An Extract from *Spirulina platensis* is a Selective Inhibitor of Herpes Simplex Virus Type 1 Penetration into HeLa Cells

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The water-soluble extract of *Spirulina platensis* achieved a dose-dependent inhibition of the replication of herpes simplex virus type 1 (HSV-1) in HeLa cells within the concentration range of 0.08–50 mg/mL. This extract proved to have no virucidal activity and did not interfere with adsorption to host cells. However, the extract affected viral penetration in a dose-dependent manner. At 1 mg/mL the extract was found to inhibit virus-specific protein synthesis without suppressing host cell protein synthesis if added to the cells 3 h before infection. In an *in vivo* experiment food containing the extract effectively prolonged the survival time of infected hamsters at doses of 100 and 500 mg/kg per day.

Keywords: *Spirulina platensis*; herpes simplex virus type 1; antiviral activity; virus penetration; animal experiment.

INTRODUCTION

Blue green algae *Spirulina platensis* has been receiving considerable attention as a protein source because the *Spirulina* contains 64–70% protein dry weight (Guerin-Dumartrait and Moyse, 1976). There are many reports on the hypocholesterolaemic effect of *Spirulina* in experimental animals (Devi and Venkataraman, 1983; Kato *et al.*, 1984; Iwata *et al.*, 1987). The antiviral effect of *Spirulina* has not previously been studied. In this paper we report the results of studies on the anti-herpes simplex virus (HSV) activity of the water-soluble extract prepared from *Spirulina platensis* and present evidence that the extract inhibits viral penetration into HeLa cells without affecting host protein synthesis and does not inhibit virus attachment to cells.

MATERIALS AND METHODS

Preparation of the extract. Freeze-dried powder of *Spirulina platensis* (10 g) grown in outdoor open tanks was obtained from Nippon Oil Company, Ltd (Yokohama, Japan) and was extracted three times with 50 mL of boiling water for 1 h. The hot water extract was freeze-dried to give a pale blue powder (0.92 g).

Cells and viruses. HeLa and Vero cells were grown in Eagle's minimal essential medium (MEM) supplemented with 6% fetal calf serum (FCS) for use in growth of virus or plaque assay. HSV-1 strain HF was propagated at a low titre and assayed by plaque titration of HeLa cell monolayers.

Cytotoxicity assay. For growth inhibition studies, 3×10^4 cells in 0.5 mL MEM plus 6% FCS were seeded into each well of 24-well plates, cultured for 24 h at 37 °C, and allowed to grow for an additional 24 h in the presence of increasing amounts of the extract. After the medium was removed, cells were trypsinized and the cell number was determined by a conventional haemocytometer using the trypan blue exclusion method. The inhibition data were plotted as dose-effect curves (not shown), from which the 50% inhibitory doses (ID_{50}) were obtained.

Antiviral activity. HeLa cell monolayers in 24-well plates were washed with phosphate-buffered saline (PBS), pH 7.2 and infected with HSV-1 at a multiplicity of infection (m.o.i.) of 0.2, adsorbed for 1.5 h at room temperature, and overlay was replaced wth maintenance medium (MEM plus 2% FCS) containing various concentrations of the extract. Cultures were incubated for 24 h at 34 °C in 5% CO_2, harvested and disrupted by three cycles of freezing and thawing. Virus yields were determined by plaque assay. Each concentration was assayed at least twice. The antiviral activity was expressed as 50% effective dose for viral replication (ED_{50}) which was the lowest drug concentration of reducing plaque numbers by 50% in the treated cultures as compared to untreated ones.

Inhibition of virus adsorption to cells. HeLa cells were grown to confluence in 60 mm dishes. The extract was added to the dishes for 3 h at 37 °C. The cells were washed with PBS and inoculated with HSV-1 at an m.o.i. of 1 for 1 h at room temperature. The inocula were recovered, dishes were washed twice with PBS, and the wash was collected. The unadsorbed viruses in these samples were assayed by plaque titration.

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Assay for rate of virus penetration. Virus penetration was measured by inactivation of unpenetrated viruses with a low-pH buffer as described by Huang and Wagner (1964) and modified by Highlander et al. (1987). HeLa cell monolayers in 60 mm dishes were pretreated with the extract for 3 h at 37 °C. After washing with PBS, the cells were inoculated with approximately 100 plaque-forming units (PFU) of HSV-1 per 0.1 mL and incubated for 1 h at 4 °C. The dishes were washed twice with PBS, added 1 mL of MEM plus 2% FCS and shifted to 37 °C. Every 30 min, each dish was treated with 1 mL of citrate buffer, pH 3.0, for 1 min. After washing twice with PBS, the monolayers were overlaid with 0.5% methylcellulose. After incubation for 2 days, the monolayers were stained with 0.06% crystal violet to count the number of plaques.

Preparation of antiserum. Rabbit anti-HSV-1 serum was prepared as follows: HeLa cells were infected with HSV-1 at an m.o.i. of 1 for 1 h at room temperature, incubated for 18 h at 34 °C, harvested, submitted to three cycles of freezing–thawing and centrifuged at $3000 \times g$ for 10 min. One half millilitre of the supernatant (10^8 PFU/mL) per animal was used as an immunogen. The immunization schedule for rabbits used a primary injection of the sample (a 1:1 emulsion of antigen in Freund's complete adjuvant) in five subcutaneous sites along the back. Booster immunizations were at 7, 14 and 21 days in Freund's incomplete adjuvant. Rabbits were bled at day 45.

Analysis of radio-labelled proteins. Vero cells with or without pretreatment with the extract for 3 h were mock-infected or infected with HSV-1 at an m.o.i. of 10 and radio-labelled immediately after infection. After washing and replenishing the monolayer with methionine-free medium, 10 μCi of Tran ^{35}S-label (1000 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA, USA) was added per 60 mm dish and incubated for 4 h at 37 °C. Infected cells were harvested, extracted with a lysing buffer containing 0.05 M Tris-HCl, pH 7.0, 0.15 M NaCl, 1% SDS and 1% Triton X-100, and centrifuged at 25 000 rpm for 1 h at 4 °C. For immunoprecipitation, an aliquot of the cell lysates was treated with rabbit antiserum and protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology, Uppsala, Sweden) overnight at 4 °C with rocking. The immunoprecipitates and the cell lysates were analysed by 8% SDS-polyacrylamide gel electrophoresis. After electrophoresis gels were soaked in 1 M sodium salicylate for 30 min, dried and exposed to x-ray films (Sambrook et al., 1989).

Animals. Four-week-old female golden hamsters (52–58 g) obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) were used.

Inoculation and treatment of animals. The extract was dissolved in distilled water and absorbed to solid food to give the doses of 100 and 500 mg/kg/day. Placebo treatment was the food containing no extract. For infection, 10 μL of virus suspension in PBS containing 2×10^5 PFU was placed on the eyes of animals. For each animal 15 linear corneal scarifications and a further 15, perpendicular to the first, were made with twenty 23-gauge needles according to the method reported previously (Hayashi et al., 1988). The experi-
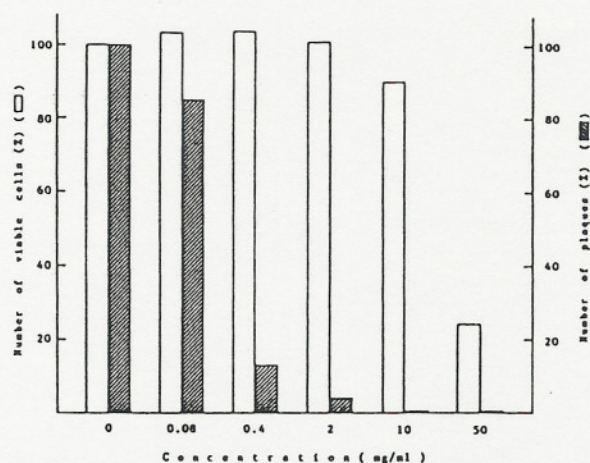

Figure 1. Inhibitory effect of the extract on cell growth and virus replication in HeLa cells. Uninfected (□) and HSV-infected (■) cells were incubated in the presence of varying concentrations of the extract. Viability of the cell was assessed by trypan blue exclusion method after 24 h incubation. Virus replication was assayed by plaque titration 24 h after infection. Each value is the mean of triplicate determinations.

ments were carried out under the 'Guide for Animal Experiments at Toyama Medical and Pharmaceutical University'. Anaesthesia was used to avoid distress to the animals. Groups of six hamsters were treated by supplying the extract-containing food ad libitum from 7 days before infection. The survival times were recorded every day.

Statistics. Statistical evaluation was done by Student's t-test.

RESULTS

Effects of the extract on cell growth and virus replication

Uninfected or HSV-infected HeLa cells were cultured for 24 h in the medium containing the extract in the range of 0.08–50 mg/mL. The extract below the concentrations of 2 mg/mL showed no inhibition of cellular growth, as shown in Fig. 1. In virus-infected cells a dose-dependent inhibition of virus replication was observed within the concentrations tested. The ID_{50} for cytoxicity was 26.3 mg/mL, while the ED_{50} for antiviral activity was 0.173 mg/mL, giving an in vitro therapeutic index of 152.

Direct virucidal activity of the extract

In order to determine the direct inactivation of virus by the extract, the extract was diluted in MEM to provide final concentrations ranging from 0.001 to 1.0 mg/mL. Virus suspension was added to the solution and incubated for 8 h at 37 °C. Samples were harvested in a small quantity every 2 h and plaque-assayed. The remaining infectivity in each treatment is shown in Table 1. The extract exerted a slight effect on the infectivity with approximately 30% reduction com-

pared with 20% reduction ($p > 0.01$) in the preparations without the extract. A dose-dependent effect was not observed in the concentrations tested.

Effect of the extract on virus adsorption and penetration

The effect on adsorption was determined by inhibition of binding of virus to host cells after pretreatment of the cells with the extract. The numbers of viruses that were not bound to cells were calculated by plaque assay and compared with those recovered from untreated control cells. As shown in Table 2, the extract did not interfere with an attachment to cell membranes at the concentrations of 0.01 to 1.0 mg/mL.

In the penetration assay employed in this experiment, the kinetics of penetration was determined by inactivating the extracellular unpenetrated viruses at various times after temperature shift from 4 °C to 37 °C with a low-pH citric acid buffer. Results are shown in Fig. 2, where each value represents the average of duplicated experiments. The cells pretreated with the extract significantly inhibited virus penetration by approximately 75% ($p < 0.01$), 65% ($p < 0.01$) and 3% ($p < 0.001$) at the concentrations of 0.01, 0.1 and 1.0 mg/mL, respectively. These data indicate that the extract exerts its effect at least in part by preventing virus penetration of the host cell membranes.

Effect of the extract on protein synthesis

As suggested in the penetration experiment described above, the viral proteins would not be synthesized in the host cells pretreated with 1.0 mg of the extract per mL before infection. It was also of interest to determine whether the host protein synthesis could be maintained at this concentration of the extract. To investigate these questions, ^{35}S-labelled protein samples from cell lysates and immunoprecipitates were used for SDS-PAGE analysis.

The protein patterns (Fig. 3) revealed following points. (i) The extract showed no inhibition of host protein synthesis at the concentration of 1.0 mg/mL (A, lane 2). (ii) When the extract was added immediately after virus infection, virus-specific protein synthesis was partially suppressed compared with that of untreated cultures (B, lanes 3 and 4). (iii) In the infected cells pretreated for 3 h and then treated with the extract throughout the incubation, host protein

Table 1. Effect of the extract on the inactivation of HSV-1

Concentration (mg/mL)	Infectivity (% inoculum) Time of incubation (h)			
	2	4	6	8
0	88 ± 4.2	80 ± 2.2	78 ± 7.1	78 ± 2.8
0.01	72 ± 2.8	68 ± 5.7	67 ± 4.2	66 ± 1.0
0.1	77 ± 6.4	66 ± 4.2	68 ± 3.6	64 ± 5.0
1	69 ± 3.6	67 ± 2.2	68 ± 6.4	71 ± 1.4

Virus suspension was added to the solution of extract to provide final titre of approximately 2×10^6 PFU/mL and incubated for 8 h at 37 °C. Samples were harvested in a small quantity of the mixtures every 2 h after incubation and assayed in HeLa cells. Each value is the mean ±SD of two experiments.

Table 2. Effect of the extract on virus adsorption

Material	Residual Infectivity (PFU/mL) Concentration (mg/mL)			
	0	0.01	0.1	1
			×10⁴	
Inoculum	60 ± 2.2	55 ± 3.6	54 ± 6.4	55 ± 1.0
Wash	2.6 ± 0.36	2.7 ± 0.10	2.6 ± 0.22	2.6 ± 0.14

HeLa cells were pretreated with the extract for 3 h at 37 °C, then washed and inoculated with 1 PFU/cell of HSV-1. After 1 h adsorption at room temperature, inocula were harvested and monolayers were washed with PBS. The inocula and the wash were subjected to plaque assay. Each value is the mean ± SD of two experiments.

synthesis level (A, lane 5) was observed to be almost equivalent to that in mock-infected cells (A, lane 1), and the virus-specific proteins were not detected almost completely (B, lane 5). In the pretreated cultures, no apparent cytopathic effect by virus infection, that is, virus-induced cell-cell fusion was observed.

Therapeutic efficacy of the extract against experimental HSV-1 corneal infection of hamsters

The efficacy of the extract at a dose of 100 or 500 mg/kg body weight per day against infection with HSV-1 was evaluated using 4-week-old hamsters. The extract feeding was started from 7 days before virus infection. The results obtained are summarized in Table 3. The extract was shown to be effective on the prolongation of the mean survival times of the infected animals. While all animals of control group died by day 8 post-infection,
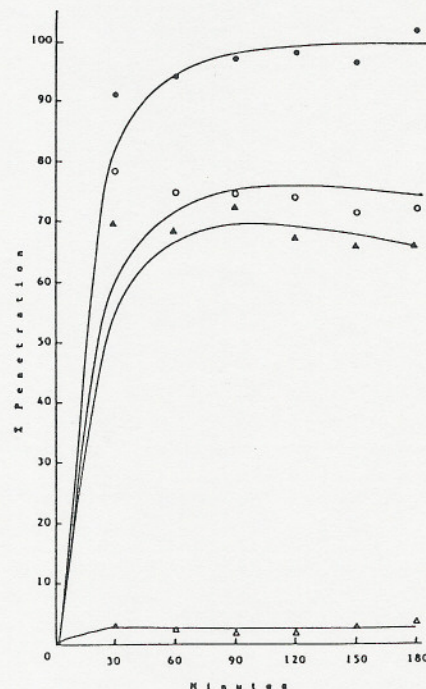

Figure 2. Effect of the extract on HSV-1 penetration. Dilutions of virus (ca. 100 PFU) were adsorbed for 1 h at 4 °C on HeLa cells pretreated for 3 h with the extract at the concentration of 0(●), 0.01(○), 0.1(▲) or 1(△) mg/mL. After washing with PBS, the cultures were shifted to 37 °C and treated with citric acid buffer (pH 3.0) for 1 min at 30 min intervals. Survived virus plaques were counted after 2 days. The results were expressed as % penetration, taking the maximum penetration of virus on untreated cells as 100%. Each point is the mean of duplicate determinations.
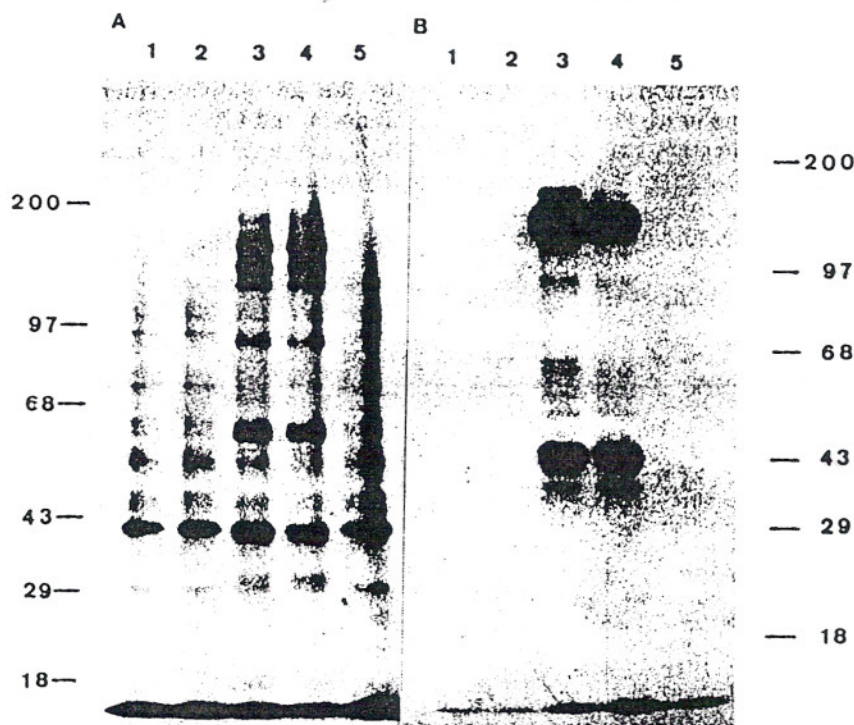
Figure 3. Autoradiographs of ^{35}S-labelled proteins of Vero cells. Mock-infected (lanes 1 and 2) or HSV-infected (lanes 3–5) cells were incubated at 37 °C in the absence (lanes 1 and 3) or presence of 1 mg/mL (lanes 2, 4 and 5) of the extract, and labelled with Tran ^{35}S-label from 0 to 4 h postinfection. Lanes 2 and 4 were treated with the extract immediately after infection, while lane 5 was treated from 3 h before infection and throughout the incubation. Solubilized cell lysates (A) and immunoprecipitates with antiserum (B) were subjected to electrophoresis on 8% polyacrylamide gel. The gels were then processed for autoradiography.

some of the animals treated with the extract remained alive with survival rate of about 15–30%: No evidence of toxicity (change in activity or appetite) was shown in the hamsters fed on the food containing the extract for more than 14 days.

DISCUSSION

At the concentrations of up to 10 mg/mL, *Spirulina* extract had a minimal effect on the growth of HeLa cells. The extract was shown to exert a significant inhibitory effect *in vitro* on the replication of herpes simplex virus type 1.

In order to elucidate the mode of action in the inhibition of HSV-1 replication, the effect of the extract was studied under various experimental conditions. It was demonstrated that the extract is not virucidal and

does not interfere with viral adsorption. On the other hand, virus penetration into host cells was reduced dose-dependently by pretreating the cells with the extract. The result that antiviral action of the extract may be attributed to the inhibition of virus penetration into cells was also supported by SDS-PAGE analysis of ^{35}S-labelled protein synthesis, where virus-induced proteins were detected in the cells without pretreatment but not in the cells pretreated with the extract. As transition from attachment to penetration of virus occurs very rapidly (Huang and Wagner, 1964), it may be essential that the extract is present in the culture medium prior to penetration in order to inhibit this step of viral replication. After virus penetration into cells, the extract might not be expected to suppress effectively the virus replication because a considerable quantity of viral protein was synthesized when the extract was added after virus infection. Furthermore, because penetration of HSV into cells is thought to involve fusion of the virion envelope with the plasma membrane (McKenzie *et al.*, 1987), the observation that the extract can inhibit the cell–cell fusion by HSV infection was reasonable in light of the extract's effect on virus penetration. Some substances have been reported to interfere with the early events surrounding viral entry into host cells. For example, apolipoprotein A-I, the major protein component of serum high density lipoproteins, has been found to inhibit HSV-induced cell fusion and virus penetration (Srinivas *et al.*, 1990). Recently, a saturated alcohol, 1-docosanol, has been suggested to inhibit HSV replication by interfering with viral fusion and/or entry (Katz *et al.*, 1991). However, so far as we know, there is no report on the agents

Table 3. Effect of the extract on experimental HSV-1 corneal infection

Dose (mg/kg/day)	Survivors /total	Time interval of death (days)
0	0/6	7–8
100	1/6	7–10
500	2/6	8–11

Hamsters were infected with 3×10^5 PFU of HSV-1 and treated with the food containing the extract which was equivalent to a dose of 100 or 500 mg/day. The food was fed from 7 days before virus infection.

derived from plants that function at the stage of virus penetration.

Although the method of penetration of HSV-1 is not thoroughly understood, Morgan *et al.* (1968) reported the hypothesis that virus replication results from the entry of virus mediated by fusion of viral envelope and plasma membranes rather than from that mediated by phagocytosis. This hypothesis is supported by the finding that penetration by endocytosis results in a nonproductive infection (Campadelli-Fiume *et al.*, 1988). It is suggested that penetration may be a multiple event involving more than one viral glycoprotein as indicated by the cumulative evidence (Manservigi *et al.*, 1977; Johnson and Ligas, 1988). Thus, it will be interesting to study the interaction between the extract and the viral glycoproteins.

From the data obtained in the *in vivo* study it will be expected that *Spirulina* may prevent herpetic encephalitis by feeding in the diet.

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