Anti HSV-1 Activity of *Spirulina platensis* Polysaccharide

Nattayaporn Chirasuwan1*, Ratana Chaiklahan1, Marasri Ruengjitatchawalya2, Boosya Bunnag2 and Morakot Tanticharoen3

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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 (IC50) 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

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**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 type 1 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
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 CHCl₃:MeOH (2:1). Then, the residue was extracted with distilled H₂O. After filtration, the filtrate was precipitated with 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 H₂O.

**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 (3x30 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 H₂SO₄ 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 H₂O. The fraction was evaporated and converted to an alditol acetate derivatives form and analyzed by
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% BaCl₂ (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 × 10⁵ cells/ml); the final volume was 200 µl. After incubation at 37°C for 72 h, under 5% of CO₂ 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.

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 IC₅₀ 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 crude 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. 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%
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>
<thead>
<tr>
<th>Fractions</th>
<th>Cytotoxicity$^{a}$ (IC$_{50}$: µg/ml)</th>
<th>Anti HSV-1$^{b}$ (IC$_{50}$: µg/ml)</th>
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</thead>
<tbody>
<tr>
<td>SHP-F1</td>
<td>&gt; 50</td>
<td>5.25</td>
</tr>
<tr>
<td>SHP-F2</td>
<td>&gt; 50</td>
<td>9.61</td>
</tr>
</tbody>
</table>

$^{a}$ maximum concentration of compound for cytotoxicity test was 50 µg/ml

compounds 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>
<thead>
<tr>
<th>Fractions</th>
<th>% Sugar composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rhamnose</td>
</tr>
<tr>
<td>SHP-F1</td>
<td>75.6</td>
</tr>
<tr>
<td>SHP-F2</td>
<td>30.4</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Composition</th>
<th>Dry weight (%)</th>
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<tbody>
<tr>
<td></td>
<td>Spirulina powder</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>21.9 ± 0.8</td>
</tr>
<tr>
<td>Protein</td>
<td>61.4 ± 1.1</td>
</tr>
<tr>
<td>Lipid</td>
<td>7.2 ± 1.3</td>
</tr>
<tr>
<td>Calcium</td>
<td>-*</td>
</tr>
<tr>
<td>Sulfate</td>
<td>-*</td>
</tr>
<tr>
<td>Ash</td>
<td>7.2 ± 0.1</td>
</tr>
</tbody>
</table>

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 (Tomane 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. (1966a) 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>
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<th>Table 4</th>
<th>Carbohydrate and protein content of crude hot water polysaccharide which was precipitated by trichloroacetic acid (TCA).</th>
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<tbody>
<tr>
<td>TCA concentration (%)</td>
<td>% w/w of crude hot water polysaccharide</td>
</tr>
<tr>
<td></td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>0</td>
<td>42.5 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>41.8 ± 1.5</td>
</tr>
<tr>
<td>20</td>
<td>45.3 ± 1.8</td>
</tr>
<tr>
<td>30</td>
<td>49.2 ± 2.3</td>
</tr>
<tr>
<td>50</td>
<td>53.1 ± 2.0</td>
</tr>
</tbody>
</table>

Mean ± standard deviation (n = 3)

<table>
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<th>Table 5</th>
<th>Anti HSV-1 activity in the crude hot water polysaccharides from <em>S. platensis</em>.</th>
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<tbody>
<tr>
<td>Sample</td>
<td>Cytotoxicity&lt;sup&gt;a&lt;/sup&gt; (IC&lt;sub&gt;50&lt;/sub&gt; : µg/ml)</td>
</tr>
<tr>
<td>Polysaccharide</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>Polysaccharide (-Ca&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>Polysaccharide (-SO&lt;sub&gt;4&lt;/sub&gt;^2-)</td>
<td>&gt; 50</td>
</tr>
</tbody>
</table>

<sup>a</sup>maximum concentration of compound for cytotoxicity test was 50 µg/ml
compound was non toxic on the growth of Vero cells if IC<sub>50</sub> > 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<sub>50</sub> value)

<sup>b</sup>% 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<sub>50</sub> 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 IC₅₀ 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.

ACKNOWLEDGEMENTS

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LITERATURE CITED


