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Evaluation of sulfated fungal β-glucans from the sclerotium of *Pleurotus tuber-regium* as a potential water-soluble anti-viral agent

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Abstract—Six water-insoluble fractions of fungal β-glucans extracted by hot alkali (TM8-1 to TM8-6) from the sclerotia of *Pleurotus tuber-regium* (PTR) having different molecular weights (M_w) were sulfated to give their corresponding water-soluble derivatives (S-TM8-1 to S-TM8-6) with the degree of sulfation (DS) ranging from 1.14 to 1.74. The in vitro anti-viral activities of the native β-glucans (TM8s) and their sulfated derivatives (S-TM8s) were evaluated by the cytopathic effect assay (CPE) and the plaque reduction assay (PRA) against four kinds of viruses, including herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), respiratory syncytial virus (RSV), and influenza A virus (Flu A). Although TM8s were inactive in inhibiting the viral replication in cell cultures, the S-TM8 fractions with the defined M_w range had potent anti-viral activity against HSV-1 and HSV-2 as shown by the CPE assay. The PRA results suggested that S-TM8 fractions seemed to exert their anti-viral effect by binding to the viral particles, preventing the latter from infecting the host cells. It was plausible that the negative charges on the polymer chain of S-TM8 could interact with the positively charged glycoproteins on the surface of HSV, minimizing the interaction between the HSV and the negatively charged host cells. The anti-viral activity of the S-TM8s might also be explained by their more extended chain conformation in solution due to an increase in one of their molecular parameter, persistence length (q), as compared to the native TM8s. The potential use of S-TM8s as a water-soluble anti-HSV agent is discussed.

Keywords: Sulfation; β-Glucans; Mushroom sclerotia; *Pleurotus tuber-regium*; Anti-viral activities

1. Introduction

Sulfated polysaccharides have been shown to have anti-viral activities[1–4] and are being investigated for the treatment of HIV, HSV-1, HSV-2, and RSV infections. Most natural sulfated polysaccharides are complex polydisperse mixtures of macromolecules, which vary in their chemical structures and biological activities. Consequently, recent research has focused on producing water-soluble anti-viral sulfated polysaccharides with defined chemical structures. Recent studies have shown that several partially synthesized sulfated polysaccharides, such as dextran sulfate, are highly inhibitory to the in vitro replication of HIV and HSV.[5]

In our previous study, water-insoluble β-glucans extracted from the sclerotia of *Pleurotus tuber-regium* (PTR) by hot alkali (TM8s) had a main chain of β-(1→3)-D-glucopyranosyl units with every third unit having a (1→6)-β-D-glucopyranosyl branch on average.[6] We had also demonstrated that TM8s had potent in vivo and in vitro anti-tumor activities, mediated probably by both the immune system and direct cytotoxicity to tumor cells.[7] In view of the anti-viral activity of some partially synthesized sulfated polysaccharides, such as dextran sulfate[4] and schizophyllan sulfate,[8] it is anticipated that chemically modified TM8s with added sulfated groups (S-TM8s) might also have potent anti-viral activities as well as improved water solubility suitable for a wider pharmacological application. Therefore, six sulfated polysaccharides (S-TM8-1 to S-TM8-6) with M_w ranging from 6.0×10^4 to 64.8×10^4 were partially synthesized by nonselective sulfation from the native

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TM8s.[9] Structural characterization of the S-TM8 fractions in our previous study had shown that S-TM8s retained the basic structure of native TM8 with glucose residues having the C-6 fully substituted while the C-2 and C-4 were partially substituted by sulfated groups.[9] The polydispersity (M_w/M_n) of each S-TM8 fraction was relatively narrow (1.6–1.8), while the degree of substitution (DS) of the six S-TM8s was found to be between 1.14 and 1.74.[9] The radius of gyration $\langle s^2 \rangle_z^{1/2}$ of S-TM8s was larger than that of native TM8s with a similar M_w, indicating that the S-TM8 fractions had adopted a stiffer chain conformation in solution than that of TM8s.[6]

In this study, four kinds of viruses including three enveloped viruses (HSV-1, HSV-2, and RSV) and one myxovirus (Flu A) were used to determine the anti-viral activities of S-TM8s using the cytopathic effect (CPE) inhibition assay. Accordingly, inhibition of HSV-1 and HSV-2 induced CPE in vero cells, RSV induced CPE in HEp-2 cells, and Flu A induced CPE in MDCK cells by S-TM8s were evaluated and the results are shown in Table 1. The IC_{50} value indicates the effective concentration of the S-TM8 fractions required to inhibit 50% virus-induced CPE. While the S-TM8 fractions were inactive in inhibiting the virus replication for Flu A, they showed a different degree of inhibitory effect toward the three enveloped viruses, which was in agreement with those reported in the literature.[10] Among these three viruses, S-TM8s showed relatively higher anti-viral activities toward HSV-1 and HSV-2 since their IC_{50} values were 10-fold lower than those for RSV (Table 1). It has been reported that the IC_{50} values determined by the CPE method for heparin and carrageenan, the two sulfated polysaccharides already known for their effectiveness against HSV-1, were 10 and 19 μg/mL, respectively.[11,12] It seemed that the partially synthesized S-TM8s in this study were potential anti-HSV agents because they had inhibition of the HSV induced CPE that was comparable to the control, Acyclovir (Table 1).

Furthermore, the plaque reduction assay (PRA) method was used to study whether the mechanism of the anti-viral activity of S-TM8s was related to the phase of viral (HSV-1 and HSV-2) replication (Table 2). All S-TM8 fractions were more effective when added simultaneously with the HSV-1 and HSV-2 at the early stage of virus infection of the host cells (treatment A), while they were less effective when added 1 h after HSV-1 infection (treatment B) (Table 2). This was in agreement with the result of dextran sulfate and carrageenan,[4] which interfered with the early stage of the HSV replication cycle by blocking the virus adsorption to the host cells due to the presence of the sulfated polysaccharides.[13,14]

Table 1. Anti-viral activities of S-TM8 fractions with different M_w against HSV-1 and HSV-2 in vero cells, RSV in HEp-2 cells, and Flu A in MDCK cells by cytopathic effect inhibition (CPE) assay

Fraction	Anti-HSV-1 activity IC_{50}[a] (μg/mL)	Anti-HSV-2 activity IC_{50}[a] (μg/mL)	Anti-RSV activity IC_{50}[a] (μg/mL)	Flu A (200 μg/mL)[b]
S-TM8-1	1.9	0.38	10	Inactive
S-TM8-2	1.9	0.625	20	Inactive
S-TM8-3	2.5	0.625	20	Inactive
S-TM8-4	2.5	0.38	10	Inactive
S-TM8-5	2.5	1.25	20	Inactive
S-TM8-6	1.9	0.38	20	Inactive
Acyclovir[c]	0.125	0.125	ND[e]	ND[e]
Ribavirin[d]	ND[e]	ND[e]	3.0	ND[e]

[a] IC_{50} is the concentration of the sample required to inhibit 50% virus-induced CPE.
[b] The anti-Flu A activities of all samples were tested at a concentration of 200 μg/mL.
[c] Acyclovir, an approved drug for the specific treatment of HSV infections in humans.
[d] Ribavirin, an approved drug for the specific treatment of RSV infections in humans.
[e] Not determined.

Table 2. Anti-HSV-1 and anti-HSV-2 activities of the S-TM8 fractions using the plaque reduction assay (PRA)

Fraction	Anti-HSV-1 activity		Anti-HSV-2 treatment A[a] IC_{50} (μg/mL)[c]
	Treatment A[a] IC_{50} (g/mL)[c]	Treatment B[b] IC_{50} (μg/mL)[c]	
S-TM8-1	0.5	3.8	0.2
S-TM8-2	1.0	3.8	0.2
S-TM8-3	0.9	6.5	0.4
S-TM8-4	1.0	6.8	0.2
S-TM8-5	0.6	6.6	0.2
S-TM8-6	0.8	3.3	0.2

[a] Treatment A: All samples were present both during and after the virus adsorption period.
[b] Treatment B: All samples were present after virus adsorption only.
[c] IC_{50} is the concentration of the sample required to inhibit 50% virus-induced PDA.

The time of the addition of S-TM8s was very important because the highest inhibitory effect (lowest IC_{50}) toward HSV-1 and HSV-2 was observed when S-TM8s were added during the attachment/adsorption period, indicating that the S-TM8s were more effective when added at the very early stages of virus adsorption (treatment A) as shown in Table 2. Moreover, the S-TM8s had a lower IC_{50} value for HSV-2 than HSV-1, indicating S-TM8s had the highest anti-HSV-2 activity among the three enveloped viruses tested.

Negatively charged sulfated polysaccharides can interact with the positively charged glycoproteins of the viral envelope so that the adsorption of the positively charged viral particles to the negatively charged host cells is inhibited. Therefore, the introduction of the charged groups (sulfated groups) along the main chain and the conformation of S-TM8s must be very important for their anti-viral activity. Although it had been suggested that at least two sulfate groups per monosaccharide unit (DS = 2) were required for dextran sulfate to exert its anti-HSV activity,[15] our results showed that all S-TM8s had potent antiviral activity even though their DS was less than 2. However, a positive correlation between the anti-HSV activity of S-TM8s and the DS was not observed in this study (data not shown).

The persistent length (q) of the polymer chain in solution is a crucial parameter that determines the conformation of polymers in solution. As reported previously, the q for TM8 and S-TM8 was 3.1 and 8.5 nm, respectively.[6,9] Figure 1 shows the chain conformation of TM8 before sulfation and that after the introduction of the sulfated groups (S-TM8). An increase in q indicated that the chain conformation had become more extended when the sulfated groups were added to the polymer chains (Fig. 1). It had been postulated that some β-glucans having anti-tumor or anti-HIV and anti-HSV activities could interact with the mannose-rich polymer chain found in tumor cells or viruses.[16] It was assumed that relatively higher M_w and more extended chain conformation of a glucan molecule would enhance the formation of additional hydrogen bonds and van der Waals interactions between the glucans and the tumor cells or viral particles. The fact that S-TM8s exhibited a more extended chain conformation in solution, which resulted in a stronger binding to the viral particles when compared with the native TM8s,[9] might explain the differences in their anti-viral activity. In our future work, the S-TM8s with a wider range of M_w and DS will be prepared for the further investigation of their structure–activity relationship.

Sulfation of the TM8 fractions, which were water-insoluble β-(1→3)-D-glucans isolated from the sclerotia of *P. tuber-regium*, produced water-soluble sulfated polysaccharides (S-TM8s) that were noncytotoxic to normal cells up to a concentration of 200 μg/mL (data not shown) and had a strong inhibitory activity against HSV with an IC_{50} value comparable with those known anti-HSV drugs. We suggested that the presence of sulfate groups and the more extended chain conformation in the S-TM8s can facilitate the binding between the sulfated polysaccharides and the HSV viruses, which interferes in the attachment of viral particles to the host cells. Thus sulfated β-glucans derived from mushrooms have the potential to be developed as an anti-HSV agent.[17]

2. Materials and methods

2.1. Materials

Sclerotia of PTR were cultivated by the Sanming Mycological Institute in Fujian province, China. Six sulfated fungal β-glucans (S-TM8-1 to S-TM8-6) with M_w from 6.0×10^4 to 64.8×10^4 were obtained from native TM8-1 to TM8-6 with M_w from 5.8×10^4 to 77.4×10^4 by procedures mentioned in our previous work.[9] All culture media and chemicals were from Gibco-BRL (Gaithersburg, MD, USA) except RPMI-1640 culture medium, which was bought from Sigma (St. Louis, MO, USA).

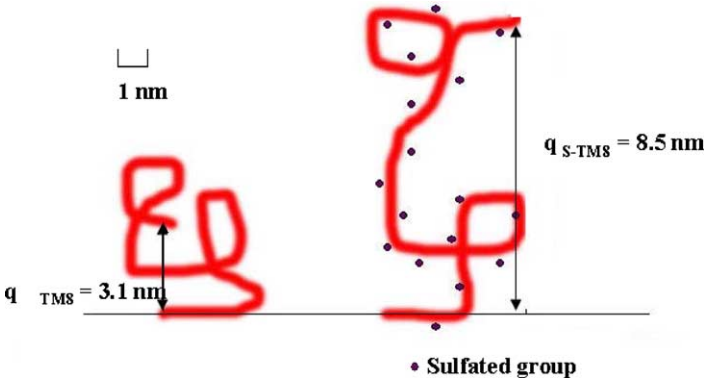

Figure 1. A diagrammatic representation of the conformational change in terms of persistence length (q) of the native TM8 and their sulfated derivatives S-TM8 in aqueous solution.

2.2. Mammalian cell and viral cultures

HEp-2 cells (human epidermoid carcinoma cells of the larynx), vero cells (Africa green monkey kidney cell line), MDCK cells (Malin Darby canine kidney cells), herpes simplex virus type 1 (HSV-1, strain 15577), herpes simplex virus type 2 (HSV-2, strain 15577), and respiratory syncytial virus (RSV, long strain) were obtained from American Type Culture Collection (ATCC). The clinical strain of influenza A virus (Flu A, H1N1) was isolated from routine clinical specimens at the Prince of Wales Hospital of The Chinese University of Hong Kong, Hong Kong, China. Vero cells (a normal mammalian cell line for comparison with other cancer cell lines) were used as the host cells for HSV-1 and HSV-2. HEp-2 cells and MDCK cells were used as the host cells for RSV and influenza A virus, respectively.

Vero cells were grown in sterile RPMI-1640 medium, containing 2 g/L sodium bicarbonate, 0.1% amphotericin B (250 μg/mL) and 1% penicillin (10,000 unit/mL)-streptomycin (10,000 μg/mL), and 10% heat-inactivated fetal bovine serum. MDCK and HEp-2 cells were grown as monolayers in EMEM supplemented with 10% inactivated calf serum and 50 μg/mL gentamycin. The above cell lines were cultured according to procedures described elsewhere.[18]

Virus titration was performed by the limiting dilution method, using a 96-well microtiter plate (Falcon Plastics Oxnard, CA) with 6-wells per dilution. The virus titer was estimated from the cytopathogenicity of cells induced by viral infection and expressed as 50% tissue culture infectious doses mL^{-1} ($TCID_{50}$/mL).[19]

2.3. Anti-viral assays

Procedures of the cytopathic (CPE) inhibition assay were mentioned elsewhere.[18] In brief, monolayers of HEp-2 cell cultures adhered at the bottom of the wells in a 96-well microtiter plate incubated for 24 h at 37 °C in a 5% CO_2 incubator. Acyclovir and Ribavirin (Sigma, USA), the current clinically used anti-HSV and anti-RSV drugs,[20] respectively, were used as the positive control in this assay. The plates were infected with RSV virus and incubated at 37 °C under 5% CO_2 for 4–5 days until the RSV virus in the control wells showed complete viral-induced CPE as observed under a light microscope. The concentrations of the S-TM8s required to inhibit 50% of the growth of RSV (IC_{50}) was estimated from the graphic plots of inhibition% of RSV growth against different concentration of S-TM8s (data not shown) in a similar manner as reported in the literature.[21] Similarly, CPE inhibition assay for HSV-1 and HSV-2 cultured in vero cells and Flu A cultured in MDCK cells were performed.

A plaque reduction assay (PRA) was performed according to the standard method described by Hill et al.[22] Briefly, monolayers of vero cells and HEp-2 cells grown on 12-well culture plates were infected with 60–70 plaque-forming unit (pfu) of HSV-1 and HSV-2, and with 30–40 pfu of RSV, respectively. After incubation for 1 h to allow viral adsorption, the inoculum was aspirated and the cultures were overlaid with a maintaining medium [EMEM with 2% fetal bovine serum (FBS) and 0.8% methylcellulose] containing dilutions of the S-TM8s. After an incubation time of 72 h for HSV-1, -2, and 120 h for RSV at 37 °C, the plates were fixed with formalin, stained with crystal violet, air-dried and the number of plaques in each plate was counted under a light microscope. Plates overlaid with methylcellulose medium without the sulfated β-glucans were used as controls. The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in control−mean number of plaques in sample)/(mean number of plaques in control)] × 100.

2.4. Cytotoxicity assay

Vero cells (10^6 cells/mL) without any viral infection were incubated separately with the S-TM8 fractions at concentrations of 50, 100, and 200 μg/mL and were allowed to grow under the same condition as mentioned above. The number of living vero cells at the end of a 72 h incubation period was determined by a colorimetric assay based on the tetrazolium salt MTT as described by Mosmann.[23] Treated samples were compared with the control in the absence of the S-TM8 fractions. All in vitro results were expressed as the ratio of inhibition of cell proliferation calculated as $[(A-B)/(A)] \times 100\%$ where A and B are the average numbers of viable cells of the control and samples, respectively. All experiments were carried out in triplicate.

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