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Fructooligosaccharides as Bulb-forming Stimulants in Onion

Meng Wang,[1] Ayana Nara,[2] Itsuki Endo,[2] Noboru Takada[1,2,*]

[1] The United Graduate School of Agricultural Science, Iwate University, Morioka, Iwate, 020-8550, Japan
[2] Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Aomori, 036-8561, Japan
* Corresponding author's e-mail address: takada@hirosaki-u.ac.jp

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Abstract: Under long days, onions form spherical bulbs and accumulate nutrients such as fructose-based oligosaccharides (FOS) in their bulbs. 1-Kestose and nystose, inulin-type FOS, have been identified as endogenous stimulants of bulb formation. According to the structure-activity-relationship study, the inulobiose moiety in 1-kestose is required for the bulb formation. Inulin-type FOS is well known as a prebiotic, which is beneficial to people's health, and does not show toxicity. FOS has not been linked to any negative crop growth effects. Thus, it could be a potential eco-friendly growth promoter.

Keywords: fructooligosaccharides, 1-kestose, nystose, *Allium cepa* L., bulb-forming stimulant.

INTRODUCTION

UNDER long day conditions (LDC), onion plants (*Allium cepa* L., Amaryllidaceae) swell the leaf sheath cells drastically and form spherical bulbs. During bulbing, onion plants store fructose-based oligosaccharides (FOS) for growth in the following spring. Inner signals that are stimulated or suppressed by the ecological environment control the bulbing.[1] A "bulbing hormone" was proposed to originate from leaves under LDC, one of the most important candidates of which is the FLOWERING LOCUS T (FT) protein.[2] The FT protein is thought to be important in flowering. The FT protein synthesized in the leaves is the primary transposable flowering indicator, florigen. Furthermore, the FT genes were found to play a role in the bulbing of *Allium* crops.[3] Three FT-like genes in onions are important in directing bulb formation (AcFT1 and AcFT4) and flowering (AcFT2).[4] Although vernalization permits flowering and is associated with AcFT2 up-regulation, an LD photoperiod is associated with AcFT4 down-regulation and AcFT1 up-regulation. Unlike *Arabidopsis* FT, these are not longdistance indicators. Although AcFTs play novel roles in bulb development in *Allium* plants, other mobile transporters with FT-like properties may also be involved. The most important endogenous compounds for modulating physiological and molecular responses are phytohormones. Phytohormones function either where they are synthesized or elsewhere in plants after they have been transported. Gibberellins (GAs) are well-organized inhibitors of bulb development,[5] and long photoperiods increase the levels of endogenous Gas.[6] Exogenous GA was applied to onions and inhibited bulbing. GA did not directly inhibit bulbing; however, it attracted several bulbing assimilates.[7] The level of zeatin riboside (ZR) increased with increasing photoperiod. On one hand, ZR is the primary endogenous determinant of shoot and root developments. Exogenous ZR, on the other hand, had no discernible effect on bulb development.[8] Jasmonic acid (JA) and related compounds are found in many higher plants and play important roles in plant growth. JAs are also effective inducers of the proteinase inhibitors of resistant proteins.[9] Because bulbing occurs in the presence of disordered microtubules, JAs are considered candidates for bulbing hormones due to their microtubule-disrupting activities and extensive transporting properties in higher plants.[10] However, exogenous JAs inhibited onion bulbing. The hormone has yet to be identified in a satisfactory way. Endogenous bulbforming stimulant identification is required to better understand the

bulb-forming mechanism and develop agrochemicals for increased harvest. Thus, this study isolated and identified endogenous bulb-forming stimulants under the supervision of an *in vitro* bioassay for bulbforming activity.

EXPERIMENTAL

Spectral Analysis

A JNM-ECA 500 spectrometer (Jeol Ltd., Tokyo, Japan) was used to record the ^{1}H (500 MHz) and ^{13}C (125 MHz) nuclear magnetic resonance (NMR) spectra in D_2O at 30 °C. In the ^{1}H NMR spectra, the remaining proton signal for HDO (δ_H 4.63 ppm) was used as an internal standard. In the ^{13}C NMR spectra, the default offset was employed and was not corrected. A NanoFrontier LD spectrometer (Hitachi High-Tech Science Corp., Tokyo, Japan) was used to collect liquid chromatography-electrospray ionization time-of-flight mass spectrometry (LC-ESI/TOFMS) spectra. A 670-IR spectrometer (Agilent Technology Japan, Ltd., Tokyo, Japan) was used to measure the fourier transform infrared (FTIR) spectra. The optically rotations were measured using a P-1030 polarimeter (Jasco Corp., Tokyo, Japan).

Chemical Reagents

Fujifilm Wako Pure Chemical Corporation (Osaka, Japan) supplied 1-kestose (code 112-00433), nystose (code 143-05983), erlose (code 635-04093), lactosucrose (code 123-03703), raffinose (code 078860), and fructooligosaccharides (code 062-02381). Neokestose[11] was purified from onion extract. The procedure and spectral data were shown in Supplementary Material. Other chemicals were obtained from commercial sources and used in their original form. A WL220 water purifier (Yamato Scientific Co. Ltd., Tokyo, Japan) was used to deionize the water used in the experiments.

In vitro Bulb-Forming Assay Protocol

Onion seeds (*Allium cepa* L. cv. Lucky) were purchased from a local seed store in Hirosaki, Aomori, Japan. In an MLR-352 growth chamber (Panasonic Healthcare Co. Ltd., Gumma, Japan), onion seedlings were incubated. In an MCV-91 Cleanbench (Sanyo Electric Co. Ltd., Osaka, Japan), onion seedlings were transplanted.

The surfaces of the onion seeds were sterilized for 4 hours with a 10 % aqueous NaClO solution (available Cl, 0.5%) and thoroughly rinsed with sterile water. Forty seeds were sown in sterile vermiculite and germinated under LDC (daytime: 16 h, 10 µmol m^{-2}·sec^{-1}, 25 °C; night: 8 h, 0 µmol m^{-2}·sec^{-1}, 15 °C) for one week. The illumination was provided by white fluorescent lamps (approximately 15,000 lux). Ten germinated seedlings were selected and grown on Murashige–Skoog (MS) plate medium with inorganic salts at half-strength (1/2 MS medium, pH 5.8).[12] Agar (0.6 %) was used to solidify the medium. The seedlings were kept in LDC for two weeks. For the assay, 3 cm segments of seedlings were excised, with the leaf tips and root removed. A 20 µL aqueous additive solution was placed in a 100 mL conical flask. After that, 20 mL of a 1/2MS medium was poured into the flask to make the plate medium. The plate medium for the bulb-forming assay contained 9 % sucrose, 3×10^{-5} M paclobutrazol (PB), and gellan gum (0.8 %). Each of the five seedling segments was transplanted into the medium and grown for four weeks under LDC. Per test sample, two flasks with five segments each were prepared. Following incubation, the segments were sampled and the bulb diameters were measured.

Isolations of 1-Kestose, Nystose, and Neokestose

In Hirosaki, Aomori, Japan, onion bulbs (9.4 kg fresh weight, FW) were purchased from a local food store. Aqueous 80 % EtOH extract of onion bulbs was washed three times with hexane, EtOAc, and ^{n}BuOH. To obtain unabsorbed H_2O fraction (760 g), the aqueous extract was chromatographed with charcoal (165 g, 50 mmID × 250 mm) using H_2O/MeOH (1 : 0 → 1 : 1 → 0 : 1; 2.6 L each). The H_2O fraction (16.2 g, 200 gFW) was separated using silica gel column chromatography (350 g, 50 mmID × 500 mm) employing $CHCl_3$/MeOH/H_2O (15 : 10 : 2) to yield fraction A (2.28 g; elution volume 1.4–1.8 L) and B (3.36 g; elution volume 1.9–4.0 L). Fraction A contained neokestose and fraction B contained 1-kestose and nystose.

The fraction A (31 mg) was further purified by HPLC (column: Wakosil 5SIL, 20 mm ID × 250 mm, solvent: MeCN/H_2O = 5/2; flow rate: 15.0 mL min^{-1}; retention time: 24–25 min) to afford neokestose (2.6 mg). The fraction B (31.0 mg, 1.8 gFW) was further purified by HPLC (High Performance Liquid Chromatography; column: Wakosil® 5SIL, 20 mmID × 250 mm, solvent: MeCN/H_2O = 5/2; flow rate: 15 mL min^{-1}; retention times: 25–26 min for 1-kestose and 27–30 min for nystose) to afford 1-kestose (3.2 mg) and nystose (0.9 mg), respectively.

The spectra of 1-kestose and nystose were identical with commercially available compounds.

Neokestose: colorless oil; R_f = 0.49 (5/5/1 $CHCl_3$/MeOH/H_2O, SiO_2); $[\alpha]_D^{21}$ = +16.8 (c = 0.060, H_2O); IR ν_{max} (KBr): = 3442, 1628, 1385, 1088 cm^{-1}. ^{1}H NMR (500 MHz, D_2O, 30 °C): δ (ppm) = 3.43 (1H, t, J = 9.7 Hz, H-4_G), 3.47 (1H, dd, J = 3.9, 10.0 Hz, H-2_G), 3.58 (2H, m, H-1'), 3.59 (1H, m, H-6_Ga), 3.62 (1H, dd, J = 6.6, 12.4 Hz, H-6a), 3.67 (1H, m, H6_Gb), 3.67 (1H, m, H-3_G), 3.69 (1H, m, H-6'a), 3.71 (1H, m, H-1a), 3.73 (1H, m, H-6'b), 3.77 (1H, m, H-6), 3.78 (1H, m, H-5), 3.80 (1H, m, H-5'), 3.85 (1H, m, H-1b), 3.86 (1H, m, H-5_G), 3.98 (1H, t, J = 8.5 Hz, H-4'), 4.05 (1H, dd, J = 7.8, 8.1 Hz, H-4), 4.10 (1H, d, J = 8.1 Hz, H3), 4.13 (1H, d, J =

8.8 Hz, H-3'), 5.32 (1H, d, J = 3.9 Hz, H-1_G); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 60.3 (t, C-6_G), 60.4 (t, C-1), 61.5 (t, C-1'), 62.36 (t, C-6), 62.41 (t, C-6'), 69.2 (d, $C4_G$), 71.0 (d, C-2_G), 71.6 (d, C-5_G), 72.5 (d, C-3_G), 74.0 (d, C-4'), 74.4 (d, C-4), 76.3 (d, C-3'), 76.9 (d, C-3), 81.2 (d, C-5), 81.4 (d, C-5'), 92.0 (d, C-1_G), 103.70 (s, C-2), 103.72 (s, C-2'); HRESIMS: *m/z* 527.1576 (calcd. 527.1588 for $C_{18}H_{32}NaO_{16}$). The NMR spectra are shown in Figures S12-S16.

Statistical Analysis

R software was used for statistical analyses (ver 3.6.1, The R Foundation, Vienna, Austria). After confirming normality using the F test, data comparisons were performed using the Tukey-Kramer test.[13]

RESULTS AND DISCUSSION

Isolation and Identification of Bulb-Forming Stimulants

An attempt was made to isolate bulb-forming stimulant (BFS) from onion bulbs under the supervision of an *in vitro* bioassay for bulb-forming activity using seedlings of *Allium cepa* L. The aqueous 80 % EtOH extract of onion bulbs was partitioned to yield H_2O, *n*-BuOH, EtOAc, and hexane fractions. After the H_2O fraction containing the activity at 10 mg mL^{-1} was subjected to a charcoal column chromatography, the activity was found in the unabsorbed H_2O fraction at 10 mg mL^{-1}. The fraction's ^{1}H NMR spectral analysis revealed that main components were glucose (30 %), fructose (15 %), and sucrose (18 %) which did not show bulb-forming activity at 10 mg mL^{-1}, respectively. The active fraction was then chromatographed using SiO_2 followed by HPLC yielding isolations of BFS A (3.2 mg) and B (0.9 mg) from 1.8 gFW of onion bulbs, respectively.

High resolution electrospray ionization mass spectrometry (HRESIMS) determined the molecular formula of BFS A to be $C_{18}H_{32}O_{16}$ at 527.1561 $[M+Na]^+$. Based on the ^{1}H-, ^{13}C-, and 2D NMR spectral data, BFS A was identified to be 1-kestose (Figure 1).[14] Similarly, the HRESIMS and NMR spectra of BFS B revealed that it was nystose.[15] At 1 mg mL^{-1}, both compounds demonstrated bulb-forming activity (Figure 2). A 1 mg mL^{-1} dose of 1-kestose, in particular, demonstrated significant activity. Commercially available 1-kestose and nystose were also used to confirm the activities. The activity recovery from the onion extract was 28 % in this purification process.

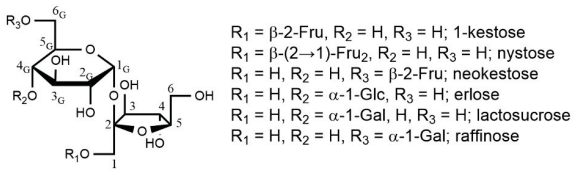

Figure 1. Structures of 1-kestose and related saccharides.

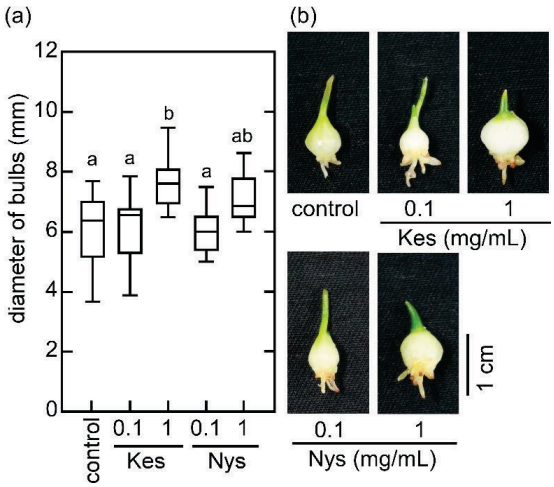

Figure 2. Bulb-forming activities of 1-kestose and nystose. (a) 1-Kestose (Kes) and nystose (Nys) were applied at concentrations of 0.1 and 1 mg mL^{-1}, respectively. The results are shown in box-plots (N = 10); horizontal lines represent medians, boxes represent the interquartile ranges, and error bars show the entire data range. Significant differences were indicated by different letters (Turkey-Kramer test, P < 0.05). (b) The representative images of onion seedlings treated with test compounds.

Structure-Activity-Relationship Study of 1-Kestose

1-Kestose is a trisaccharide which contains a sucrose core connecting additional fructose at sucrose's C-1 position, whereas nystose is a tetrasaccharide with an inulobiose side chain at the same position. As previously stated, sucrose did not exhibit activity at 10 mg mL^{-1}, while 1kestose and nystose exhibited activity at 1 mg mL^{-1}. The inulobiose moiety in 1-kestose was expected to be required for bulb formation. The bulb-forming activities of other trisaccharides with sucrose cores, such as neokestose and erlose, were compared with that of 1-kestose (Figure 3). Their structures were listed in Figure 1. At 1 mg mL^{-1}, none of the related trisaccharides showed bulb-forming activity, as expected. Surprisingly, neokestose did not exhibit the bulbforming activity. Both 1-kestose and neokestose are FOS, and both are important assimilates in onion bulbs.[16] FOS comes in a variety of chemical structures with varying degrees of polymerization and chemical-bonding states. FOS in onions are classified as inulin or inulin neo-type. 1-Kestose belongs to the inulin group, while neokestose belongs to the inulin neo group. It has been proposed that onion plants recognize only inulin-type FOS such as 1-kestose and nystose as bulb-forming stimulants. To

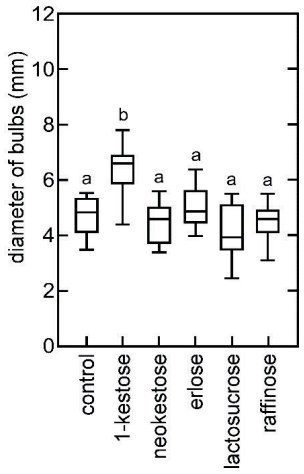

Figure 3. Comparisons of bulb-forming activities between 1-kestose and related trisaccharides. 1-Kestose, neokestose, erlose, lactosucrose, and raffinose were all added at 1 mg mL^{-1}, respectively. The results are shown in box-plots (N = 10); horizontal lines represent medians, boxes show the interquartile ranges, and error bars show the entire data range. Significant differences are indicated by different letters (Turkey-Kramer test, $P < 0.05$).

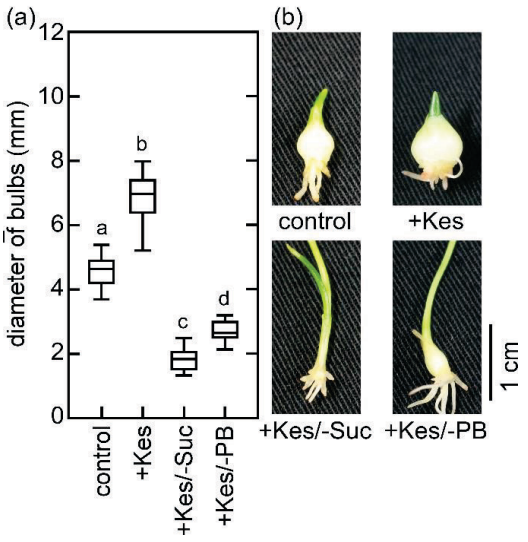

Figure 4. Comparisons of bulb-forming activities in the presence/absence of sucrose/paclobutrazol. (a) 1-Kestose was used at a concentration of 1 mg mL^{-1}. The results are shown in box-plots (N = 10); horizontal lines represent medians, boxes show the interquartile ranges, and error bars show the entire data range. Significant differences are indicated by different letters (TurkeyKramer test, $P < 0.05$). (b) The representative images of onion seedlings treated with test compounds.

further clarify the structure-activity-relationship of 1-kestose, it will be necessary to examine the activities of related saccharides, such as 6-kestose and 1,1,1-kestopentaose, which were not examined in this study due to their unavailability.

1-Kestose is a Bulb-Forming Stimulant, not Involved in GA Action

A sucrose-enriched medium is required for the bulbing of onion seedlings *in vitro*, as sucrose is an essential nutrient for bulbing. In this study, the culture medium contained 9 % sucrose as a nutrient; however, 1-kestose should not be considered as a nutrient because the amount of 1-kestose (1 mg mL^{-1}, 0.1 % in medium) was negligible as a nutrient when compared with sucrose, and the same amount of additional sucrose did not cause noticeable swelling. Furthermore, in the absence of sucrose, 1-kestose could not stimulate bulbing (Figure 4). It should be considered that 1-kestose acts as a stimulant in bulbing, rather than as a nutrient.

The cortical microtubule orientation in leaf sheath cells is required for bulb development. Indeed, microtubules oriented transversely to the cell axis under short days vanish during bulb development under LDC.[17] GAs have been shown to stabilize microtubules oriented transversely in onion leaf sheath cells.[5] Paclobutrazol (PB), a GA biosynthesis inhibitor, was used in this study to scramble the microtubule orientation. Even when 1-kestose was added to the culture medium, onion seedlings could not develop bulbs *in vitro* in the absence of PB (Figure 4). 1-Kestose was unable to replace PB, implying that 1-kestose has no effect on GA biosynthesis or microtubule orientation. Bulb development also requires sugar accumulation and FOS metabolism. 1-Kestose may aid in sugar accumulation and/or FOS metabolism. Given that inulin-type FOS, such as 1-kestose, are bulbing stimulants, onion plants would have a positive feedback mechanism via inulin-type FOS recognition. A detailed FOS biosynthetic pathway, which includes the key metabolic enzymes as well as the chemical composition and structure of each type of FOS, has also been reported.[18] To demonstrate the positive feedback mechanism in onion, the FOS profile in onion must be examined during 1-kestose treatment. The remaining issue is still present.

Onion plants transport the sucrose photosynthesized in the leaves to leaf sheath cells, where FOS is biosynthesized in the vacuole. In this study, FOS in the culture media was administered exogenously to onion seedlings via the vessel. The identification of location and the process of inulin-type FOS recognition will present new challenges for understanding the bulb development mechanism.

Potential of Inulin-Type FOS as an Eco-Friendly Growth Enhancer

Commercially available inulin-type FOS are from various reagent vendors, with 1-kestose and nystose as major

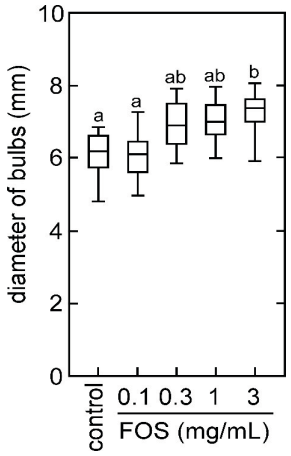

Figure 5. Bulb-forming activities of commercially available inulin-type FOS. The inulin-type FOS was treated at concentrations of 0.1, 0.3, 1, and 3 mg mL^{-1}, respectively. The results are shown in box-plots (N = 10); horizontal lines represent medians, boxes show the interquartile ranges, and error bars show the entire data range. Significant differences are indicated by different letters (Turkey-Kramer test, $P < 0.05$).

components. The bulb-forming activity of commercially available inulin-type FOS was investigated (Figure 5). As a result, at concentrations greater than 0.3 mg mL^{-1}, inulin-type FOS demonstrated bulb-forming activity. The inulin-type FOS are soluble, nondigestible dietary fibers that are modified by the gut microbiota at the end of ileum and improve the digestive and immune systems.[19] Therefore, they act as prebiotics, which are beneficial to people's health.[20] FOS has not been linked to any negative crop growth effects. Thus, inulin-type FOS are potentially eco-friendly growth promoters. More research into the use of FOS in onion plants is currently being conducted.

CONCLUSION

Under long days, onions form spherical bulbs that accumulate FOS. This study was carried out to identify endogenous bulb-forming stimulants to provide 1-kestose and nystose, inulin-type FOS. Prebiotics, such as inulin-type FOS, are well known for their health benefits. As a result, FOS was demonstrated to be potentially eco-friendly growth promoter. Our next goal is to clarify the positive feedback mechanism of FOS in onion plants, which leads to bulb formation.

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Supplementary Information. Supporting information to the paper is attached to the electronic version of the article at: https://doi.org/10.5562/cca4087.

PDF files with attached documents are best viewed with Adobe Acrobat Reader which is free and can be downloaded from Adobe's web site.

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