Sulfated Fibroin, a Novel Sulfated Peptide Derived from Silk, Inhibits Human Immunodeficiency Virus Replication in Vitro

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We prepared two kinds of sulfated silk fibroins, ScFib30 and ScFib31, which contain different amounts of sulfate. These sulfated silk fibroins have anti-HIV-1 activity in vitro, apparently due to interference with the adsorption of virus particles to CD4+ cells, and completely blocked virus binding to the cells at a concentration of 100 μg/ml. Sulfated fibroins also abolished cell-to-cell infection-induced syncytium formation upon cocultivation of MOLT-4 and MOLT-4/HIV-1m cells, suggesting that they would interfere with gp120 and prevent the formation of gp120/CD4 complex. Silk is used in biomaterials such as surgical sutures and is believed to be a safe material for humans. In accordance with low anticoagulant activity and high anti-HIV-1 activity against both X4 HIV-1 and R5 HIV-1 strains, sulfated silk fibroins have potential as antiviral material such for a vaginal anti-HIV formulation.

Key words: AIDS; silk fibroin; sulfated peptides; binding inhibitor; anti-HIV formulation

Human immunodeficiency virus type 1 (HIV-1) is the causative retrovirus of acquired immunodeficiency syndrome (AIDS). Although some anti-retroviral drugs, such as nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and HIV-specific protease inhibitors, have shown to prolong survival and improve the quality of life of patients with advanced HIV infection, serious toxicity and side effects and the emergence of drug-resistant HIV strains have been reported.1-3) Therefore, there is an urgent need to develop new types of anti-HIV reagents, with different mechanisms of action from the anti-retroviral drugs which are presently being used.

The main three routes of HIV transmission are through unprotected sex, contaminated needles, and from mother to child. These three types of transfer account for over 99% of all new infections in the past year. It is important to not only consider the treatment of patients already infected with HIV, but also chemoprophylaxis and protection from new infection. Heterosexual transmission of HIV is responsible for the large majority of infections and it is therefore, essential to develop effective prevention strategies to combat further spread of the epidemic through sexual contact.

Both sulfated polysaccharides and acamitid group-containing polysaccharides have attracted much attention since it was found that the former had high anti-HIV activity. We have reported that lentinian sulfate,4) curdlan sulfate,5) and several sulfated alkyl oligosaccharides6)7 had high anti-HIV activity, but low anticoagulant activity. During serial studies for evaluation of anti-HIV compounds, we found that sulfate residues might play a key role in blocking HIV infection, because the addition of sulfate residues to glycyrhrizin apparently endowed the compound with high antiviral activity.8) We have also been able to convert several non-sulfated compounds into effective anti-HIV substances in vitro.9) However, most sulfated polysaccharides, such as dextran sulfate, have not been used clinically as therapeutic agents for HIV infection because of their anticoagulant activity, poor absorbance, and instability.10-12)

Recently, the sequence of the Bombyx mori silk fibroin gene was reported, showing that the fibroin coding region is composed of a repeating bipartite unit sequence.13) Silk is a natural product and the core element is characterized by repeats of a highly conserved 18-bp sequence coding for perfect repeats of

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the unit peptide Gly-Ala-Gly-Ala-Gly-Ser. Silk is also used in biomaterials such as surgical sutures and is believed to be a safe material for humans. We had synthesized sulfated fibroin and investigated its bioavailability. In accordance with the anti-HIV compounds development strategy of sulfated polysaccharides, antiviral activity and/or anticoagulant activity might be related to sulfate contents and repeated structures. Therefore, we investigated the anti-HIV activity and anticoagulant activity of sulfated fibroins. We also address their practical applications as medical products and the potential of a vaginal anti-HIV formulation.

Materials and Methods

Compounds and reagents. Silk fibroin was prepared from cocoons of the silkworm, Bombyx mori. Cocoons were cut in to small pieces and washed with toluene and methanol. Degreased cocoons were boiled in 0.5% sodium carbonate solution to remove sericin, and washed with hot water three times. The degummed silk was dried at 60°C in a vacuum for 12 h. Sulfation of silk fibroin was done by chlorosulfonic acid in pyridine. Two mL of chlorosulfonic acid was mixed with 20 mL of pyridine and added to 1 g of silk fibroin immersed previously in pyridine. Sulfation proceeded at 70°C for 8 h (SclfFib30) and 3 h (SclfFib31). After sulfation, the solution was neutralized with NaOH and the sulfated silk fibroin was precipitated with ethanol. The precipitate was collected by centrifugation and dissolved in distilled water. Finally, the sulfated fibroin solution was dialyzed against distilled water to remove salt, and lyophilized. Sulfate group contents of SclfFib30 and SclfFib31 were approximately 0.8 mmol/g and 0.5 mmol/g, respectively.

The following reagents were obtained from the indicated companies: dextran sulfate (MW 8 kDa) (Kowa Co. Ltd, Tokyo, Japan); curdlan sulfate (MW 79 kDa) (Ajinomoto Co. Ltd, Tokyo, Japan); RPMI 1640 medium (Gibco, Grand Island, NY); fetal calf serum (FCS) (Whittaker Bioproduct, Walkersville, MD); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Wako Pure Chemical, Osaka, Japan); fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Cappel Organon Teknika Co., West Chester, PA).

Cells and viruses. A human T-cell leukemia virus type I (HTLV-I) positive T-cell line, MT-4, and a lymphoblastoid T-cell line, MOLT-4 (clone no. 8), were subcultured twice a week at a concentration of 3 × 10⁵ cells/mL in RPMI 1640 medium with 10% (V/V) heat inactivated FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. MAGI-CCR5 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone, 300 μg/mL l-glutamine, 200 μg/mL G418, and 100 μg/mL hygromycin B. The X4 HIV-1 strain, (HIV-1NL4-3), and the R5 HIV-1 strains, (HIV-1JR-CSR and HIV-1JR-FL) were obtained from the culture supernatant of COS-7 cells transfected with molecular clone viruses pNL4-3, pYK-JRCSF, and pJR-FL, respectively. HIV-1HXB, an X4 HIV-1 strain, was prepared from a culture supernatant of MOLT-4/HIV-1HXB cells, which are persistently infected with HIV-1HXB. The infectious titer of virus stocks was measured by MAGI assay, and all virus stocks were stored at −80°C until use.

Antiviral assay. The anti-HIV activity of the test compounds was based on the inhibition of virus-induced cytopathogenicity in MT-4 cells as measured by the MTT method, and the inhibition of virus-specific antigen expression as measured by an indirect immunofluorescence method. To estimate the antiviral activity of both X4 and R5 HIV-1 strains, multinuclear activation of the galactosidase indicator (MAFI) assays were also done. MAGI-CCR5 cells were placed in 24-well plates at 4 × 10⁵ cells/well in RPMI 1640 medium containing 10% FCS and antibiotics. After 24 hours, the culture medium was removed from each well and replaced with fresh medium containing different concentrations of the test compounds and also the HIV-1HXB, HIV-1NL4-3, HIV-1JR-FL, or HIV-1JR-CSR virus solution (MOI = 0.2), giving a total volume of 400 μL. Two days later, the medium was removed and the monolayer was fixed with 500 μL of a solution of 1% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min at room temperature. The cells were then washed three times with PBS and incubated for 50 min at 37°C in 400 μL of a solution containing 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM MgCl₂, and 400 μg/mL of 5-bromo-4-chloro-3-indolyllaβ-d-galactoside (X-gal). Removing the staining solution and washing the cells twice with PBS stopped the reaction and the blue cells were counted under a microscope at a magnification of × 100.

Syncytium formation assay. MOLT-4 cells (5 × 10⁵) were cultured with an equal number of MOLT-4/HIV-1HXB cells in a 96-well cell culture plate containing various concentrations of the test substance. At 20 h of cocultivation, the number of viable cells was measured by the trypan blue dye exclusion method, and the fusion index (FI) was calculated as described previously.

Assay for virion binding to MT-4 cells. The inhibitory effect of the test compounds on virion binding to CD4-positive cells was measured by an indirect immunofluorescence-laser flow cytofluorographic method. MT-4 cells were exposed to highly con-
centrated HIV-1_{HIV} virions in the presence or absence of 100 μg/ml of SclFib30, SclFib31 or curdian sulfate. The cells were incubated for 60 min at 37°C and washed twice in PBS to remove the unbound virus particles. A high-titer polyclonal antibody obtained from a patient with AIDS-related complex (diluted 1/500 in PBS) was then added. After 60 min of incubation at 37°C, the cells were washed twice with PBS. The cells were then incubated with FITC-conjugated F(ab')2; fragments of rabbit anti-human IgG (diluted 1/30 in PBS) for 60 min at 37°C, washed twice in PBS, resuspended in 1 ml of 0.5% paraformaldehyde in PBS, and analyzed by a FACScan (Becton Dickinson, Sunnyvale, CA).

Prothrombin time (PT) and activated partial thromboplastin time (APTT). PT and APTT of plasma from a healthy volunteer were evaluated in the presence of the test substance by using an automated machine (CA-5000, Toa Medical Electronics). The test substance (40 μl) was mixed with 360 μl of normal human plasma and then PT and APTT were measured.

Results

In vitro anti-HIV activity of sulfated fibroin. The anti-HIV-1 activities of sulfated fibroins, SclFib30 and SclFib31, and non-sulfated silk fibroin were assessed by the protection from the HIV-1-induced cytopathic effects (CPE) and inhibition of virus-specific antigen expression in MT-4 cells in vitro (Fig. 1). While untreated HIV-1-infected MT-4 cells did not remain viable 5 days after virus infection, both SclFib30 (Fig. 1(A)) and SclFib31 (Fig. 1(B)) showed cell-protective activities against HIV-1-induced CPE and inhibition of HIV-1 antigen expression in a dose-dependent manner. The median anti-HIV-1 effective concentrations (EC_{50}) of SclFib30 and SclFib31 were 13.2 μg/ml and 13.1 μg/ml, respectively. No cytotoxic effects were observed up to 1000 μg/ml in both SclFib30 and SclFib31. However, no anti-HIV-1 activity was observed in non-sulfated silk fibroin treated cells (Fig. 1(C)). The anti-HIV-1 activities of SclFib30 and SclFib31 were confirmed by MAGI assay using X4 HIV-1 strains, HIV-1_{HIV}, HIV-1_{NL4-3}, and R5 HIV-1 strains, HIV-1_{JRFL} and HIV-1_{CSR}. As demonstrated in Fig. 2, both SclFib30 and SclFib31 inhibited not only X4 strains but also R5 HIV-1 strains.

Inhibition of syncytium formation. Cocultures of persistently HIV-1-infected MOLT-4 cells (MOLT-4/ HIV-1_{HIV}) and uninfected MOLT-4 cells were used for estimation of inhibition activity against cell-to-cell infection-induced syncytium formation. Both SclFib30 and SclFib31 effectively inhibited syncytium formation as effectively as curdlan sulfate, used as a positive control (Fig. 3).

Inhibition of virus binding to MT-4 cells. To discover the mechanism of action of sulfated fibroin, SclFib30 and SclFib31 were evaluated to find whether they were able to inhibit the binding of HIV-1 particles to CD4+ cells, as assessed by laser flow cytometry analysis. As demonstrated in Fig. 4, 100 μg/ml of SclFib30 and SclFib31 completely inhibited HIV-1 binding to MT-4 cells. These inhibitory activities were comparable to that of curdlan sulfate.

Anticoagulant activity of sulfated fibroin. The effects of sulfate fibroin on PT and APTT of human plasma were analyzed using an automated machine. SclFib30 did not significantly prolong PT at concentrartions up to 300 μg/ml. At 30–300 μg/ml of SclFib30, APTT was prolonged. The anticoagulant activity of SclFib30, however, was much weaker than that of heparin and dextran sulfate; they markedly

![Fig. 1](attachment:image.png)  
**Fig. 1.** Anti-HIV-1 Activity and Viral Antigen Inhibition in MT-4 Cells by SclFib30 (A), SclFib31 (B), and Non-sulfated Silk Fibroin (C). The viability of HIV-1_{HIV} infected MT-4 cells (shaded columns) and mock-infected MT-4 cells (open columns) was estimated by the MTT methods 5 days after infection. The number of viable cells is expressed as a percentage of mock-infected compound-free control cells. HIV-1 antigen positive cells were detected by the indirect immunofluorescence, using a polyclonal antibody as a probe. The number of viral antigen positive cells is expressed as a percentage of the HIV-1_{HIV} infected compound-free control.)
Fig. 2. Anti-HIV-1 Activity of ScFib30 (A) and ScFib31 (B) in MAGI-CCR5 Cells.
Anti-HIV-1 activity in different HIV-1 infected MAGI-CCR5 cells was estimated by MAGI-assay. The values are represented as a percent inhibition ratio compared to the number of blue cells in the compound-free control. ■: HIV-1_{TR}, □: HIV-1_{NL-A}, ■ HIV-1_{TR-FL}, □: HIV-1_{TR-CSI}.

![Graph A](image1)

![Graph B](image2)

Table 1. Effects of Sulfated Fibroin Prothrombin Time and Activated Partial Thromboplastin Time

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration mg/ml</th>
<th>PT* (sec)</th>
<th>APTT* (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fibroin #30</td>
<td>300</td>
<td>14.0</td>
<td>300.0</td>
</tr>
<tr>
<td>(µg/ml)</td>
<td>100</td>
<td>10.1</td>
<td>97.9</td>
</tr>
<tr>
<td>sulfate</td>
<td>30</td>
<td>12.6</td>
<td>50.8</td>
</tr>
<tr>
<td>(µg/ml)</td>
<td>10</td>
<td>11.7</td>
<td>34.6</td>
</tr>
<tr>
<td>Dextran</td>
<td>300</td>
<td>35.3</td>
<td>&gt;600</td>
</tr>
<tr>
<td>sulfate</td>
<td>100</td>
<td>20.1</td>
<td>&gt;600</td>
</tr>
<tr>
<td>(µg/ml)</td>
<td>10</td>
<td>12.1</td>
<td>65.6</td>
</tr>
<tr>
<td>Heparin</td>
<td>3</td>
<td>12.4</td>
<td>37.8</td>
</tr>
<tr>
<td>(U/ml)</td>
<td>1</td>
<td>11.1</td>
<td>39.4</td>
</tr>
<tr>
<td>PBS control</td>
<td>—</td>
<td>10.5</td>
<td>29.5</td>
</tr>
</tbody>
</table>

* Prothrombin time of normal human plasma in the presence of compounds.
* Activated partial thromboplastin time of normal human plasma in the presence of compounds.

Fig. 3. Inhibitory Effects of ScFib30, ScFib31, and Curdian Sulfate on Syncytium Formation in a Coculture of MOLT-4 and MOLT-4/HIV-1_{TR} Cells.
The number of syncytia was measured 20 h after cocultivation in the presence of various concentrations of test compounds and the percentage of fusion inhibition was calculated. □: ScFib30, ■: ScFib31, ◼: Curdian sulfate.

![Graph](image3)

prolonged PT and APTT (Table 1). No anticoagulant activity was observed in non-sulfated silk fibroin (Tamada, unpublished data).

Discussion

This study indicated that sulfated silk fibroins, ScFib30 and ScFib31, have anti-HIV-1 activity in vitro, apparently due to interference with the adsorption of virus particles to CD4+ cells. The infection of T lymphocytes and macrophages by HIV-1 is mediated by the binding of the HIV-1 envelope glycoprotein gp120 to the cell surface receptor of CD4 molecules, which is an integral membrane glycoprotein of CD4+ cells.\textsuperscript{3,12} Both ScFib30 and ScFib31 completely blocked virus binding to the cells at a concentration of 100 µg/ml (Fig. 4). This concentration was subtoxic to the target cell and did not influence the binding of monoclonal antibodies against cellular membrane surface proteins. Sulfated fibroins also abolished cell-to-cell infection induced syncytium formation upon cocultivation of MOLT-4 and MOLT-4/HIV-1_{TR} cells, suggesting they would interfere with gp120 and prevent the formation of gp120/CD4 complex.
The highest HIV infection-risk groups are men with a history of homosexual or bisexual activity, individuals who have sexual contact with homosexual or bisexual men, intravenous drug users, and persons receiving contaminated blood or blood products. Documentation of the transmission of HIV to steady heterosexual partners of intravenous drug users, bisexual men, and persons infected by contaminated blood or blood products suggests that any person engaging in unprotected sexual activity with persons with a known history of risk or unknown history is at some risk. This risk may increase with increasing numbers of sexual contacts or in areas where there is a higher prevalence of HIV infection. Therefore, it is important to develop effective prevention strategies to combat the spread of HIV infection through sexual contacts.

One plausible target for such agents is at HIV-1 entry into target cells. Earlier studies demonstrated that sulfated polysaccharides could be considered for a vaginal anti-HIV formulation because these compounds block the binding of gp120 and CD4 receptor at the HIV-1 adsorption to CD4 T cells. Other targets for virus entry inhibitors include two coreceptors on the cell surface, CCR5 and CXCR4, which together with CD4 mediate virus binding and membrane fusion. Macrophage-tropic strains of HIV, now classified as R5 viruses, replicate in macrophages and in primary CD4 T cells and use the CCR5 receptor, and T-tropic HIV-1 isolates (referred to as X4 viruses) replicate in primary or established CD4 lymphocytes and use the CXCR4 receptor. The pathogenic mechanisms responsible for CD4 T-cell decline in AIDS patients are related to not only quantitative but also qualitative changes in HIV-1. R5 strains are predominant during the initial transmission and asymptomatic stages of HIV-1 infection and X4 HIV-1 strains are also becoming prevalent, concomitant with the decline of CD4+ T cells, in the symptomatic stages. Thus, it might be required to inhibit R5 HIV-1 infection at the initial transmission stage. Sulfated silk fibroins, ScFib30 and ScFib31, inhibit HIV-1 cell-to-cell transmission and cell-free virus infection of both X4 HIV-1 and R5 HIV-1 strains. Anticoagulant activity of sulfated fibroins was much weaker than that of heparin and dextran sulfate. The anticoagulant activity of sulfated polysaccharides might be related with high density and repeated sulfated structures. Compared with heparin and dextran sulfate, sulfated fibroins which we used in this study have low sulfate contents. High anti-HIV-1 activity, low cytotoxicity and weak anticoagulant activity of sulfated fibroins, they might be AIDS-preventing compounds as a vaginal anti-HIV formulation, permissively. Further studies on pharmacokinetics, spermicidal activity, tolerance by genital mucosa, and effects on the normal vaginal flora are demanded in considering the clinical development of sulfated silk fibroin.

Acknowledgments

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References


