Antiviral Substance from Silkworm Faeces: Purification and Its Chemical Characterization

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In a previous paper, we reported that an extract of silkworm faeces has a marked antiviral activity on enveloped viruses, but not on a non-enveloped virus, and that it inhibits the synthesis of a viral specific gene of HVJ (Sendai virus) without affecting the viral adsorption and entry into the host cell. In this paper, we accomplished the purification of an antiviral active substance by extraction of a hydrophobic substance and thin layer chromatography. The active substance was found to be a chlorophyll-like substance with a molecular mass of about 530. This substance shows clear antiviral activity against HVJ, HSV (herpes simplex virus type-1), and HIV (human immunodeficiency virus type-1). Its antiviral activity was dependent on light irradiation and temperature. Furthermore, it also possesses a strong hemolytic activity under light.

Keywords  silkworm faeces; chlorophyll-like substance; enveloped virus; HVJ (Sendai virus); light irradiation

For the study of cell structures and functions, it is one effective way to observe cellular responses on biological stimulation. For this, we have tried to obtain a such biologically active substance as an effective tool and have already isolated a new lectin from silkworm faeces.1) The morphogenesis of an enveloped virus is accomplished on the cell surface, or nuclear envelope, after the transport of synthesized proteins into the membrane, and assembled there. Therefore, to investigate the mechanism of enveloped virus morphogenesis is useful in understanding cell functions such as intracellular transportation and in sorting out glycoproteins on biological membranes. In the course of the investigation of enveloped virus morphogenesis in the host cell, we found that an extract of silkworm faeces suppresses viral production from the host cell. In a previous report, this active substance, inhibiting viral proliferation, was partially purified from the extract and its biological characteristics were examined.2) The antiviral activity was seen on enveloped viruses such as HVJ (Sendai virus), HSV (herpes simplex virus type-1), and HIV (human immunodeficiency virus type-1), but not on poxvirus, a non-enveloped virus. The active substance directly affects virion itself and inhibits the synthesis of a viral specific gene without affecting viral adsorption or entry into the host cell in an HVJ–LLC-MK2 cell system.

In this paper, we further purified and isolated the active antiviral substance by the extraction of a hydrophobic fraction with 1:1 chloroform–methanol and thin layer chromatography (TLC) from the partially purified active substance, then chemically characterized the active substance. From analysis of the visible absorption spectrum, fluorescence spectrum, mass spectrum and atomic absorption, the active substance was found to be a chlorophyll-like substance with a molecular mass of about 530, containing a magnesium ion. Biologically, this substance has unique characteristics. Its antiviral activity appears under light irradiation but disappears in the dark. Further, this activity was dependent on temperature. Under light irradiation, this substance also had marked hemolytic activity. In addition, its activity was found to be lost in the presence of serum.

MATERIALS AND METHODS

Purification of an Active Substance  An active antiviral substance was purified from SF-2 (a partially purified active substance). SF-2 was partially purified from silkworm faecal extract as described previously.2) Namely, dried silkworm faeces were extracted with phosphate-buffered saline (PBS(−); NaCl 8 g/l, KCl 0.2 g/l, Na2HPO4·12H2O 2.9 g/l, KH2PO4 0.2 g/l) at 60°C, and precipitated with a 50% saturation of solid ammonium sulfate. Lyophilized precipitate was dissolved in 1 N NaOH solution (10 mg/ml) for 24 h. After adjusting the pH to 7.4 by HCl, the active fraction was obtained by gel filtration within the void volume. This active fraction, termed SF-2, was kept at 4°C until use.

For the extraction of hydrophobic substances, the Bligh and Dyer method was used.3) Namely, 2.5 ml of methanol and 1.25 ml of chloroform were added to 1 ml of sample. After vigorous shaking for 2 min, 1.25 ml of chloroform was added to this solution, and the mixture was shaken again. Further, 1.25 ml of PBS(−) was added and shaken, followed by centrifugation at 3000 rpm for 5 min, and the chloroform layer was collected. The remaining layers, water-soluble and fluff layers, were rinsed with 1.5 ml of chloroform, then the chloroform layer was collected. After mixing each chloroform layer, 2 ml of 3:48:47 chloroform–methanol–water was added. The mixture was then shaken and centrifuged, and the chloroform layer was collected. To remove the hydrophilic substance completely, washing with 3:48:47 chloroform–methanol–water was done again. The collected chloroform layer was evaporated with N2 or aspirated at 30°C, then dissolved in chloroform. This fraction was stored at 4°C in the dark, with replacement by N2 until use.

A TLC was performed as described below. Extraction of the active spot from the thin layer chromatoplate was performed as follows: Each spot, collected from chromatoplates, was suspended in 1:2 chloroform–methanol, and silica gel was removed by a glass filter.

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Then, the silica gel was rinsed with 6:5:1 chloroform-methanol-water. These filtrates were mixed and evaporated. After the addition of 1 ml of 1:1 chloroform-methanol and 0.45 ml of water, the mixture was shaken and centrifuged, then the chloroform layer was collected and evaporated completely. For biological experiments, the analysis of antiviral or hemolytic activity, this mixture was dissolved in a small quantity of dimethyl sulfoxide (DMSO), then dissolved in PBS(−) (2% DMSO in PBS(−)) finally. This solution was stored at 4 °C in the dark, with replacement by N₂ until use. Under this condition, its antiviral activity was maintained for at least four months.

Chlorophyll-a and -b were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Sigma Chemical Co. (CA, U.S.A.), respectively. For analysis of antiviral activity, these chlorophylls were dissolved in DMSO at 4 mg/ml, then dissolved in PBS(−).

The concentration of L4-1 (lipid fraction 4-1), the isolated active substance, was determined by measuring optical density (OD₆₇₀) and comparing it with that of chlorophyll-a as follow:

\[
\text{concentration of L4-1 (µg/ml)} = 36 \times \text{OD}_{670} \text{ in 2% DMSO in PBS(−)}
\]

**TLC Analysis** TLC was performed at room temperature as follows: A glass plate (5 cm × 20 cm or 20 cm × 20 cm) was applied with a 0.25 mm thickness of silica gel H (Kieselgel 60H, Merck). The first development solvent used was 65:25:4 chloroform-methanol-water. For two-dimensional development, 80:20:2:1 diethyl ether-benzene-acetone-acetic acid was used as a secondary solvent. All spots on the chromatoplate were detected by the vapor of I₂ or by heating at 120 °C after spraying it with 100% H₂SO₄. The Rf (rate of flow) value was expressed as follows:

\[
R_f = \text{(moved length of substance/moved length of developing solvent)}
\]

**Cells and Viruses** LLC-MK2 and Vero line cells, derived from monkey kidney, were grown in minimum essential medium (MEM) supplemented with 10% calf serum (CS) as described previously.⁴⁵

MT-4 cells, the human CD4+ T cell line, were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 µg/ml of streptomycin and 100 U/ml of penicillin.

Hemagglutinating virus of Japan (HVJ, Sendai virus), Z strain, was propagated in embryonated eggs as described before, and the chorioallantoic fluid was harvested and stored at 4 °C until use.

HSV (herpes simplex virus type-1), G strain, was prepared by infection to Vero cells. After the appearance of complete cytopathic effects (CPE) by HSV infection, the culture fluids were harvested by low speed centrifugation, then stored at −80 °C until use.

HIV (human immunodeficiency virus type-1) strain JMH-1 was propagated in MT-4 cells, and the clarified supernatant fluid was stored at −80 °C until use.

**Viral Infection and Infectivity Titration** HVJ infection to LLC-MK2 cells was performed as described previously.⁵¹ Namely, HVJ was inoculated onto LLC-MK2 cell monolayers at 100 MOI (multiplicity of infection) (100 virions/cell) (approximately 8 HAu [hemagglutination units] per 35 nm plate) in an ice bath for 30 min. After viral adsorption, the cells were washed twice with PBS(−) and cultured in 3 ml of DMEM (Dulbecco’s modified Eagle’s medium) with 10% CS. The 0.5 ml samples of culture media were harvested at 24 and 48 h after infection, then virus production was measured by hemagglutinating activity (HA) using the procedure of Salt.⁵⁹

HSV infection to Vero cells was performed as described previously.²¹ Briefly, 0.2 ml of virus samples (about 50 PFU [plaque forming unit]/0.2 ml) were inoculated onto Vero cell monolayers in 60 mm plates and incubated for 1 h at 37 °C. Then, the monolayers were washed twice with PBS(−). MEM with 10% CS containing 5% anti-HSV human serum was added to the plates. The number of plaques as HSV infection was counted at 72 h after infection.

HV infection to MT-4 line cells was conducted as described previously.²¹ Namely, an aliquot of HV was diluted 20-fold with PBS(−). The volume of 0.1 ml of the dilution was 10-fold serially diluted with the culture medium in wells of 96-well microplates, and 0.02 ml of each dilution was dispersed into 3 wells. The volume of 0.1 ml of MT-4 cell suspension (2 × 10⁶ cells/0.1 ml) was dispersed into wells. The plates were then incubated in a 5% CO₂ incubator. The cultures in each well were subcultured into wells of 24-well plates after 4 d, and CPE was examined after another 3 d. Virus infectivity was determined by the Reed-Muench method.

**Treatment of Viruses with L4-1** On the HVJ–LLC-MK2 plating cell system, equal volumes of 94 HAu/ml HVJ solution in PBS(−) and 50, 100 and 200 ng/ml of L4-1 were mixed before infection and incubated for 15 min in an ice bath. Then, 0.17 ml of the mixture was inoculated onto LLC-MK2 cell monolayers in a 35 mm plate at 100 MOI of HVJ.

In the case of HSV, equal volumes of 2.5 × 10⁶ PFU/0.1 ml of HSV stock preparation and 80 or 200 ng/0.1 ml of L4-1 were mixed before infection and incubated for 15 min in an ice bath. The mixtures were diluted 5 × 10⁶-fold with PBS(+) (PBS(−) containing 2 mm CaCl₂) to prevent the effect of a free L4-1. Next, 0.2 ml of the diluted mixtures were inoculated onto Vero cell monolayers.

In the case of HIV, an aliquot of HIV was diluted 10-fold with PBS(−), and 0.1 ml of the dilution was mixed with 55 ng/0.1 ml of L4-1. The mixture was then kept at 4 °C for 15 min before infection.

Pretreatment of L4-1 with CS was performed as follows: Equal volumes of 800 ng/ml of L4-1 and 20% CS in PBS(−) were mixed for 15 min in an ice bath, then equal volumes of the mixture (400 ng/ml of L4-1 in 10% CS in PBS(−)) and 94 HAu/ml of HVJ were mixed for an additional 15 min in an ice bath before infection.

For pretreatment of L4-1 with bovine serum albumin (BSA: albumin bovine, F-V, Nakalai Tesque, Kyoto, Japan) or rabbit immunoglobulin G (IgG; purified from the serum of a non-immunized rabbit using an Ampure PA Kit as an affinity column of protein A (Amersham Japan Co., Tokyo, Japan)), equal volumes of 800 ng/ml of L4-1 and 10 mg/ml of BSA or IgG in PBS(−) were
mixed for 15 min in an ice bath, then equal volumes of the mixture (400 ng/ml of L4-1 in 5 mg/ml of BSA or IgG) and 94 HAU/ml of HVJ were mixed for an additional 15 min in an ice bath before infection.

**Indirect Immunofluorescent Staining** Indirect immunofluorescent staining was performed as described previously. Namely, after inoculation of HVJ to cells grown on glass coverslips on an ice bath for 30 min, the cells were washed with PBS (−), then stained with rabbit anti-serum against HVJ envelope (anti-HVJ envelope antibody) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin. For observation of infected cells 24 h after infection, the cells were fixed with 1:1 acetone–methanol for 12 min at −20°C. Fluorescence was examined using an Axiophoto (Carl Zeiss, Germany).

**Immunoelectron Microscopy** All procedures were performed as described previously. Namely, after infection of HVJ on an ice bath, the cells were cultured for 10 min at 37°C, then the cells were treated with anti-HVJ envelope antibody for 30 min in an ice bath. After being washed 3 times with PBS (+), the dishes were treated with ferritin-conjugated goat anti-rabbit immunoglobulin (Sigma Chemical Co., CA, U.S.A.) for 30 min in an ice bath, then washed 6 times with PBS (+) before fixation. Monolayer cultures of infected cells in 60 mm plastic plates were embedded in Quotol 812 (Nisshin EM Co., Tokyo, Japan) as described previously. Thin sections were double-stained with 2% uranyl acetate and 1% lead citrate, and observed with a JEOL 2000EXII electron microscope (Tokyo, Japan) at 80 kV.

**Spectral Analysis** For visible spectrum analysis, a visible wavelength of L4-1 in 100% acetone was scanned with a Beckman DU 640 spectrophotometer (Beckman, U.S.A.).

For fluorescence spectrum analysis, excitation and emission wavelengths of L4-1 in 100% acetone were measured by a spectrofluorophotometer RF-5000 (Shimadzu, Kyoto, Japan).

Infrared spectrum was measured on a FTIR-8100A Fourier Transform Infrared Spectrophotometer (Shimadzu, Kyoto, Japan). For measurement, L4-1 was dissolved in 100% chloroform.

Atomic absorption analysis was performed with a flame emission spectrophotometer AA-640-12 (Shimadzu, Kyoto, Japan).

Fast atom bombardment-mass spectrometry (FAB-MS) was performed with a JEOL JMS-SX-102AQ hybrid mass spectrometer. The sample in chloroform–methanol was dissolved in a thioglycerol matrix.

1H-NMR analysis was performed with a Varian XL-300 NMR Spectrometer. For this, L4-1 was dissolved in CDCl3, and TMS (tetramethylsilane) was used as a standard substance.

**Photodynamic Treatment of HVJ with L4-1** After equal volumes of HVJ solution (94 HAU/ml) and L4-1 (400 ng/ml) were mixed, the mixture in a glass tube (17 mm diameter) was exposed to visible light for 15 min on an ice bath before infection. A 15 watt white lamp was used as the light source at a distance of 10 cm from the sample. During the viral infection, the virus samples were as shielded from the light as possible.

**Hemolytic Activity of L4-1** Equal volumes of 2% chick red blood cells (c-RBC) and L4-1 (400 ng/ml) were mixed, then the mixture in a glass tube (14 mm diameter) was exposed to visible light (a 15 watt white lamp, 10 cm distance from the sample) for 15 min on an ice bath. At the indicated time, the supernatant was obtained by centrifugation at a low speed and was measured at OD0.575 as the release of hemoglobin.

**RESULTS**

**Purification of an Active Substance** As reported previously, the active substance (SF-2) that was partially purified with alkali treatment and gel filtration from silkworm faeces extract reveals glycoprotein-like characters with a molecular mass of about 25 and 14 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and lectin blotting, respectively. In addition, it is considerable that the active site is not protein, because its activity is heat-stable and not lost by protease digestion.

We first tried to purify the active substance with ion exchange and hydroxyapatite column chromatography by monitoring its antiviral effect on viral production with a HA assay in the HVJ–LLC-MK2 cell system. However, the isolation was not successful. Further, for sugar purification, lectin column chromatography was conducted. Again, the active substance was not isolated. From these results, it was considered that the active site may exist in lipid. Thus, the lipid fraction was extracted with 1:1 chloroform–methanol and its antiviral activity was examined. Activity was detected in the lipid fraction of

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**Fig. 1.** TLC Analysis of Hydrophobic Fraction

The total hydrophobic substance, extracted from the partially purified active substance (SF-2), was developed by TLC. (a) After development, only a brownish spot, the antiviral active spot, was observed under visible-ray. (b) The antiviral active spot was a red colored fluorescence under UV-ray (302 nm). (c) Developed plate was colored by heating at 120°C after spraying 100% H2SO4. 6 spots were detected under visible-ray. The same observation was obtained with coloration by a vapor of I2. Each arrow in a–c indicates the antiviral active spot.
SF-2. Further purification of the lipid fraction was achieved by TLC using chromatoplate on silica gel H. For this, the total hydrophobic substances were extracted from SF-2 with 1:1 chloroform–methanol. First, the chromatoplate was developed with 65:25:4 chloroform–methanol–water. As shown in Fig. 1a, only one spot, a brownish spot, was observed (arrow in Fig. 1a). For detection of all spots, on coloring this plate by heating at 120 °C after spraying it with 100% H₂SO₄, 6 spots were detected (Fig. 1c). The fourth spot from the top (Rf = 0.68), a brownish spot before coloration, possessed antiviral activity (arrow in Fig. 1c), whereas the others did not. Interestingly, on observation under UV-ray (302 nm), this active spot produced a red fluorescence (arrow in Fig. 1b). For further purification, two-dimensional development was performed with 80:20:2:1 diethyl ether–benzene–acetone–acetic acid. The antiviral active spot was further separated into 3 spots (arrows in Figs. 2a and b). Spot 4-1 had marked antiviral activity. On the other hand, weak activity was observed with spot 4-2, and no antiviral activity was observed with spot 4-3. Spot 4-1, the marked active antiviral spot, was named L4-1, and its chemical and biological characteristics were examined.

Effect of L4-1 on Virus Infections To start the antiviral effect of L4-1 on viral production was examined using an HVJ-LLC-MK2 line cell system by assay of HA. In a previous report, it was clearly indicated that the active substance directly interacts with virion itself for the activity. Hence, HVJ virions were pretreated with L4-1 and infected as described in Materials and Methods. As shown in Fig. 3, HA as viral production was suppressed dependent on the L4-1 concentration. It is noteworthy that viral production was completely inhibited with a very low concentration (200 ng/ml) of L4-1. Its activity was about 10²-fold higher in comparison with SF-2, a partially purified active fraction.

Viral infection of HVJ pretreated with L4-1 was further confirmed by indirect immunofluorescent staining and indirect immunoelectron microscopy using an anti-HVJ envelope antibody which specially reacts with HANA (hemagglutinin-neuraminidase) and F (fusion) glycoproteins. At 0 h after infection (before incubation at 37 °C), viral adsorption with granular stainings of fluorescence was seen with focus on the apical regions of the cells by indirect immunofluorescent staining (Fig. 4b). In general, after viral adsorption, HVJ enters into the host cell with envelope fusion between the viral envelope and the cell membrane. At 10 min after incubation, viral entry into the cell with envelope fusion was confirmed by immunoelectron microscopy, and already, viral antigens integrated into the cell membrane began to incorporate by endocytosis with a coated pit (asterisk in Fig. 4e). Thus, viral adsorption and entry into the host cell were not affected by L4-1. In spite of viral entry into the cell, the synthesis of viral specific proteins was not observed at 24 h by indirect immunofluorescent staining (Fig. 4c). These observations were identical to those of SF-2-treated HVJ infected cells, as described previously.

The antiviral effect of L4-1 on other enveloped viruses was also tested with HSV and HIV. On pretreating HSV (2.5 x 10⁶ PFU/0.1 ml) with 200 ng/0.1 ml of L4-1, the
Fig. 4. Observation of HIVJ Antigens in the Infected Cells by Fluorescence Microscopy and Immunoelectron Microscopy

L4-1 pretreated HIVJ infected cells were observed by indirect fluorescence microscopy (a, b) or immunoelectron microscopy (c, d), using anti-HIVJ envelope antibody. After viral infection on an ice bath (a, b), the cells were cultured for 10 min (c) and 24 h (c, d) at 37°C. At 0 h after infection (before culture at 37°C), viral adsorption with granular staining of fluorescence was seen on the cell surface (b). After culture for 10 min, viral entry into the cell with envelope fusion was observed, and viral antigens integrated into the cell membrane began to incorporate by endocytosis with the coated pit (asterisk in c). After culture for 24 h, however, the cells did not express newly synthesized viral specific proteins in the cells (c). (a) shows the cells phase contrast image of (b). (d) shows the cells infected with non-treated HIVJ at 24 h after culture. A virion labeled with ferritin grains is seen on the cell surface (arrow head in e). (a, b) bar: 10 µm. (c) bar: 0.5 µm.

Table 1. Antiviral Effect of L4-1 to HSVa

<table>
<thead>
<tr>
<th>Amount of L4-1 (ng/0.1 ml)</th>
<th>Number of plaques/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>63 (100%)</td>
</tr>
<tr>
<td>80</td>
<td>43 (67%)</td>
</tr>
<tr>
<td>200</td>
<td>7 (11%)</td>
</tr>
</tbody>
</table>

a) Equal volumes of HSV stock preparation (2.5 × 10⁶ PFU/0.1 ml) and L4-1 were mixed. After incubation in an ice bath for 15 min, the mixture was diluted 5 × 10⁶-fold with PBS (+). Then 0.2 ml of the diluted mixture was added to Vero cell monolayers. Cytotoxicity of L4-1 to Vero cells was not observed at 85.

HIVJ infection was inhibited by L4-1. Consequently, it was confirmed that L4-1 also had marked antiviral activity to HSV and HIV.

Table 2. Antiviral Effect of L4-1 to HIVa

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (non-treatment)</th>
<th>Treatment with L4-1 (55 ng/0.1 ml)</th>
<th>1 × log 10⁶ TCID₅₀/ml</th>
<th>1 × log 10⁴ TCID₅₀/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>63 (100%)</td>
<td>1 × log 10⁶ TCID₅₀/ml (55 ng/0.1 ml)</td>
<td>67%</td>
<td>67%</td>
</tr>
<tr>
<td></td>
<td>43 (67%)</td>
<td>1 × log 10⁴ TCID₅₀/ml (55 ng/0.1 ml)</td>
<td>11%</td>
<td>11%</td>
</tr>
</tbody>
</table>

a) Before infection to MT-4 cells, equal volumes of HIV preparation diluted with PBS (−) and L4-1 were mixed, then incubated at 4°C for 15 min. Cytotoxicity of L4-1 to MT-4 cells was not observed.

Chemical Characterization of L4-1 TLC analysis of L4-1 was already shown in Fig. 2a. Under a visible ray, L4-1 was colored green on a chromatoplate (data not shown). However, under UV-ray (302 nm), L4-1 produced red fluorescence (Fig. 2b). This red fluorescence is a characteristic color of porphyrins under UV-ray. To obtain more precise characteristics of the active substance,
we conducted visible absorption and fluorescence spectra analysis of L4-1. As shown in Fig. 5a, L4-1 in acetone showed primary absorbance at 408 and 663 nm, mainly. On the other hand, on fluorescence spectral analysis of L4-1, its excitation wavelength was 408 nm and emission wavelength 662 nm (Fig. 5b). These visible and fluorescence spectra were similar to those of chlorophylls, a kind of porphyrin. To clarify this point, infrared absorption spectral analysis of L4-1 was conducted. The main bands were detected at 2928, 1730, 1719, and 1601 cm\(^{-1}\), which are phytol, ester, ketone, and C=C, respectively. These are characteristic bands of chlorophylls. Chlorophyll-a and -b, typical chlorophylls, possess Mg\(^{2+}\) in the molecule. Then, atomic absorption analysis was conducted to confirm Mg\(^{2+}\). As a result, Mg\(^{2+}\) was detected in L4-1. The molecular mass of L4-1 was found to be about 530, as judged by mass spectrometry.

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Fig. 5. (a) Visible Absorption Spectrum of L4-1 in 100% Acetone was Measured. Main absorption peaks were observed at 408 and 663 nm. 
(b) Fluorescence Spectrum of L4-1 in 100% Acetone was Measured with a Spectrofluorophotometer.

Maximum excitation and emission wavelengths were 408 and 662 nm, respectively.

Fig. 6. Mass Spectrum Analysis of L4-1
L4-1 was analyzed by positive FAB-MS using a thiglycerol matrix. A main peak, assumed to be a mass of L4-1, was observed at 535 m/z.

Fig. 7. \(^1\)H-NMR Analysis of L4-1
L4-1 dissolved in CDCl\(_3\) was analyzed by \(^1\)H-NMR spectrometry.
Further, $^1$H-NMR spectral analysis of L4-1 was conducted, and this result is shown in Fig. 7.

Chemically and biologically, L4-1 was unstable. When the developed chromatoplate was allowed to stand in contact with air at room temperature under light, the green color of L4-1 was gradually changed to brown. Subsequently, its brown color became weak and finally disappeared for a few days. During this same time, the reddish fluorescence of L4-1 under UV-ray was also changed to a white yellowish fluorescence. Biologically, the antiviral activity was lost within about one week under the same conditions.

**Relation of L4-1 to Chlorophylls** From the results of visible absorption, fluorescence, and infrared absorption spectral analysis, it was indicated that L4-1 may be a chlorophyll derivative. Therefore, we next tested the antiviral activities of chlorophyll-a and -b as typical, standard chlorophylls. For this, the effects of chlorophyll-a and -b on HVJ production were examined in a HVJ-LLC-MK2 cell system, as shown in Fig. 3. On pretreating virions (94 HAU/ml) with chlorophyll-a at the same concentration as L4-1 (200 ng/ml), viral production was not inhibited. However, on pretreating virions with a higher concentration of chlorophyll-a (200 $\mu$g/ml), viral production was inhibited, so the antiviral activity was detected (Fig. 8). On the other hand, on pretreating virions with 200 $\mu$g/ml of chlorophyll-b, viral production at 48 h was about half that of the control. Thus, chlorophyll-a surely possessed antiviral activity, and its activity appeared to be higher than that of chlorophyll-b. However, its activity was about $10^3$-fold lower than that of L4-1. Further, the effect of chlorophyll-a on HSV and HIV was tested, and no antiviral effects of chlorophyll-a on HSV and HIV were observed at concentrations of 800 and 200 $\mu$g/ml, respectively.

Next, chlorophyll-a was chemically compared with L4-1. For this, TLC analysis of L4-1 and chlorophyll-a was conducted as shown in Figs. 1a and b. The mobility of L4-1 ($R_f$ = 0.68) was apparently different from that of chlorophyll-a ($R_f$ = 0.92) on the chromatoplate (data not shown). Furthermore, spectral patterns of L4-1 with $^1$H-NMR and mass spectrometry were clearly different from those of chlorophyll-a.

**Hemolytic Activity of L4-1** It has been reported that some decomposition products of chlorophyll-a and -b have strong hemolytic activity under visible light; however, chlorophyll-a and -b have little such activity. We next tested whether L4-1 has the hemolytic activity. The experiment was conducted in the dark or under light. As shown in Fig. 9, when the sample was irradiated with visible light, L4-1 (final concentration 200 ng/ml) markedly hemolyzed c-RBC after 3 h. In contrast, no hemolysis was observed in the dark. Thus, L4-1 had hemolytic activity by light irradiation, as did the decomposition products of chlorophyll-a and -b, as already known.

**Antiviral Activity of L4-1 Dependent on Light** As described above, it was suggested that L4-1 is a chlorophyll derivative which belongs to the porphyrin family. It has been reported that porphyrins inactivate enveloped viruses by light irradiation. Therefore, we tested whether the antiviral activity of L4-1 is affected by light. For this, pretreatment of HVJ with L4-1 was conducted in the dark or under light, as shown in Fig. 9. The viral production was examined by HA assay, as shown in Fig. 3. As shown

![Fig. 8. Antiviral Effects of Chlorophyll-a and -b to HVJ](image)

Before infection, equal volumes of 94 HAU/ml of HVJ and 100 ng/ml of chlorophyll-a or 100 ng/ml of chlorophyll-b were mixed in an ice bath for 15 min, respectively. As a control, PBS (-) was mixed with HVJ (●).

![Fig. 9. Hemolytic Activity of L4-1](image)

Equal volumes of L4-1 (400 ng/ml) and 2% c-RBC were mixed, and the mixture was placed on an ice bath under light (●) or in the dark (▲). As a control, only 1% c-RBC solution was placed on the ice bath under light (▲). After 1, 2, and 3 h, the supernatant of the mixtures was measured at OD$_{540}$.

![Fig. 10. Effect of Light on Antiviral Activity of L4-1](image)

Equal volumes of HVJ solution (94 HAU/ml) and L4-1 (400 ng/ml) were mixed and placed on an ice bath for 15 min in the dark or under light. After viral infection, HA as viral production was assayed at 24 h (▲) and 48 h (●).
Table 3. Effect of Light on Anti-HSV Activity of L4-1<sup>a</sup>

<table>
<thead>
<tr>
<th>Light irradiation</th>
<th>Number of plaques/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (non-treatment)</td>
<td>69 (100%)</td>
</tr>
<tr>
<td>+</td>
<td>51 (74%)</td>
</tr>
<tr>
<td>Pretreated with L4-1 (200 ng/0.1 ml)</td>
<td>69 (100%)</td>
</tr>
<tr>
<td>+</td>
<td>10 (14%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Before infection to Vero cell monolayers, equal volumes of HSV stock preparation (2.5 x 10<sup>6</sup> PFU/0.1 ml) and L4-1 were mixed on an ice bath for 15 min in the dark or under light. During HSV infections, the virus samples were shielded from the light as possible.

In Fig. 10, L4-1 did not inhibit the viral production if pretreatment was conducted in the dark. However, when pretreatment was conducted under light, viral production was strongly inhibited by L4-1.

The effect of light on the anti-HSV activity of L4-1 was also tested. As shown in Table 3, the plaque formation as HSV infection was not suppressed by L4-1 if pretreatment of virions with L4-1 was conducted in the dark. This result indicates that the antiviral effect of L4-1 on HSV was also lost in the dark as well.

Furthermore, the antiviral activity of L4-1 was also dependent on temperature. When the pretreatment of HVJ with L4-1 was conducted at 37 °C for 15 min, unlike in an ice bath as in Fig. 3, HA as viral production was completely inhibited with a lower concentration (25 ng/ml) of L4-1 than in the case of in an ice bath (200 ng/ml) (data not shown). This result indicates that the activity of L4-1 at 37 °C is about 10-fold higher in comparison with that under the ice bath condition.

Inhibition of Antiviral Activity of L4-1 with Serum During the experiments, we unexpectedly found that the antiviral activity of L4-1 was lost in the presence of serum. Therefore, we tested the effect of serum on the antiviral activity of L4-1. In the HVJ–LLC-MK2 cell system, L4-1 was mixed with CS (final concentration 10% CS) before treatment with HVJ virions, as described in Materials and Methods. The result is shown in Fig. 11. On mixing of L4-1 with CS, its HA as viral production was the same as the non-treatment sample (without both L4-1 and CS). Thus, the antiviral activity of L4-1 was lost in the presence of CS.

Since albumin and globulin are the main components of serum, we tested whether BSA or IgG inhibits the activity of L4-1. As a consequence, both BSA and IgG did not affect the activity of L4-1. These results suggest that some component(s) of serum, other than albumin or globulin, inhibits the antiviral activity of L4-1.

DISCUSSION

In this paper, we purified the antiviral active substance of silkworm faeces by TLC and examined its chemical and biological properties. The active substance (L4-1) was found to be a porphyrin type from the observation under UV-ray. The results of visible absorption, fluorescence, and infrared absorption spectra analyses indicated that L4-1 is a chlorophyll-like substance with a molecular mass of about 530 as judged by mass spectrometry.

Silkworms mainly eat mulberry leaves. Accordingly, it seems that silkworm faeces include chlorophylls derived from mulberry leaves. Interestingly, however, L4-1 was clearly different from chlorophyll-a or -b, which are typical chlorophylls. It has been reported that some decomposition products of chlorophylls were found in silkworm excrement. From these points, it is suggested that L4-1 is one such metabolic product of chlorophyll in silkworm body. In addition, it is considered that spots 4-2 and 4-3, obtained by two-dimensional TLC, may be degradation products of L4-1.

“Pyropheophorbide-a,” a degradation product of chlorophyll-a (molecular mass of 534), is known to be obtained from silkworm excrement and it possesses the most marked hemolytic activity among degradation products of chlorophylls. On considering chemical characteristics, L4-1 seems to be one of these “pyropheophorbide-a” derivatives. However, L4-1 possesses Mg<sup>2+</sup> in the molecule, unlike the “pyropheophorbide-a.” Accordingly, it is apparently different from “pyropheophorbide-a.”

It has been reported that some porphyrin derivatives inactivate enveloped viruses by light irradiation. It is well known that porphyrins and chlorophylls produce active oxygen by light irradiation, and the active oxygen causes damage to the lipid membrane, proteins, and nucleic acids. In practice, Lewin et al. and Schnipper et al. reported that hematoporphyrin derivative inhibited viral adsorption and penetration into the host cell with light irradiation in HSV–Vero cell systems. Therefore, it is considered that the anti-HSV activity of porphyrins may be derived from damage of viral envelope protein and/or lipid, possibly by the active oxygen. L4-1, which we purified from silkworm faecal extract, was a chlorophyll-like substance, and its antiviral activity was also dependent on light irradiation. Consequently, L4-1 may also inactivate the virions by producing active oxygens. However, to be noteworthy, L4-1 does not inhibit...
HVJ adsorption or entry into the host cell. This point apparently differs from the observations of Lewin et al., and Schnipper et al. in the HSV–Vero cell system. Also, it was recently reported that a mixture of different porphyrin types from silkworm faeces (CpD-D) photo-dynamically inhibit the reverse transcriptase activity of Gross leukemia virus, in vitro. However, L4-1 was effective not only upon a retrovirus but also on other enveloped viruses such as HVJ and HSV, at a much lower concentration than CpD-D. Moreover, L4-1 did not show cytotoxicity to culture cells, unlike CpD-D. From the above observations, L4-1 is a new derivative of porphyrin from silkworm faeces, and it has strong antiviral activity.

The antiviral activity of L4-1 was inactivated with serum. It was not caused by albumin or globulin, the main components of serum. Further, the activity was dependent on temperature. Although details of these mechanisms are unknown at present, these are interesting phenomena. Now, analysis of the mechanisms of the inactivation by serum and the temperature-dependent activity of L4-1 are in progress at the molecular level.

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