Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Anti-influenza A virus effects of fructan from Welsh onion (Allium fistulosum L.)

Jung-Bum Lee,*, Sachi Miyake, Ryo Umetsu, Kyoko Hayashi, Takeshi Chijimatsu, Toshimitsu Hayashi

*Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, 2630 Sugitani, Toyama, Toyama 930-0194, Japan
bSasaki Food Company Limited, 276 Sakai, Bungotakada, Oita 879-0615, Japan
cShizenshokken Company Limited, Oita 879-0615, Japan

A R T I C L E   I N F O

Article history:
Received 10 January 2012
Received in revised form 12 March 2012
Accepted 4 April 2012
Available online 13 April 2012

A B S T R A C T

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 residues (65.3%) with 1,6-linked β-D-Glc 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.

© 2012 Elsevier Ltd. All rights reserved.

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 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).
Oseltamivir phosphate (Tamiflu) was purchased from F. Hoffmann-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$_2$O (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$_2$O and then dialysed against H$_2$O (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$_2$O (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$_2$O. Fractions of 20 ml were collected and monitored by phenol-H$_2$SO$_4$ 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 GMPWXL 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, methoxymine 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. The methylated polysaccharide was hydrolysed with 0.5 M TFA at 90°C for 1 h. After drying under N$_2$ stream, methoxyamine hydrochloric acid revealed that the polysaccharide consisted of fructose and glucose (Mw/Mn) was 1.18.

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 a standard 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$^6$ 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$_2$O) 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 7000g 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 13,000g 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$_2$O to fractionate to non-dialyze (WH) and dialyze (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$^4$ 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 Fruf (65.3%), terminal Fruf (21.0%), and 1,6-disubstituted...
Glc (13.7%) residues. These data revealed that the polysaccharide might be an oligofructan that consists mainly of eight fructosyl and one glucosyl residues. 1H- and 13C-NMR spectra of the polysaccharide also revealed that it was a fructan-type polysaccharide (Fig. 1). The chemical shifts of 1H and 13C 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 × 10^3 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 reinfe-
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 mice. Similarly, fructan administration significantly enhanced the production of neutralising antibodies in sera. Thus, we investigated the effects of fructan on the immune system for responding to the invasion of pathogenic organisms. 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; Chandrashhekar et al., 2011; Goodridge, Wolf, & Underhill, 2009; Jaime, Martin-Cabrejas, Moll, 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 antiviral fructans is that they have a branched structure, whereas the fructan from A. fistulosum in the present study is a linear polysaccharide. The antiviral activities of the former were thought to be dependent on their branched structure, and the later actually showed no antiviral 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 (Sche petitkin & 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.

Acknowledgement

This study was supported in part by Grants-in-Aid for Scientific Research (C) (#232617006) supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Akaike, T., & Maeda, H. (2000). Nitric oxide and virus infection. Immunology, 101, 305–308.

Aoyama, S., & Yamamoto, Y. (2007). Antioxidant activity and flavonoid content of Welsh onion (Allium fistulosum) and the effect of thermal treatment. Food Science and Technology Research, 13, 67–72.

Baumgartner, S., Dax, T. C., Praznik, W., & Falk, H. (2000). Characterisation of the high-molecular weight fructan isolated from garlic (Allium sativum L.). Carbohydrate Research, 328, 177–183.

Cérantola, S., Kervarec, N., Pichon, R., Magné, C., Bessieres, M.-A., & Deslandes, E. (2004). NMR characterisation of inulin-type fructooligosaccharides as the major water-soluble carbohydrates from Matriaria maritima (L.). Carbohydrate Research, 339, 2445–2449.

Chandrashekar, P. M., Prashanth, K. V. H., & Venkatesh, Y. P. (2011). Isolation, structural elucidation and immunomodulatory activity of fructans from aged garlic extract. Phytochemistry, 72, 255–264.

Chen, X., Liu, Y., Bai, X., Wen, L., Fang, J., Ye, M., & Chen, J. (2009). Hypoglycemic polysaccharides from the tuberous root of Liriope spicata. Journal of Natural Products, 72, 1988–1992.

Ciucanu, I., & Caprita, R. (2007). Per-O-methylation of neutral carbohydrates directly from aqueous samples for gas chromatography and mass spectrometry analysis. Analytica Chimica Acta, 585, 81–85.

Dawood, F. S., Jain, S., Finelli, L., Shaw, M. W., Lindstrom, S., Garten, R. J., Guabareva, L. V., et al. (2009). Emergence of a novel swine-origin influenza A (H1N1) virus in humans. The New England Journal of Medicine, 360, 2605–2615.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, 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.

Fujishima, M., Furuyama, K., Ishihiro, Y., Onodera, S., Fukushima, E., Benkeblia, N., & Shimoi, N. (2009). Isolation and structural analysis in vivo of newly synthesized fructooligosaccharides in onion bulbs tissues (Allium cepa L.) during storage. International Journal of Carbohydrate Chemistry, 2009, 1–10.

Goodridge, H. S., Wolf, A. J., & Underhill, D. M. (2009). Beta-glucan recognition by the innate immune system. Immunological Reviews, 230, 38–50.

Iciek, M., Kwiecien, I., & Wlodek, L. (2009). Biological properties of garlic and garlic-derived organosulfur compounds. Environmental and Molecular Mutagenesis, 50, 247–265.

Jaline, L., Martín-Cabejeras, M. A., Mollá, E., López-Andréu, F. J., & Esteban, R. M. (2001). Effect of storage on fructan and fructooligosaccharide of onion (Allium cepa L.). Journal of Agricultural and Food Chemistry, 49, 982–988.

Kyung, K. H. (2011). Antimicrobial properties of allium species. Current Opinion in Biotechnology, 23, 1–6.

Leach, D. J., & Sobolik, K. D. (2010). High dietary intake of prebiotic inulin-type fructans in the prehistoric Chihuahuan Desert. The British Journal of Nutrition, 103(11), 1558–1561.

Lee, J.-B., Fukai, T., Hayashi, K., & Hayashi, T. (2011). Characterization of fructan from Chikuyo-Sekko-To, a Kampo prescription, and its antitherpetic activity in vitro and in vivo. Carbohydrate Polymers, 85, 408–412.

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

O’Donoghue, E. M., Somerfield, S. D., Shaw, M., Bendall, M., Hedderly, D., Eason, J., & Sims, I. M. (2004). Evaluation of carbohydrates in Pukekohe Longkeeper and Grano cultivars of Allium cepa. Journal of Agricultural and Food Chemistry, 52, 5383–5390.

Ohta, Y., Lee, J.-B., Hayashi, K., Fujita, A., Park, D. K., & Hayashi, T. (2007). In vivo anti-influenza virus activity of an immunomodulatory acidic polysaccharide isolated from Cordyceps militaris grown on germinated soybeans. Journal of Agricultural and Food Chemistry, 55, 10194–10199.

Perez, C. M., Ferres, M., & Labara, J. A. (2010). Pandemic (H1N1)2009 reinfection, Chile. Emerging Infectious Diseases, 16, 156–157.

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

Roxas, M., & Jurenka, J. (2007). Cold and influenza: A review of diagnosis and conventional, botanical, and nutritional considerations. Alternative Medicine Review, 12, 25–46.

Sang, S., Lao, A., Wang, Y., Chin, C.-K., Rosen, R. T., & Ho, C.-T. (2002). Antifungal constituents from the seeds of Allium fistulosum L. Journal of Agricultural and Food Chemistry, 50, 6318–6321.

Sche petitkin, I. A., & Quinn, M. T. (2006). Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. International Immunopharmacology, 6, 317–333.

Stahl, B., Linos, A., Karas, M., Hillenkamp, F., & Steup, M. (1997). Analysis of fructans from higher plants by matrix-assisted laser desorption/ionization mass spectrometry. Analytical Biochemistry, 246, 195–204.

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, 46, 217–225.

Thakur, M., Weng, A., Fuchs, H., Sharma, V., Bhargava, C. S., Chauhan, N. S., Dixit, V. K., et al. (2012). Rasyana properties of Ayurvedic herbs: Are polysaccharides a major contributor. Carbohydrate Polymers, 87, 3–15.

Yaguchi, S., McCallum, J., Shaw, M., Pither-Joyce, M., Onodera, S., Shimoi, N., Yamauuchi, N., et al. (2008). Biochemical and genetic analysis of carbohydrate accumulation in Allium cepa L. Plant and Cell Physiology, 49, 730–739.