
	Rhamnose	Ribose	Arabinose	Glucose	Mannose	Galactose	Xylose
SHP-F1	75.6	13.4	11.0	-	-	-	-
SHP-F2	30.4	27.1	10.0	18.2	7.5	4.5	2.3

Table 3 Composition of *S. platensis* powder and crude hot water polysaccharide.

Composition	Dry weight (%)	
	Spirulina powder	Crude hot water polysaccharide
Carbohydrate	21.9 ± 0.8	42.5 ± 0.3
Protein	61.4 ± 1.1	31.0 ± 0.8
Lipid	7.2 ± 1.3	0
Calcium	-*	0.123 ± 0.0006
Sulfate	-*	1.44 ± 0.03
Ash	7.2 ± 0.1	12.9 ± 0.4

Mean ± standard deviation (n = 3)

* It was not determined

compound exerted weak anti HSV-1 activity when compared with that of the crude hot water polysaccharide, whereas, in the absence of sulfate groups in polysaccharide, no significant anti HSV-1 activity was detected in this compound (Table 5).

DISCUSSION

This study found that the hot water extract polysaccharide exhibited anti HSV-1 activity, while the cold extract of the polysaccharide did not. Previous studies found that the majority of potential antiviral algal polysaccharides were extracted from tissues by hot water, dilute acid or alkali solution (Damonte *et al*., 1994; Hoshino *et al*., 1998). Crude hot water polysaccharide still contained a high level of protein which may co-precipitate when CTAB is used for polysaccharide precipitation (Tomanee *et al*., 2004).

Partial purification of the hot water polysaccharide by gel-filtration on Sepharose 6B column gave 2 fractions (SHP-F1 and SHP-F2), both fractions effectively inhibited HSV-1 activity. Results reported by Hayashi *et al*. (1996a) revealed 3 fractions (SP-H-1, SP-H-2 and SP-H-3) but only a SP-H-2 fraction had anti HSV-1 activity. The sugars found in SHP-F1 and SHP-F2 fractions in this study are almost the same as previously reported by Hayashi et al. (1966a) except for arabinose which was found in this study instead of fructose which was reported by the same researchers.

Calcium ion and sulfate groups in the hot water polysaccharide were important for the anti HSV-1 activity. This result was supported by Hayashi's study that when the calcium-free

Table 4 Carbohydrate and protein content of crude hot water polysaccharide which was precipitated by trichloroacetic acid (TCA).

TCA concentration (%)	% w/w of crude hot water polysaccharide	
	Carbohydrate	Protein
0	42.5 ± 0.3	31.0 ± 0.8
10	41.8 ± 1.5	28.1 ± 2.1
20	45.3 ± 1.8	25.4 ± 1.3
30	49.2 ± 2.3	22.3 ± 1.5
50	53.1 ± 2.0	18.0 ± 0.8

Mean ± standard deviation (n = 3)

Table 5 Anti HSV-1 activity in the crude hot water polysaccharides from *S. platensis.*

Sample	Cytotoxicity[a] (IC_{50} : µg/ml)	Anti HSV-1[b] (IC_{50} : µg/ml)
Polysaccharide	> 50	21.3
Polysaccharide (-Ca^{2+})	> 50	38.4
Polysaccharide (-SO_4^{2-})	> 50	Inactive

[a] maximum concentration of compound for cytotoxicity test was 50 µg/ml
compound was non toxic on the growth of Vero cells if IC_{50} >50 µg/ml
(if compound was toxic on the growth of Vero cells, the compound will be subjected to the serial dilution for determination of IC_{50} value)

[b] % inhibition of HSV-1; < 25%= inactive, 25-35%= weakly active, >35-50% = moderately active, > 50%= active (the compound will be subjected to the serial dilution for determination of IC_{50} value)

spirulan (H-SP), and a desulfated compound from Ca-SP were subjected to cytotoxicity and antiviral assay, both compounds exerted strong toxicity to the growth of host cell (HeLa cells) and weakly inhibited HSV-1 (Hayashi *et al*., 1996a). Ca-SP was found to inhibit replication of several enveloped virus and selectively inhibited the penetration of virus into host cell (Hayashi *et al*., 1996b). Loya *et al*. (1998) postulated that the negatively charged (e.g., sulfonate vs. sulfate) may interact with the positively charged side chains on the DNA polymerase and Witvrouw *et al*. (1994) assumed that sulfated polysaccharides disruption of ionic interactions between positively charged regions of viral surface glycoproteins and cellular membrane phospholipids.

CONCLUSION

Results from this study demonstrated the significant potential of *S. platensis* polysaccharide for activity against HSV-1. The hot water extract polysaccharide which contained rhamnose as the main sugar component showed anti HSV-1 activity at IC_{50} 21.3 μg/ml. Calcium ion and sulfate groups in the polysaccharide had major roles in the anti HSV-1 activity. *S. platensis*, is a possible source for new drugs in the treatment of HSV-1 and other viral diseases.

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