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Anti-influenza A virus effects of fructan from Welsh onion (*Allium fistulosum* L.)

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ABSTRACT

A fructan that acts as an anti-influenza A virus substance was isolated from hot water extract of the green leafy part of a Welsh onion (*Allium fistulosum* L.). The structure of the fructan was characterised and elucidated by chemical and spectroscopic analyses. The fructan was composed of terminal (21.0%) and 2,1-linked β-D-Fru*f* residues (65.3%) with 1,6-linked β-D-Glc*p* residues (13.7%). The molecular weight of the polysaccharide and polydispersity was estimated to be 1.5×10^3 and 1.18, respectively. Although the fructan did not show anti-influenza A virus activity *in vitro*, it demonstrated an inhibitory effect on virus replication *in vivo* when it was orally administered to mice. In addition, the polysaccharide enhanced the production of neutralising antibodies against influenza A virus. Therefore, the antiviral mechanism of the polysaccharide seemed to be dependent on the host immune system, i.e., enhancement of the host immune function was achieved by the administration of the polysaccharide. From our observations, the fructan from Welsh onions is suggested to be one of the active principles which exert an anti-influenza virus effect.

1. Introduction

Many organisms cause respiratory tract infections. The common cold is a self-limiting infectious disease caused by more than 100 different viruses (Roxas & Jurenka, 2007). Of these, rhinoviruses and coronaviruses are responsible for approximately 50–70% of all colds. On the other hand, flu is an acute respiratory illness caused by influenza viruses (serotypes A and B). In particular, influenza A viruses (IFV-As) cause recurrent epidemics with substantial human morbidity and mortality, and are associated with pandemics. In fact, a novel swine-origin IFV-A (H1N1) emerged as the first influenza pandemic of the 21st century (Dawood et al., 2009). In addition, H5N1 viruses ('avian flu'), which are also currently circulating, are extremely virulent in humans but have not yet acquired the ability for efficient human-to-human transmission. In order to overcome these respiratory infectious diseases, development of novel methodologies for preventing or curing such 'slight' condition is very important.

Allium vegetables are important plants which are cultivated worldwide. Together with their nutritional benefits, they have received attention for their potencies as medicinal and functional foods. Indeed, numerous studies have been conducted to evaluate their biological activities, including their antioxidant, antifungal and antimicrobial effects (Aoyama & Yamamoto, 2007; Iciek, Kwiecien, & Wlodek, 2009; Kyung, 2011; Sang, Lao, Wang, Chin, Rosen, & Ho, 2002). Among them, the Welsh onion (*Allium fistulosum* L.) is a very popular vegetable in East Asian countries, and it has been recorded as a crude drug in oriental medical dictionaries for abdominal pain and phlegmon. In addition, Welsh onion has been used as a folk remedy for the common cold in Japan. These traditional usages of *A. fistulosum* suggest that it might contain active substances that contribute to the prevention and/or cure of respiratory infectious diseases, including flu.

The central goal of our study is to test the medicinal effects of such edible plants and obtain evidence of such effects at the molecular level. With this in mind, we evaluated the antiviral potency of a hot water extract of the green leaf part from *A. fistulosum*, because it showed antiviral effects through its oral administration in mice. This result prompted us to isolate the active property in the hot water extract from Welsh onion, and thus the obtained results are reported in the present paper.

2. Materials and methods

2.1. Materials

Welsh onion (*A. fistulosum* L.) was purchased from Totsuka Seed Garden (Kusatsu, Shiga, Japan). DEAE 650 M, Toyopearl HW-55 and HW-40 were obtained from Tosoh Corp. (Tokyo, Japan).

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Oseltamivir phosphate (Tamiflu®) was purchased from F. Hoffman-La Roche Ltd. (Basel, Switzerland) and other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Isolation of fructans from Welsh onion

Welsh onions (the green leafy parts) were cut into pieces and then extracted with four volumes of EtOH overnight at room temperature. After filtration, the residue was extracted with H_2O (Four volumes) for 1 h under reflux. The extract was concentrated *in vacuo* and lyophilised to give a hot water extract (W, yield, 0.5%). W was dissolved in H_2O and then dialysed against H_2O (MWCO, 14,000). The non-dialyzable and dialyzable portions were concentrated and lyophilised to give high (WH, 18.3%) and low molecular weight fractions (WL, 77.5%), respectively. WH was applied to a DEAE 650 M anion exchange column chromatography (5 × 15 cm), and the pass-through fraction was collected by eluting with H_2O (WH-1, 14.6%), then eluted with 0.5 M NaCl to give WH-2 (82.3%). WH-1 was subjected to a Toyopearl HW-55 gel filtration column chromatography (4.4 × 100 cm) and eluted with H_2O. Fractions of 20 ml were collected and monitored by phenol–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). WH-1a (10.9%) and -1b (42.4%) were obtained on the basis of elution profile. WH-1b was purified by Toyopearl HW-40 gel filtration (2.2 × 95 cm) to give a purified a polysaccharide (40.6%).

2.3. Characterisation of fructan

The molecular weight of the isolated polysaccharides was estimated by HPLC analysis. The sample was applied on TSK $GMPW_{XL}$ gel filtration columns (7.6 mm × 300 mm × 2; Tosoh, Tokyo, Japan) and eluted with 0.1 M $NaNO_3$ at 0.6 ml/min. Commercially available pullulans (Shodex P-52; Showa Denko K.K., Tokyo, Japan) were used as standard molecular markers. Mw and polydispersity were calculated using the GPC software supplied by Shimadzu Corp. (Kyoto, Japan).

Sugar composition was analysed as follows: the polysaccharide was hydrolysed with 0.5 M trifluoroacetic acid (TFA) at 60 °C for 1 h. After drying under N_2 stream, methoxyamine hydrochloric acid in pyridine (20 mg/ml) was added and heated at 70 °C for 1 h. Then, oximes were trimethylsilylated with TMSI-H reagent (GL Science Inc., Tokyo, Japan), and the obtained derivative was analysed by GC using an SPB-1 fused silica capillary column (30 m × 0.32 mm i.d., Supelco, MA, USA) at an oven temperature of 170–210 (2 °C/min).

Methylation of the polysaccharide was performed according to Ciucanu's method (Ciucanu & Caprita, 2007). The methylated polysaccharide was hydrolysed with 0.5 M TFA at 90 °C for 1 h. After reduction with $NaBD_4$ and acetylation, alditol acetates were analysed by GC using an SP-2330 fused silica capillary column and GC–MS using a DB-5MS fused silica capillary column. Identification of partially methylated alditol acetates was carried out on the basis of relative retention time to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and its mass fragmentation patterns. The peak area was corrected using published molar response factors (Sweet, Shapiro, & Albersheim, 1975).

NMR spectra were recorded at 300 K on a Varian Unity 500 plus spectrophotometer.

2.4. Cells and viruses

MDCK cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% foetal bovine serum (FBS) and kanamycin (60 mg/l). RAW 264.7 cells were grown in Dulbecco's modified MEM (DMEM) supplemented with 10% FBS. Influenza A virus (IFV-A, A/NWS/33 strain, H1N1 subtype) was propagated on MDCK cells, and viruses were stored at −80 °C until use. An aliquot of the virus stock was titered by plaque assay.

2.5. Biological activities of fructan

Cell growth inhibition studies and antiviral tests *in vitro* were performed as described previously (Lee, Fukai, Hayashi, & Hayashi, 2011). *In vivo* anti-influenza A virus effects were evaluated as follows: Female BALB/c mice (5 weeks old) were obtained from Japan SLC Inc., Shizuoka, Japan, and an adaptation period of 1 week was set before starting the experiments. 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 (n = 10 per group) were inoculated intranasally with 50 μl of viral suspension (2×10^4 PFU/mouse). Fructan (0.5 or 1.5 mg/day/mouse) was orally administered twice a day (every 12 h) from 3 days before virus inoculation to 7 days post-inoculation (p.i.). Control mice were treated orally with 0.2 ml of vehicle (H_2O) alone. Body weight change and mortality were monitored for 14 days after virus inoculation. Blood, bronchoalveolar lavage fluid (BALF) and the lung were individually collected after scarification of the animals at 3 (n = 5) and 14 days (n = 5) p.i. Blood samples were centrifuged at 7000*g* for 10 min, and sera were stored at −80 °C. Lung samples were sonicated for 10 s after the addition of 1 μl of PBS/mg of lung tissue and centrifuged at 13,000*g* for 30 min to separate the supernatants, which were then stored at −80 °C. BALFs were prepared by four washes with 0.8 ml of ice–cold PBS *via* a tracheal cannula and centrifuged at 1500 rpm for 10 min to obtain the supernatants, which were then stored at −80 °C. Virus titres at 3 days p.i. and neutralising antibody titres at 14 days p.i. were determined by plaque assay as described elsewhere (Ohta, Lee, Hayashi, Fujita, Park, & Hayashi, 2007).

Determination of stimulatory effects of NO production on RAW 264.7 cells was performed as described elsewhere (Lee et al., 2011).

2.6. Statistical analyses

The data are presented as the mean ± S.D. The differences between groups were analysed by one-way analysis of variance (ANOVA), and correction for multiple comparisons was made using Tukey or Dunnett's multiple-comparison tests.

3. Results

3.1. Isolation and characterisation of fructan from Welsh onion

Defatted Welsh onion was extracted with hot water and the obtained soluble extract (W) was dialysed against H_2O to fractionate to non-dialyzate (WH) and dialyzate (WL). Since WH showed antiviral effects in the preliminary evaluation (data not shown), the former fraction was subjected to further fractionation. WH was applied to a DEAE 650 M anion exchange chromatography, and it gave non-adsorbed (WH-1) and adsorbed (WH-2) fractions. This time, WH-1 was undertaken to obtain an antiviral property, and it was subsequently purified by gel filtration on a Toyopearl HW-55 and HW-40 to give abundant purified polysaccharide. Analytical GFC showed that the weight-averaged molecular weight (Mw) of the obtained polysaccharide was 1.5×10^3 and its polydispersity (Mw/Mn) was 1.18.

The GC analysis of the hydrolysed products of the polysaccharide revealed that the polysaccharide consisted of fructose and glucose with an approximate ratio of 8:1. In addition, methylation analysis indicated that the polysaccharide consisted of 2,1-disubstituted Fru*f* (65.3%), terminal Fru*f* (21.0%), and 1,6-disubstituted

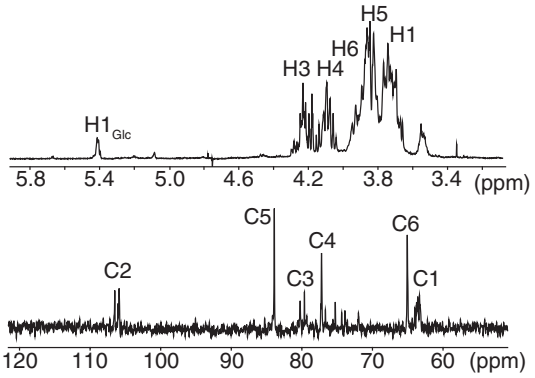

Fig. 1. ^{1}H- and ^{13}C-NMR spectra of the fructan from *A. fistulosum*.

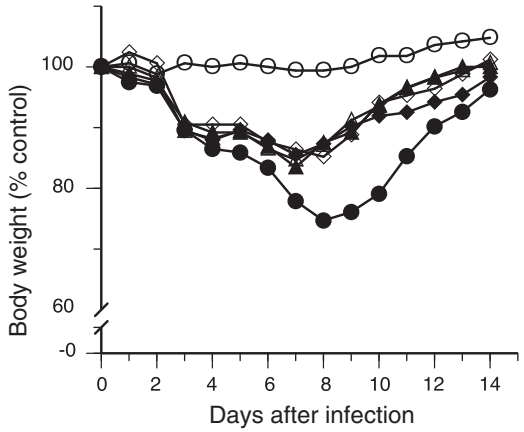

Fig. 2. Effects of oral administration of WH and fructan on body weight loss in mice infected with influenza A virus. BALB/c mice were treated with vehicle (closed circle), oseltamivir (open circle), WH (closed triangle; 0.5 mg/day, open triangle; 1.5 mg/day) or fructan (closed diamond; 0.5 mg/day, open diamond; 1.5 mg/day) from 3 days prior to viral infection to 7 days post-infection.

Glc*p* (13.7%) residues. These data revealed that the polysaccharide might be an oligofructan that consists mainly of eight fructosyl and one glucosyl residues. ^{1}H- and ^{13}C-NMR spectra of the polysaccharide also revealed that it was a fructan-type polysaccharide (Fig. 1). The chemical shifts of ^{1}H and ^{13}C we observed were compatible with those of reported values by others (Cérantola, Kervarec, Pichon, Magné, Bessieres, & Deslandes, 2004; Chandrashekar, Prashanth, & Venkatesh, 2011; Chen et al., 2009; Fujishima et al., 2009). From these results, the isolated polysaccharide was found to be an inulin-type fructan.

3.2. Biological activities of fructan

When we evaluated the anti-IFV-A effect of the fructan *in vitro*, the polysaccharide did not show any marked inhibitory effects on virus replication (data not shown). However, we have frequently seen that some types of polysaccharides possess antiviral effects *in vivo* and yet have no antiviral effects *in vitro*. In addition, the hot water extract from Welsh onion showed inhibitory effects on virus replication in an animal model in our preliminary experiment. Therefore, we attempted to evaluate the anti-IFV-A effects of the fructan in animal experiments.

The efficacy of oral administration of the fructan and WH against influenza A virus infection was evaluated on the basis of body weight loss (Fig. 2). Fructan and WH (0.5 or 1.5 mg/day) were given orally twice per day from 3 days before inoculation to 7 days after inoculation. Mice (n = 5 per group) were infected with IFV-A (2×10^4 PFU) *via* the intranasal route, and no mice died in any group throughout the experiments under these conditions. As shown in Fig. 2, control mice treated with vehicle showed marked reduction in body weights (74.7% at 8 days p.i.), whereas those who underwent 0.2 mg of oseltamivir (Tamiflu®) administration showed no body weight loss during the experimental period of 14 days. WH or fructan-treated mice showed continuous loss of body weight from day 3 to day 7 post infection and then recovered after day 9 p.i.. However, these animals showed moderate protection from body weight loss resulting from IFV-A infection when compared with those of no-drug control groups. Of the WH and fructan-administered mice, there were no differences between the low (0.5 mg/day) and high dose groups (1.5 mg/day) of WH and fructan.

In order to examine whether or not fructan could suppress the virus loads, virus titres in the bronchoalveolar fluid (BALF) and lung were determined at 3 days p.i.. As shown in Fig. 3, WH and fructan significantly decreased virus titres of both lung and BALF samples when compared with those of the no-drug control group ($p < 0.001$). Both samples showed inhibitory effects in a dose-dependent manner. Oseltamivir markedly suppressed virus production in both the BALF and lung samples of infected mice.

Production of neutralising antibodies is one important host defence mechanism to prevent the reinfection of infectious diseases in the form of the primarily infected strain or closely related strains. Exposure of viruses induces the production of neutralising antibodies; however, it has been reported surprisingly that reinfec-

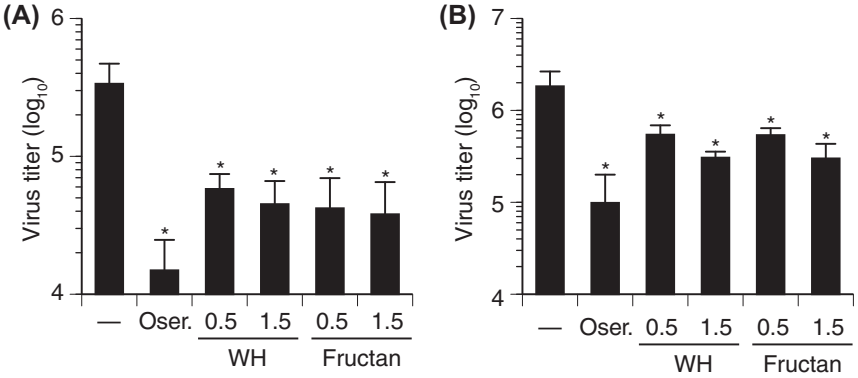

Fig. 3. Effects of WH and fructan on the virus replication in the mice infected with influenza A virus. Mice were treated as described in Fig. 2. Virus titres in the bronchoalveolar lavage fluid (BALF) (A) and lung (B) were determined at 3 days after virus infection. Data are shown as mean ± SD. Asterisks indicates statistically significant differences as compared with vehicle controls: $^{*}p < 0.001$.

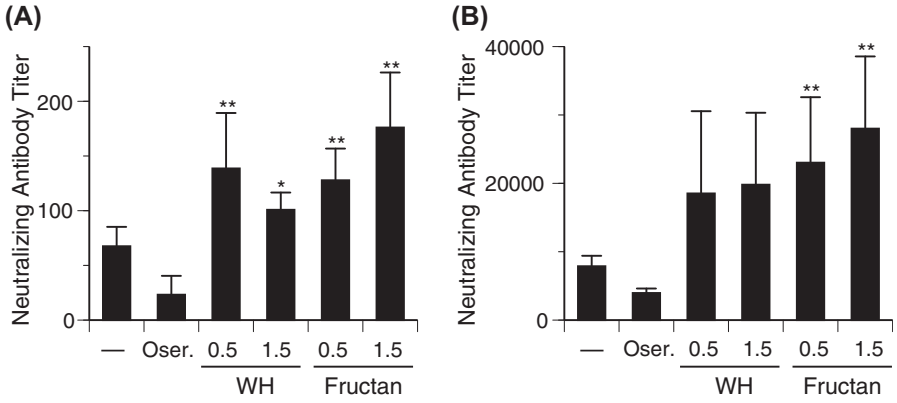

Fig. 4. Effects of WH and fructan on the production of neutralising antibodies in BALFs and sera. Mice were treated as described in Fig. 1. Neutralising antibody titres in the BALF (A) and sera (B) were determined at 14 days after virus infection. Data are shown as mean ± SD. Asterisks indicates statistically significant differences as compared with oseltamivir-treated group: $^{*}p < 0.05$, $^{**}p < 0.001$.

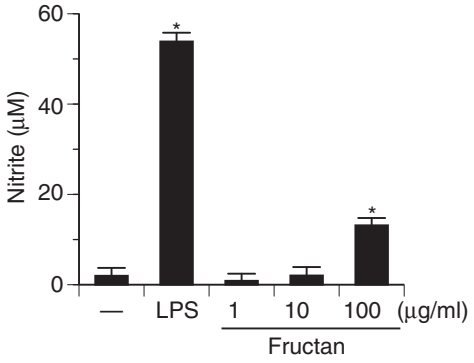

Fig. 5. Effect of fructan on NO production in RAW 264.7 cells. No sample control (–) and lipopolysaccharide (LPS, 0.1 μg/ml) were also assayed. Data are shown as mean ± SD of triplicate cultures. Asterisks indicates statistically significant differences as compared with vehicle control: $^{*}p < 0.05$, $^{**}p < 0.001$.

tion of pandemic H1N1 virus was occurred after successful treatment with oseltamivir (Perez, Ferres, & Labarca, 2010). In order to prevent reinfection, it is important to enhance the production of neutralising antibodies after first exposure to viruses. Fig. 4 shows the effects of tested samples on the systemic antibody response to IFV-A. At 14 days p.i., neutralising antibody titres of oseltamivir-treated mice were lower than those of control mice. On the other hand, the titres in the BALFs were markedly increased in WH- and fructan-treated mice when compared with those of oseltamivir-treated animals. Similarly, fructan administration significantly enhanced the production of neutralising antibodies in sera.

As described above, fructan was regarded to be one of key antiviral properties of the Welsh onion. Its antiviral mechanism was considered to be a host-mediated one because the polysaccharide showed no direct inhibitory effect on IFV-A replication *in vitro*. Although host defence mechanisms are complex, it is known that macrophages are key participants in the function of the innate immune system for responding to the invasion of pathogenic organisms. Thus, we investigated the effects of fructan on the activation of macrophages by measuring NO production which is an antiviral mediator (Akaike & Maeda, 2000). RAW 264.7 murine macrophage cells were incubated with the fructan for 24 h, and NO concentrations in the culture supernatants were measured using the Griess reaction. As shown in Fig. 5, fructan showed a stimulatory effect on NO production in a dose-dependent manner.

4. Discussion

In the present study, a 2,1-linked linear fructan was isolated from the green leafy part of *A. fistulosum*, and it was regarded as of the inulin type. It was obtained from a non-charged fraction (WH-1). Other polysaccharides are anticipated to be present in WH-2. Currently, we are progressing the isolation of these other polysaccharides from WH-2, and we will publish their chemical characteristics and biological activities of them in the near future.

There are numerous papers reporting on the linear fructans from *Allium* sp. (Baumgartner, Dax, Praznik, & Falk, 2000; Chandrashekar et al., 2011; Goodridge, Wolf, & Underhill, 2009; Jaime, Martín-Cabrejas, Mollá, López-Andréu, & Esteban, 2001; Leach & Sobolik, 2010; O'Donoghue et al., 2004; Stahl, Linos, Karas, Hillenkamp, & Steup, 1997). *Allium cepa* (onion) and *Allium sativum* (garlic) are important vegetables that form bulbs, whereas *A. fistulosum* does not. Fructans accumulate in bulbs in the case of onions and garlic as storage polysaccharides. On the other hand, *A. fistulosum* stores the polysaccharide in the thickened sheath of its bladeless leaves (Yaguchi et al., 2008). Our result is consistent with the data previously reported.

In general, the antimicrobial activity of *Allium* species has long been recognised with allicin and other thiosulfinates (Kyung, 2011). However, there are no reports that water soluble fractions and substances from *Allium* species possess antiviral effects, including anti-IFV-A properties. Therefore, our present report is the first to point out the usefulness of fructan from *Allium* sp. for infectious viral diseases.

Inulins are used as functional food ingredients that offer unique biological effects such as immune modulation and the reduction of disease risks (Roberfroid, 2007). In addition, fructan has been thought to be one of the important ingredients of ayurvedic herbs (Thakur et al., 2012). Although there are many studies on the biological effects of inulins, only two reports have shown their antiviral effects against herpes viruses (Lee et al., 2011; Liu, Liu, Meng, Yang, & He, 2004). A common characteristic of these antiherpetic fructans is that they have a branched structure, whereas the fructan from *A. fistulosum* in the present study is a linear polysaccharide. The antiherpetic activities of the former were thought to be dependent on their branched structure, and the later actually showed no antiherpetic effect *in vitro* (data not shown). From these observations, the antiviral effect of the fructan from *A. fistulosum* in an animal model is suggested to be mediated by host immune functions.

In order to provide supporting evidence for the hypothesis, we tested whether the fructan stimulated macrophages (Fig. 5). In the

mucosal region, the innate immune system is the most important mechanism in 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 the early stages of viral replication, and thus prevent viral spread, promoting viral clearance and recovery of the host. Interestingly, the administration of the fructan induced NO production in RAW 264.7 murine macrophage cells. Similarly, a fructan from aged garlic has also been shown to possess stimulatory effects on NO production from macrophages (Chandrashekar et al., 2011). Our results on NO release from macrophages are similar to those seen with other immunomodulating polysaccharides (Schepetkin & Quinn, 2006).

In conclusion, the immunostimulating potency of the fructan from *A. fistulosum* might contribute at least in part to anti-IFV-A effects *in vivo*.

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