Carbohydrate Polymers 85 (2011) 408–412

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Characterization of fructan from Chikuyo-Sekko-To, a Kampo prescription, and its antiherpetic activity *in vitro* and *in vivo*

Jung-Bum Lee, Tomoko Fukai, Kyoko Hayashi, Toshimitsu Hayashi*

Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, 2630 Sugitani, Toyama, Toyama 930-0194, Japan

ARTICLE INFO

Article history:
Received 6 January 2011
Received in revised form 16 February 2011
Accepted 28 February 2011
Available online 5 March 2011

Keywords:
Fructan
Chikuyo-Sekko-To
Herpes simplex virus type 2
Antiviral effect
Immunostimulation

ABSTRACT

A fructan (CSH1-1, Mw = 4.0×10^3) was isolated as an antiviral principle by combination of column chromatographies from Chikuyo-Sekko-To (CST), which is a traditional Japanese herbal (Kampo) medicine for infectious diseases. Chemical and spectroscopic analyses revealed that CSH1-1 was a highly branched fructan consisted of terminal (19.0%), 2,1- (61.9%), 2,6- (4.9%) and 1,2,6-linked β-D-Fru*f* residues (12.3%) with 1,6-linked β-D-Glc*p* residues (1.9%). CSH1-1 showed anti-herpes simplex virus type 2 (HSV-2) effects *in vitro* and *in vivo*. The polysaccharide had stimulating effects of nitric oxide production and induction of several cytokine mRNA expression including IL-1β, IL-6, IL-10 and TNF-α on RAW264.7 cells. From these results, a fructan from CST was suggested to be a candidate as an anti-HSV-2 agent.

1. Introduction

Recently, there are increasing risks of infectious disease worldwide. Herpes simplex virus type 2 (HSV-2) is an ubiquitous pathogen and major cause of genital herpes. Immunocompetent people with genital HSV infection can have frequent, painful, and recurrent genital lesions associated with much psychosocial distress. Over the past two decades, HSV-2 infection has also been linked to three times higher risk of sexually acquired human immunodeficiency virus (HIV) (Freeman et al., 2006). Therefore, treatment and prophylaxis of the infection of HSV-2 should reduce the likelihood of HIV infection. Acyclovir is the most commonly used chemotherapy as a very effective treatment for HSV infection, however, it is not always tolerated and drug resistant mutants are rapidly emerging, particularly in immunocompromised patients (Whitley & Roizman, 2001). Thus, it is demanded to develop new anti-HSV drugs with novel mode of action.

In Japan, many Kampo prescriptions based on traditional Chinese medicine have been regarded as useful for the treatment of various infectious diseases. Among them, Chikuyo-Sekko-To (CST), which consists of seven crude drugs including Lophatheri Folium (aerial parts of *Lophatherum gracile*), Ginseng Radix (roots of *Panax ginseng*), Glycyrrhizae Radix (roots of *Glycyrrhiza glabra* or *Glycyrrhiza uralensis*), Ophiopogonis Tuber (tuberous roots of *Ophiopogon japonicus*), Pinelliae Tuber (tuberous roots of *Pinellia ternata*), Oryzae Semen (seeds of *Oryza sativa*) and Gypsum Fibrosum (gypsum), has been used to treat the people who have weak energy with strong cough, breathing difficulty, thirst and perspiration. In other words, diseases which are susceptible to treatment of CST are flu, measles, pneumonia, bronchitis, asthma and tuberculosis. Therefore, CST has been suggested to contain active principles which might be useful for the treatment of various infectious diseases, and prompted us to isolate and characterize the antiviral principles in CST. In the present paper, we describe the characterization of a bioactive polysaccharide from CST and the evaluation of its biological activities *in vitro* and *in vivo*.

2. Materials and methods

2.1. Isolation of polysaccharide

Chikuyo-Sekko-To was purchased from Tochimoto Tenkaido Co. Ltd. (Osaka, Japan). It was decocted twice with H_2O for 30 min. The combined decoction was concentrated *in vacuo* and lyophilized to give brownish powder (CS, 114.2 g). CS was dissolved in H_2O and centrifuged to remove insoluble portion. The soluble part was dialyzed against H_2O with seamless cellulose tube (MWCO: 14,000, Wako Pure Chemical Industries Ltd., Osaka, Japan), and the dialyzate and nondialyzate were lyophilized to give CSL (32.9%) and CSH (55.7%), respectively. CSH was applied to a DEAE 650 M anion exchange chromatography (5 i.d. × 14 cm, Tosoh Corp., Tokyo, Japan), which was successively eluted with H_2O, 0.5 M NaCl, 1 M NaCl and 0.1 M NaOH to obtain CSH1, CSH2, CSH3 and CSH4, respectively. The yields of the eluates were CSH1 (76.6%), CSH2 (5.8%),

* Corresponding author. Tel.: +81 76 434 7560; fax: +81 75 434 5170.
E-mail address: hayashi9@pha.u-toyama.ac.jp (T. Hayashi).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.02.044

CSH3 (0.6%) and CSH4 (2.8%). CSH1 was subjected to a Sepharose 6B gel filtration chromatography (4.4 i.d. × 93 cm, GE Healthcare Japan, Japan) and eluted with 0.01 M citrate buffer (pH 7) containing 0.1 M NaCl. Fractions of 15 ml were collected and monitored by the phenol–H_2SO_4 method (Dubois, Gilles, Hamilton, Revers, & Smith, 1956) to obtain CSH1-1 (81%).

2.2. Estimation of molecular weight of CSH1-1

High performance gel filtration chromatography analysis was carried out as described elsewhere (Lee, Koizumi, Hayashi, & Hayashi, 2010).

2.3. Chemical analyses of CSH1-1

Sugar composition was determined as follows; CSH1-1 was hydrolyzed with 0.2 M TFA at 80 °C for 30 min and immediately cooled. After removal of TFA under N_2 gas, the hydrolyzates were converted to oxime derivatives by treatment with methoxyamine (20 mg in 1 ml of pyridine) at 70 °C for 1 h. The derivatives were trimethylsilylated with TMSI-H reagent (GL Sciences Inc., Tokyo, Japan), and they were analyzed by GC using a SPB-1 fused silica capillary column (30 m × 0.32 mm i.d.; Supelco Inc., MA, USA) on a GC-353 gas chromatograph equipped with FID. Oven temperature program was as follows: 170–210 °C (2 °C/min), then rose to 300 °C (30 °C/min). Methylation of CSH1-1 was performed by the Ciucanu's method (Ciucanu & Kerek, 1984). The methylated polysaccharide was hydrolyzed with 0.5 M TFA at 90 °C for 1 h, reduced with $NaBD_4$, and acetylated. The partially methylated alditol acetates were analyzed GC using a SP-2330 fused silica capillary column (Supelco, 30 m × 0.32 mm i.d.) and GC–MS (Shimadzu QP-5000, Kyoto, Japan) using a DB-5MS fused silica capillary column (30 m × 0.32 mm i.d.; Agilent Technologies Inc., CA, USA). Identification of partially methylated alditol acetates was carried out based on relative retention time to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and its mass fragmentation patterns (Carpita & Shea, 1988). Peak area was corrected using published molar response factors (Sweet, Shapiro, & Albersheim, 1975).

2.4. Spectroscopic analyses of CSH1-1

IR spectrum of CSH1-1 was recorded with a FT/IR-460plus spectrophotometer (Jasco Corp., Tokyo, Japan). NMR spectra were recorded at 303 K on a Varian Unity 500 *plus* spectrophotometer, and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as internal reference.

2.5. Cells and viruses

Vero and MDCK cells were grown in Eagle's MEM supplemented with 5% fetal bovine serum (FBS) and kanamycin (60 mg/l). RAW 264.7 cells were grown in DMEM supplemented with 10% FBS. HSV-2 (UW268 strain) and influenza A virus (IFV-A) (A/NWS/33 strain, H1N1) were propagated on Vero and MDCK cells, respectively. Those viruses were stored at −80 °C until use. An aliquot of the virus stock was titered by plaque assay.

2.6. Antiviral activity of CSH1-1

Cell growth inhibition studies and antiviral tests *in vitro* were performed as described previously (Lee et al., 2010a). *In vivo* antiherpetic effects was evaluated as follows. Female BALB/c mice (5–6 weeks old) were obtained from Japan SLC Inc., Shizuoka, Japan. All experiments were conducted in accordance with the animal experimentation guidelines of the University of Toyama under the permission of the Animal Care Committee at the University of Toyama. Mice were subcutaneously injected with medroxyprogesterone 17-acetate (3 mg) at 6 days and 1 day before virus inoculation, and then were infected vaginally with HSV-2 (1×10^4 PFU). Phosphate-buffered saline (PBS) (no drug control), CSH1-1 (0.2 or 1 mg/20 μl) or acyclovir (ACV, 0.2 mg/20 μl) were administrated intra-vaginally twice a day from 3 days before infection to 7 days after infection. In the control group, mice were treated with same volume of PBS. Viral shedding was determined by washing the vaginal cavity with PBS (100 μl) on 3 days post-infection. The virus yields were titered by plaque assay on Vero cell monolayers.

2.7. NO production

Accumulated nitrite, which is a stable oxidized products of NO, in the culture media of RAW 264.7 cells was measured using a colorimetric assay based on the Griess reaction (Green et al., 1982). Briefly, the cells (2×10^5) were seeded in a 96-well plate and incubated in the absence or presence of sample at 37 °C for 24 h. The culture supernatants were reacted with Griess reagent at room temperature for 10 min, and then nitrite concentration was determined by measuring the absorbance at 550 nm. The standard curve was obtained using the known concentrations of sodium nitrite.

2.8. Expression of cytokine mRNAs

RT-PCR of cytokine mRNAs were performed as described elsewhere (Lee, Ohta, Hayashi, & Hayashi, 2010).

2.9. Statistical analysis

The data are presented as the mean ± S.D. The differences between groups were analyzed by one-way analysis of variance (ANOVA), and correction for multiple comparisons was made using Dunnett's multiple-comparison test. A comparison between the two groups was made using Student's *t*-test. In animal experiments, Kaplan–Meier survival curves were assessed by log-rank test.

3. Results

3.1. Isolation of CSH1-1

The freeze-dried decoction of Chikuyo-Sekko-To (CS) was dialyzed against H_2O to fractionate to non-dialyzate (CSH) and dialyzate (CSL). Since preliminary examination showed antiviral effects of CSH was higher than those of CSL, the former fraction was subjected to further fractionation. Then, CSH was applied to a DEAE 650 M anion exchange chromatography and fractionated four fractions. Among the fractions, CSH1 eluted with H_2O, which was the most abundant fraction (yield, 76.6%), was separated by a Sepharose 6B gel filtration chromatography to give CSH1-1. Analytical GFC showed that the obtained polysaccharide was eluted as a single peak (Mw: 4.0×10^3) and its polydispersity (Mw/Mn) was 1.33.

3.2. Chemical characterization of CSH1-1

The FT-IR spectrum of CSH1-1 showed strong OH stretching bands (3400 cm^{-1}) and no carbonyl bands (data not shown). It was essentially identical to those of inulin reported elsewhere (López-Molina et al., 2005). Neutral sugar analysis confirmed that CSH1-1 consisted of fructose (95.8%) and glucose (4.2%). Therefore, CSH1-1 was suggested to be a fructan-type polysaccharide. Then, CSH1-1 was applied to a methylation analyses in order to elucidate the linkage of monosaccharides. Table 1 summarizes the all detected partially methyl alditol acetates. The major derivative of Fru*f* was (2 → 1)-linked residue, because peak 4

Table 1
Methylation analysis of CSH1-1.

Peak	Deduced linkage	Mol%
Fru*f*		
1, 2	Terminal	19.0
4, 5	2,1-Linked	61.9
3	2,6-Linked	4.9
7, 8	1,2,6-Linked	12.3
Glc*p*		
6	1,6-Linked	1.9

Table 2
^{1}H and ^{13}C NMR spectral data of CSH1-1 in D_2O at 300 K.

Residue	1		2	3	4	5	6	
(2→1)	62.9		105.9	79.6	76.9	83.5	64.6	
	3.75	3.67		4.22	4.09	3.86	3.84	3.74
Terminal	62.5		106.4	79.0	77.7	83.5	65.0	
	3.83	3.68		4.17	4.10	3.86	3.81	3.63
1,6-di	63.4		106.5	79.0	77.5	82.6	65.7	
	3.87	3.70		4.17	4.05	3.93	3.93	3.71

and 5 were identified to be 1,2,5-tri-*O*-acetyl-(2-deuterio)-3,4,6-tri-*O*-methyl-glucitol and -mannitol, respectively. In addition, significant amounts of 3,4-dimethylated (peaks 7 and 8) and 1,3,4,6-tetramethylated derivatives (peaks 1 and 2) were also found and assigned to be 1,6-di- and terminal Fru*f* residues, respectively. In addition, peak 3 was identified to be a 2,5,6-tri-*O*-acetyl-(2-deuterio)-1,3,4-tri-*O*-methylmannitol, and it revealed that the presence of (2→6)-linked Fru*f* residue. Reduction of methylated derivatives with sodium borodeuteride introduces asymmetry into (2→1)- and (2→6)-linked Fru*f* that otherwise would yield identical mass fragment ions (Mancilla-Margalli & López, 2006). In the peak 6, two glucitol derivatives were found to be present since characteristic two pairs of fragment ions such as (*m*/*z* 189 and 162) and (*m*/*z* 190 and 161) derived from 1,2,5-tri-*O*-acetyl-(2-deuterio)-3,4,6-tri-*O*-methylgluciton and 2,5,6-tri-*O*-acetyl-(2-deuterio)-1,3,4-tri-*O*-methylgluciton, respectively, were observed. Finally, peak 6 was identified as 1,5,6-tri-*O*-acetyl-(1-deuterio)-2,3,4-tri-*O*-methylglucitol and it revealed the presence of 1,6-linked Glc*p* residues in CSH1-1. On the other hand, there is a trace amount of peak that derived from terminal glucopyranosyl residues. Therefore, Glc*p* residues were suggested to be mainly present as internal residues. Quantification of the amount of each derivative was determined as the *m*/*z* 161 and 162 ratio (Carpita & Shea, 1988).

3.3. NMR analyses of CSH1-1

The ^{1}H and ^{13}C NMR spectra of CSH1-1 were shown in Fig. 1. ^{13}C NMR spectrum showed similarity to the spectra reported for fructans from *Agave tequilana* (Lopez, Mancilla-Margalli, & Mendoza-Diaz, 2003) and *Agave americana* (Ravenscroft et al., 2009). Therefore, assignments were made by comparison with literature data of inulins (Chen & Tian, 2003; Lopez et al., 2003; Mancilla-Margalli & López, 2006; Ravenscroft et al., 2009; Wu et al., 2006) and related fructans and supported by 2D experiments. In the ^{1}H NMR spectrum, only one broad anomeric proton resonances were observed at δ 5.39 ppm, and it was assigned to be a α-D-Glc*p* residues. Other proton resonances were appeared in a region between 3.5 and 4.3 ppm. On the other hand, at least three anomeric carbon resonances of β-D-Fru*f* residues were observed at δ 105.9, 106.4 and 106.5 ppm, and it revealed that there are three types of δ-D-Fru*f* residues. The broad resonance at δ 105.9 ppm were typical for C-2 of (2→1)-linked β-D-Fru*f* residue, those at δ 106.4 and 106.5 ppm were deduced to be terminal and 1,6-di-β-D-Fru*f* residues, respectively. Two resonances at δ 82.9 and 83.8 ppm were deduced to be C-5 of δ-D-Fru*f* residues carrying a substituent at *O*-6 or not, respectively (Sims, Cairns, & Furneaux, 2001). Together with methylation data, these signals strongly supported the occurrence of 1,6-di-β-D-Fru*f* residues in CSH1-1. Other chemical shifts were summarized in Table 2, and they were well agreed with reported values. Therefore, CSH1-1 was suggested to be a highly branched fructan closely resembled to *Agave* inulin (Lopez et al., 2003; Ravenscroft et al., 2009).

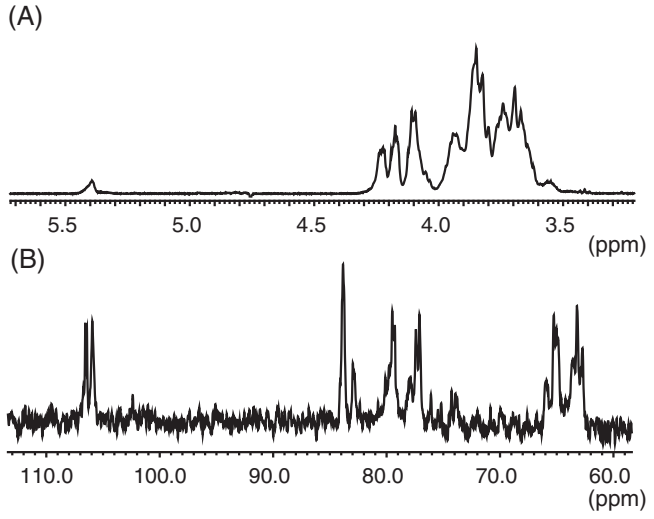

Fig. 1. ^{1}H NMR (A) and ^{13}C NMR (B) spectra of CSH1-1. Spectra were recorded in D_2O at 300 K at 500 MHz using 2,2-dimethyl-2-silapentane-5-sulfonate as internal reference.

3.4. Antiviral effect of CSH1-1

CSH1-1 was evaluated for its antiviral potencies against HSV-2 and IFV-A. In general, a sample was regarded as possessing antiviral activity when its selectivity index (SI, CC_{50}/IC_{50}) was higher than 10. As shown in Table 3, CSH1-1showed a low inhibitory effect against cell growth, with CC_{50} values being higher than >10,000 μg/ml. The IC_{50} values against HSV-2 replication were 550 and >600 μg/ml, respectively, when it was added to the medium during infection and throughout the incubation (A) or immediately after viral infection (B). Therefore, CSH1-1 showed antiviral activity against HSV-2. No marked inhibitory effect was observed against IFV-A replication. Since the SI value in experiments A was higher than that in experiment B, it was suggested that the main antiviral target of CSH1-1 might be early stages of viral replication including virus adsorption and/or penetration step(s) on host cell surface.

In order to investigate whether CSH1-1 could protect mice from HSV-2 infection, the animals pretreated with medroxyprogesterone were given the polysaccharide intravaginally from 3 days before vaginal challenge with the virus (Fig. 2). No toxicity was observed due to the administration of the compound (data not shown). The mean titers of virus shed on day 3 were reduced from 275.5×10^2 PFU/mouse in untreated control mice to 137.4×10^2, 102.2×10^2 and 1.2×10^2 PFU/mouse in mice that received CSH1-1 (0.2 mg), CSH1-1 (1 mg) and ACV (0.2 mg), respectively. Therefore, virus titers in vaginal region were decreased in a dose-dependent manner by the administration of CSH1-1. As predicted from viral shedding, CSH1-1 could elongate the survival period at the dose of 1 mg/day when compared with that of untreated control ($P = 0.0397$, log-rank test).

Finally, we examined whether CSH1-1 is an immunostimulating polysaccharide or not. The polysaccharide was administered to RAW 264.7 cells, which is a murine macrophage like cell, and evaluated its potency of NO production. As shown in Fig. 3, the

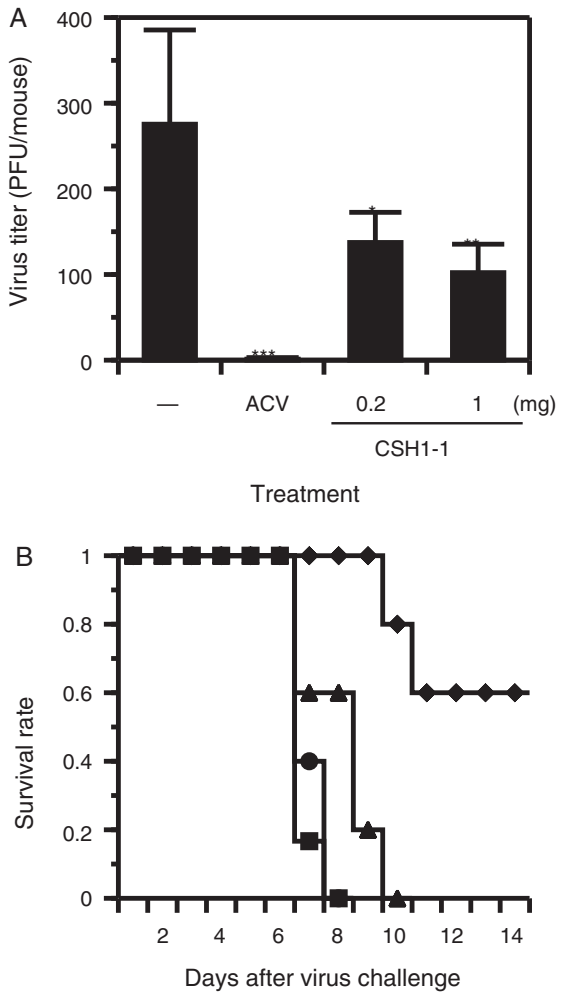

Fig. 2. Effect of CSH1-1 on experimental genital herpes infection. Mice ($n = 5$) were given PBS, CSH1-1 or ACV intravaginally twice a day from 3 days before infection to 7 days after infection. (A) Vaginal washings were obtained at 3 days of infection to be plaque-titrated. (B) The survived animal were recorded for 14 days after virus infection. Squares, PBS; diamonds, 0.2 mg of ACV per 20 μl; circles, 0.2 mg of CSH1-1 per 20 μl, and triangles, 1 mg of CSH1-1 per 20 μl. Asterisks indicate statistical difference compared with control (PBS) group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

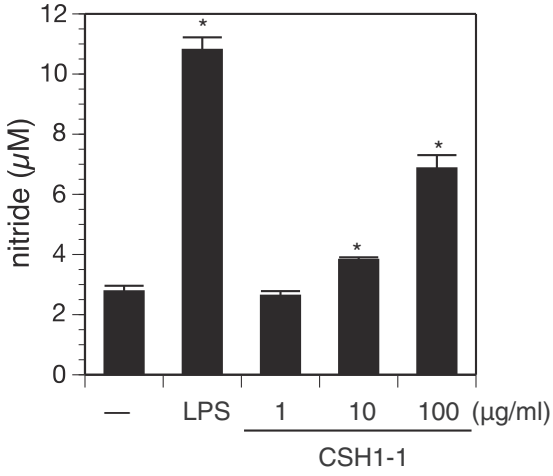

Fig. 3. Effect of CSH1-1 on NO production in RAW 264.7 cells. The cells were incubated with CSH1-1 for 24 h. The NO production was determined by measuring the accumulation of nitrite in the culture medium. No sample control and LPS (0.1 μg/ml) were also assayed. Any significant differences were determined using the Dunnett's test versus a control group. Data are expressed as mean ± S.D., $n = 3$, $^{*}p < 0.01$.

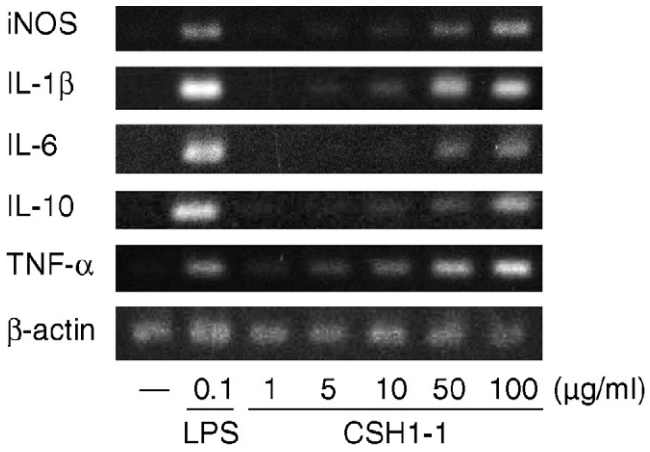

Fig. 4. Effect of CSH1-1 on iNOS and cytokine mRNA expression in RAW264.7 cells. The cells were cultured in the absence or presence of LPS (0.1 μg/ml) or CSH1-1 (1, 5, 10, 50 or 100 μg/ml) for 6 h. mRNA expression was detected by RT-PCR. β-Actin was used as a control.

polysaccharide could stimulate the production of NO in a dose-dependent manner. In addition, CSH1-1 also induced expression of mRNAs of iNOS, TNF-α, IL-1 β, IL-6 and IL-10 (Fig. 4). Therefore, CSH1-1 might enhance the production of both pro-inflammatory (IL-1, IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokines.

4. Discussion

We have shown that a fructan was isolated from Kampo formula, Chikuyo-Sekko-To (CST), and evaluated its anti-HSV-2 effects *in vitro* and *in vivo*. Fructan-producing plants are commonly present among the grasses, whereas 15% of flowering plants produce fructans in significant amounts. They are widely spread within the Liliaceae and Compositae (Franck & De Leenheer, 2004). In general, inulins are typically linked by β(2 → 1)-Fru*f* linkages with a Glc residue typically resident at the end of almost each Fru*f* chain, and contain a small branched-chain (Franck & De Leenheer, 2004; Watzl, Girrbach, & Roller, 2005). However, there are some reports that fructans from *Agave* and *Dasylirion* species consist of a complex mixture of fructooligosaccharides containing principally β(2 → 1)-Fru*f* linkages, but also β(2 → 6)- and branch Fru*f* residues (Lopez et al., 2003; Mancilla-Margalli & López, 2006). In the present study, our fructan consisted of large amounts of β(2 → 1)-

Table 3
Antiviral activities of CSH1-1 *in vitro*.

Virus	Host cells	Cytotoxicity (CC_{50}, μg/ml)	Antiviral activity (IC_{50}, μg/ml)		Selectivity index (CC_{50}/IC_{50})	
			*A**	*B*	*A*	*B*
HSV-2	Vero	>10,000	550	>600	>18	><17
IFV-A	MDCK	>10,000	>1000	>1000	>10	><10

A: Sample was added to the medium during infection and throughout the incubation thereafter.
B: Sample was added to the medium immediately after viral infection.

Fru*f* residues with terminal and 1,6-di-β-D-Fru*f* residues. However, the amount of β(2 → 6)-Fru*f* residues was significantly lower than that of reported values of *Agave* fructans. On the other hand, CST is a mixture of crude drugs, such as Lophatheri Folium, Ginseng Radix, Glycyrrhizae Radix, Ophiopogonis Tuber, Pinelliae Tuber, Oryzae Semen and Gypsum Fibrosum. Among these crude drugs, there are no reports about isolation of fructans from crude drugs in CST except for *Ophiopogon japonicus* (Tomoda, Nagumo, & Nakatsuka, 1973; Wu et al., 2006). Wu et al. (2006) reported that fructans from *O. japonicus* consisted of β(2 → 1)- and β(2 → 6)-Fru*f* residues as backbone with 1,6-di-β-D-Fru*f* residues. When compared the composition of sugar residues, contents of β(2 → 6)-Fru*f* residues were seemed to be higher than that of our fructan. Furthermore, they are reported that α-D-Glc*p* was present at terminal position, whereas α-D-Glc*p* was present as internal Glc*p* residues in CSH1-1. Although it is still uncertain whether CSH1-1 has been derived from *O. japonicus* or not, the fructan type polysaccharide, CSH1-1, is one of the constituent of CST.

On the other hand, fructans including inulins are well known to be storage polysaccharides and used as a functional food ingredients that offer unique biological effects such as immune modulation and reduction of disease risks (Roberfroid, 2007). However, there is only one report that fructan and its fragments from *Polygonatum cyrtonema* possess antiherpetic effects (Liu, Liu, Meng, Yang, & He, 2004). Since the structure of the fructan from *P. cyrtonema* has been reported to be a highly branched fructan like CSH1-1, the characteristic features might be important for antiherpetic activity. During the past decades, many polysaccharides have been reported to possess antiherpetic effects, and most of them are sulfated polysaccharides derived from various kinds of algae. In addition, some polysaccharides from higher plants have also often been reported to possess antiviral effects although their antiviral potencies were lower than those of the sulfated polysaccharides. In general, the mechanisms by which polysaccharides inhibit can be explained by the inhibition of virus binding to host cells and subsequent virus–cell fusion step (Ghosh et al., 2009). Antiviral targets of CSH1-1 were also seemed to be the interference with the same steps of viral replication. In the present study, *in vivo* anti-HSV-2 effect of CSH1-1 was evaluated using HSV-2 genital infection model, and therapeutic efficacy of CSH1-1 was confirmed in this murine model (Fig. 2). In addition, it is noteworthy that CSH1-1 also showed immunostimulating effects on macrophages (Figs. 3 and 4). In the mucosal region, innate immune system is the important mechanism to proper the first line of host defence, and antigen-presenting cells like macrophages and dendritic cells possess a pivotal role in responding to infectious pathogens. In particular, macrophages orchestrate a multitude of antiherpetic actions during the first hour of the attack (Ellermann-Eriksen, 2005). NO may inhibit an early stage in viral replication, and thus prevent viral spread, promoting viral clearance and recovery of the host. TNF-α, IL-6 and IL-1β were important for responding to infections, whereas IL-10 might act in a negative feedback mechanism to prevent potential detrimental effects from excessive macrophage activation during inflammation. Therefore, it was suggested that the immunostimulating effects of CSH1-1 might contribute at least in part to its anti-HSV-2 effects *in vivo*.

References

Carpita, N. C., & Shea, E. M. (1988). Linkage structure of carbohydrates by gas chromatography-mass spectrometry (GC–MS) of partially methylated alditol acetates. In C. J. Biermann, & G. D. McGinnis (Eds.), *Analysis of carbohydrate by GLC and MS* (pp. 157–216). Boca Raton, FL: CRC Press.

Chen, X. M., & Tian, G. Y. (2003). Structural elucidation and antitumor activity of a fructan from *Cyathula officinalis* Kuan. *Carbohydrate Research*, *338*, 1235–1241.

Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, *131*, 350–356.

Dubois, M., Gilles, K. A., Hamilton, J. K., Revers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Ellermann-Eriksen, S. (2005). Macrophages and cytokines in the early defence against herpes simplex virus. *Virology Journal*, *2*, 59.

Franck, A., & De Leenheer, L. (2004). Inulins. In S. De Baets, E. Vandamme, & A. Steinbüchel (Eds.), *Polysaccharides II. Polysaccharides from eukaryotes Biopolymers* (pp. 439–479). Weinheim, Germany: Wiley-VCH Verlag, GmbH & Co. KGaA.

Freeman, E. E., Weiss, H. A., Glynn, J. R., Cross, P. L., Whitworth, J. A., & Hayes, R. J. (2006). Herpes simplex virus 2 infection increases HIV acquisition in men and women: Systematic review and meta-analysis of longitudinal studies. *AIDS*, *20*, 73–83.

Ghosh, T., Chattopadhyay, K., Marschall, M., Karmakar, P., Mandal, P., & Ray, B. (2009). Focus on antivirally active sulfated polysaccharides: from structure–activity analysis to clinical evaluation. *Glycobiology*, *19*, 2–15.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry*, *126*, 131–138.

Lee, J.-B., Koizumi, S., Hayashi, K., & Hayashi, T. (2010). Structure of rhamnan sulfate from the green alga *Monostroma nitidum* and its anti-herpetic effect. *Carbohydrate Polymers*, *81*, 572–577.

Lee, J.-B., Ohta, Y., Hayashi, K., & Hayashi, T. (2010). Immunostimulating effects of a sulfated galactan from *Codium fragile*. *Carbohydrate Research*.

Liu, F., Liu, Y., Meng, Y., Yang, M., & He, K. (2004). Structure of polysaccharide from *Polygonatum cyrtonema* Hua and the antiherpetic activity of its hydrolyzed fragments. *Antiviral Research*, *63*, 183–189.

Lopez, M. G., Mancilla-Margalli, N. A., & Mendoza-Diaz, G. (2003). Molecular structures of fructans from *Agave tequilana* Weber var. azul. *Journal of Agricultural and Food Chemistry*, *51*, 7835–7840.

López-Molina, D., Navarro-Martínez, M. D., Rojas-Melgarejo, F., Hiner, A. N. P., Chazarra, S., & Rodríguez-López, J. N. (2005). Molcular properties and prebiotic effect of inulin obtained from artichoke (*Cynara scolymus* L.). *Phytochemistry*, *66*, 1476–1484.

Mancilla-Margalli, N. A., & López, M. G. (2006). Water-soluble carbohydrates and fructan structure patterns from *Agave* and *Dasylirion* species. *Journal of Agricultural Food Chemistry*, *54*, 7832–7839.

Ravenscroft, N., Cescutti, P., Hearshaw, M. A., Ramsout, R., Rizzo, R., & Timme, E. M. (2009). Structural analysis of fructans from *Agave americana* grown in South Africa for spirit production. *Journal of Agricultural Food Chemistry*, *57*, 3395–4003.

Roberfroid, M. B. (2007). Inulin-type fructans: Functional food ingredients. *Journal of Nutrition*, *137*, 2493S–2502S.

Sims, I. M., Cairns, A. J., & Furneaux, R. H. (2001). Structure of fructans from excised leaves of New Zealand flax. *Phytochemistry*, *57*, 661–668.

Sweet, D. P., Shapiro, R. H., & Albersheim, P. (1975). Quantitative analysis by various g.l.c. response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydrate Research*, *40*, 217–225.

Tomoda, M., Nagumo, S., & Nakatsuka, S. (1973). Water-soluble carbohydrates of Ophiopogonis Tuber. III. Isolation and characterization of a new inulin-type fructan. *Chemical and Pharmaceutical Bulletin*, *21*, 659–662.

Watzl, B., Girrbach, S., & Roller, M. (2005). Inulin, oligofructose and immunomodulation. *British Journal of Nutrition*, *93*, S49–S55.

Whitley, R. J, & Roizman, B. (2001). Herpes simplex virus infections. *Lancet*, *357*, 1513–1518.

Wu, X. M., Dai, H., Huang, L., Gao, X. M., Tsim, K. W., & Tu, P. F. (2006). A fructan, from *Radix ophinopogonis*, stimulates the proliferation of cultured lymphocytes: Structural and functional analyses. *Journal of Natural Products*, *69*, 1257–1260.