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Research Article

Isolation and Structural Analysis In Vivo of Newly Synthesized Fructooligosaccharides in Onion Bulbs Tissues (*Allium cepa* L.) during Storage

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Fructooligosaccharides are involved in physiological activities and quality attributes of onion bulbs. This work describes structures of newly synthesized oligosaccharides formed by fructose moieties in onion bulb tissues during storage. Onion bulbs were stored for four weeks at 10°C. HPAEC-PAD analysis showed that saccharide 1 was eluted after 1-kestose while saccharide 2 was eluted after nystose (**4a**). Saccharides 1 and 2 have R-sucrose values of 1.55 and 2.15 by HPAEC, a reducing terminal, a reducing sugar-to-fructose ratio of 0.5 and 0.3, and a degree of polymerization of 2 and 3 by TOF-MS, respectively. GLC analysis of the methyl derivatives and NMR measurement of the saccharides confirmed the presence of two different structures: the structure of saccharide 1 is composed by two fructose moieties and linked by $\beta(2 \rightarrow 1)$ linkage and was identified as inulobiose [β-D-fructofuranosyl-$(2 \rightarrow 1)$-β-D-fructopyranose]. The structure of saccharide 2 consists of three units of fructose linked by $\beta(2 \rightarrow 1)$ linkage and was identified as inulotriose [β-D-fructofuranosyl-$(2 \rightarrow 1)$-β-D-fructofuranosyl-$(2 \rightarrow 1)$-β-D-fructopyranose]. The spectra also showed that 70 to 80% of the terminal fructose residue of the two saccharides is of pyranosyl form, while 20 to 30% is of furanosyl form. This finding demonstrated that these newly produced saccharides, catalyzed by onion-purified 6G-FFT, were synthesized by the action of 1-FFT fructosyltransfer from 1-kestose to free fructopyranose yielding inulobiose and sucrose, while elongation of fructofuranosyl units occurs at this transferred fructofuranosyl residue to produce inulooligosaccharide having an additional unit of fructofuranose.

1. Introduction

About 80% of onion bulb (*Allium cepa*) dry matter consists of nonstructural carbohydrates [1]. The predominant of these nonstructural carbohydrates consist of glucose, fructose, sucrose, and low-molecular-weight fructooligosaccharides [1, 2]. Fructans, fructooligosaccharides (FOSs) and polyfructosylsucroses of varying molecular size, are the main carbohydrate reserve in onion, as well as in other vegetative organs and plants including alliaceous organs (bulbs). Fructans accumulate during bulbing, and are then catabolized during the regrowth and the sprout development of the bulbs [3].

1-Kestose [**3a**, 1^F-β-D-fructofuranosylsucrose, 1-kestotriose], Neokestose [**3b**, 6^G-β-D-fructofuranosylsucrose, 6G-kestotriose], nystose [**4a**, 1^F $(1$-β-D-fructofuranosyl$)_2$ sucrose, 1,1-kestotetraose], **4b** [6^G $(1$-β-D-fructofuranosyl$)_2$ sucrose, 1, 6G-kestotetraose], and **4c** [1^F, 6^G-di-β-fructofuranosyl sucrose, 1 and 6G-kestotetraose] constitute part of the different FOS found in onion bulb tissues and their contents vary between 4 and 5%, 1.5 and 2.5% , 1.5 and 2%, 4 and 4.5%, as well as 3 and 4% of total carbohydrates for DP **3a**, **3b**, **4a**, **4b,** and **4c**, respectively [2–5]. During the past decade, FOS have received considerable interest as food ingredients. They are used as nondigestible dietary fiber and texturing properties in many food stuff [6–8].

Some studies have been focused on the FOS properties with respect to their polydispersities, and few take into account their degradation parameters in vitro [9, 10] but only two referenced works have reported their in vivo hydrolysis parameters during the storage of the bulbs under different temperature regimes [11, 12].

During the postharvest life and the onset of sprouting, which is characterized by a catabolism of the FOS reserve, the activities of fructan:fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100) and fructan:fructan 6^G-fructosyltransferase (6G-FFT) involved in fructan biosynthesis were also observed simultaneously [13]. These two activities link one fructosyl residue by $\beta(2\rightarrow1)$ and β $(2\rightarrow6)$ initiating the formation of inulins and inulin neoseries FOS, respectively. Indeed, the involvement of two different enzymatic proteins (6G-FFT and 1-FFT) has been established in other vegetables, that is, asparagus, however, recent proteomic and genomic studies carried out on 6G-FFT and 1-FFT activities have demonstrated that both activities in onion bulbs are assigned to a single and unique enzymatic protein [14, 15].

Although it was established that net fructan accumulation only occurs when the rate of synthesis exceeds the rate of degradation in the vacuole [16], an imbalance occurs during the postharvest life which lead to the exhausting of the FOS reserve in onion tissues. Beside these biosynthetic activities, up to this date, no work reported the biosynthesis of new FOS in onion bulbs during storage, or in any other liliaceous plants as well. However, Van den Ende et al. [17] first succeeded to synthesize in vitro new fructosyl-only oligosaccharides using purified FFT of chicory roots. Later, Timmermans et al. [18] confirmed the presence of three new series of oligosaccharides in forced chicory roots (one disaccharide, one trisaccharide, and one tetrasaccharide) which contain neither $\beta(2\rightarrow1)$ nor $\beta(2\rightarrow6)$ linkages. Ernst et al. [19] reported later the presence of two inulo-n-ose series without a terminal glucose from species of the *Allium* genus.

The present work is devoted to the study of the isolation and structural analysis of newly formed short-chain FOS in stored onion bulbs.

2. Materials and Methods

2.1. Materials. Onion bulbs (*Allium cepa* cv. Tenshin, summer cultivar), which had been freshly harvested and dried (cured) in the field for two weeks, were obtained from the local farm of Rakuno Gakuen University, Ebetsu, Hokkaido, Japan. The bulbs were sorted for uniformity and absence of defects, and then packed in commercial plastic (PVC) trays of 10 kg each. Five trays were kept in store as described in what follows. FOS were analyzed immediately after harvesting and one month later in onion bulbs stored at 10°C and 70% RH.

2.2. Fructooligosaccharides Standards. 1-Kestose [**3a**, 1^F -β-D-fructofuranosylsucrose; 1 kestotriose] and nystose [**4a**, 1^F (1-β-D-fructofuranosyl)$_2$ sucrose; 1,1 kestotetraose] were previously prepared in our laboratory as described by Takeda et al. [20]. Neokestose [**3b**: 6^G-β-D-fructofuranosylsucrose; 6G-kestotriose], **4b** [6^G (1-β-D-fructofuranosyl)$_2$ sucrose; 1, 6G-kestotetraose], **4c** [1^F, 6^G-di-β-D-fructofuranosyl sucrose; 1 and 6G-kestotetraose], **5a** [1^F (1-β-D-fructofuranosyl)$_3$ sucrose; 1,1,1-kestopentaose], **5b** [6^G (1-β-D-fructofuranosyl)$_3$ sucrose; 1,1,6G-kestopentaose], **5c** [1^F (1-β-D-fructofuranosyl)$_2$ -6^G-β-D-fructofuranosyl sucrose; 1,1 and 6G-kestopentaose], **5d** [1^F-β-D-fructofuranosyl- 6^G (1-β-D-fructofuranosyl)$_2$ sucrose; 1 and 1,6G-kestopentaose], **6b**[6^G (1-β-D-fructofuranosyl)$_4$ sucrose; 1,1,1,6G-kestohexaose], **6c** [1^F (1-β-D-fructofuranosyl)$_3$ -6^G-β-D-fructofuranosyl sucrose; 1,1,1 and 6G-kestohexaose], **6d**$_1$ [1^F-β-D-fructofuranosyl-6^G (1-β-D-fructofuranosyl)$_3$ sucrose; 1 and 1,1,6G-kestohexaose], **6d**$_2$ [1^F (1-β-D-fructofuranosyl)$_2$-6^G (1-β-D-fructofuranosyl)$_2$ sucrose; 1,1 and 1, 6G-kestohexaose], **7** [1^F (1-β-D-fructofuranosyl)$_m$-6^G (1-β-D-fructofuranosyl)$_n$ sucrose $(m+n=5)$], **8** [1^F (1-β-D-fructofuranosyl)$_m$-6^G (1-β-D-fructofuranosyl)$_n$ sucrose $(m+n=6)$], and X ≥ 9 [1^F (1-β-D-fructofuranosyl)$_m$-6^G (1-β-D-fructofuranosyl)$_n$ sucrose $(m+n\geq7)$] were obtained from asparagus roots as described in previous papers [21–23]. The standards **6a** [1^F (1-β-D-fructofuranosyl)$_4$ sucrose; 1,1,1,1-kestohexaose], **7a** [1^F (1-β-D-fructofuranosyl)$_5$ sucrose; 1,1,1,1,1-kestoheptaose] were prepared from Jerusalem artichoke tubers in our laboratory. Because the fructan nomenclature is not simple since the structures are very complex, the nomenclatures for FOS proposed by Lewis [24] and Waterhouse and Chatterton [25] were used. All isolated and synthesized standards are of high-grade purity (≥99.8%).

2.3. Fructooligosaccharides Extraction. Fructooligosaccharides (FOSs) were extracted by the method of Shiomi [26]. Tissues (10 g) were homogenized in 80 mL of aqueous ethanol (70%) using a small amount of calcium carbonate (0.5 g L^{-1}). The homogenate was boiled under reflux in a water bath for 10 minutes. Homogenate was filtered and the residue extracted three times with aqueous ethanol and one time with water in the same conditions. The filtrates were combined and made up to 500 mL with distilled water. An aliquot of the filtrate (10 mL) was concentrated to dryness under vacuum at 35°C using a Büchi rotavapor (B*ü*chi labortechnik AG, Flawil, Switzerland). This dry concentrate was redissolved in one mL of water, filtered through a 0.45 μm filter, and analyzed by high-performance anion exchange chromatography (HPAEC Dionex, Sunnyvale, Calif, USA). All processes were run in triplicate.

2.4. Fructooligosaccharides Analysis. FOS were separated on an HPLC-carbohydrate column PA1, Carbo Pack (Sunnyvale, Calif, USA) with a Dionex Bio LC series HPLC (Sunnyvale, Calif, USA) and pulsed amperometric detector (PAD). The gradient was established by mixing eluent A (150 mM NaOH) with eluent B (500 mM acetate—Na in 150 mM NaOH) in two ways: System I: 0-1 minute, 25 mM; 1-2 minutes, 25–50 mM; 2–20 minutes, 50–200 mM, 20–22 minutes, 500 mM; 22–30, 25 mM; System II: 0-1 minute, 25 mM; 1-2 minute(s), 25–50 mM; 2–14 minutes, 50–500 mM, 14–22 minutes, 500 mM; 22–30, 25 mM. The established gradient of mixing eluent A with eluent B

was 0-1 minute, 95% A-5% B; 1-2 minute(s), 80% A-20% B; 2–20 minute(s), 60% A-40% B; 20–22 minute(s), 100% B, 22–30 minute(s), 95% A-5% B. The flow rate through the column was 1.0 mL min^{-1}. The applied PAD potentials for E1 (500 ms), E2 (100 milliseconds), and E3 (50 milliseconds) were 0.01, 0.60, and –0.60 V, respectively, and the output range was 1 μC. Samples (25 μL) were injected using an autosampler (Intelligent autosampler, model AS-4000, HITACHI Ltd, Tokyo, Japan). FOS were estimated by comparison with standards peaks.

2.5. Isolation of the Newly Formed FOS. The FOS were purified and isolated as shown in Figure 1. The stored onion bulbs (2 kg) were extracted in 2000 mL 70% ethanol containing a small amount of calcium carbonate (0.5 g L^{-1}) in a boiling water bath. The treatment was repeated three times. The resulting extract was concentrated under vacuum at 35°C to 700 mL, which, after addition of basic lead acetate, was allowed to stand over night. After the precipitate was removed by filtration, the filtrate was bubbled with hydrogen sulfide gas, and the resulting precipitate was filtered off. The solution was degassed, neutralized with 0.5 N NaOH, concentrated under vacuum, and lyophilized to give a white sugar powder (86 g). After the sugar powder was solubilized in water, the sugar solution was loaded onto a carbon-Celite column (6 × 50 cm) and eluted with water, 10%, 15%, and 30% ethanol successively. Because 10% ethanol eluate fraction contained di-, tri- and tetrasaccharides, the 10% ethanol fraction was re-chromatographed with carbon-Celite column (5 × 75 cm) by successive elution with water, 3 and 5% ethanol. Saccharides 1 and 2 were eluted with 3% and 5% ethanol, respectively. Saccharides 1 and 2 fractions were concentrated under vacuum and lyophilized to give white powders, 25 and 10 mg, respectively. During these different purification processes, the fractions were identified using the method described earlier.

2.6. In Vitro Biosynthesis of Saccharides 1 and 2 by Purified Onion 6G-FFT. Saccharides 1 and 2 were synthesized in vitro using 6G-FFT purified from onion bulbs as described previously [15] and isolated as shown in Figure 2. A mixture of onion 6G-FFT (4 U), 0.2 M fructose, and 0.2 M 1-kestose in McIlvaine buffer (pH 5.5, 60 mL) was incubated at 30°C for 168 hours. After the reaction was stopped by heating at 100°C in a boiling water bath for 3 minutes, the reaction mixture was loaded on a carbon-Celite column (6 × 75 cm) and eluted with water, 3 and 5% ethanol successively. Saccharides 1 and 2 were eluted with 3 and 5% ethanol, respectively. Saccharides 1 and 2 fractions were concentrated under vacuum and lyophilized to give white powders, 182 and 53 mg, respectively,

2.7. Gas Liquid Chromatography (GLC) Analysis of Methanolyzates of Permethylated Saccharides 1 and 2. Methylation of the oligosaccharides was carried out by the method of Hakomori [27]. The permethylated saccharides were methanolysed by heating with 1.5% methanolic hydrochloric acid at 92°C for 10 minutes. The reaction mixture was treated with Amberlite IRA-410 (OH) to remove hydrochloric acid, and evaporated under vacuum to dryness. The resulting methanolysate was dissolved in a small volume of methanol and analyzed using gas chromatography (Hitachi 063 Type Gas Chromatograph Apparatus; stainless column (3 mm × 1 m) packed with 15% butane-1, 4-diol succinate polyester on acid-washed Celite at 175°C and fitted with an FI detector, the carrier gas was nitrogen at the flow rate 40 mL min^{-1}).

2.8. Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS). MALDI-TOFF-MS: MALDI-TOF-MS spectra were measured using a Shimadzu-Kratos mass spectrometer (with KOMPACT Probe) in positive ion mode with 2.5%-dihydroxybenzoic acid as a matrix. Ions were formed by a pulsed UV laser beam (nitrogen laser, 337 nm). Calibration was done using nystose as an external standard.

2.9. Nuclear Magnetic Resonance (NMR) Measurements of Saccharides 1 and 2. Saccharides **1** (5 mg) and **2** (5 mg) were dissolved in 0.5 mL D_2O. NMR spectra were recorded at room temperature with a Bruker AMX 500 spectrometer (^1H 500 MH_Z, ^{13}C 125 MH_Z) equipped with a 5 mm diameter C/H dual (1D spectra) and TXI probe (2D spectra). Chemical shifts of ^1H (δ_H) and ^{13}C (δ_C) in ppm were determined relatively to the external standard of sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl) propanoate in D_2O (δ_H0.00 ppm) and 1,4-dioxane (δ_C67.40 ppm) in D_2O, respectively. ^1H-^1H COSY and HSQC were obtained using gradient selected pulse sequences. The phase-sensitive HSQC-TOCSY spectra were determined with the sequence including inversion of directly resonance (IDR). HMBC spectra were obtained using the pulse sequences of CT-HMBC.

3. Results and Discussion

The formation of the two new FOS was observed in onion bulb tissues four weeks after harvesting and storage at 10°C as shown by the chromatograms (Figure 3(a)). We noted that retention time of saccharides 1 and 2 was 6.87 and 9.52 minutes by HPAEC, respectively, and saccharide 1 was eluted after 1-kestose while saccharide 2 was eluted after nystose (**4a**). When synthesized in vitro by 6G-FFT, saccharides 1 and 2 also showed similar retention times, 6.89 and 9.54 minutes, and were eluted after the same FOS, that is, 1-kestose and nystose (Figure 3(b)). We also found at first that the presence of 1-kestose as sole substrate did not initiate the reaction, while in the presence of fructose and 1-kestose, the reaction was initiated. In vivo, the fructose is provided by the hydrolysis of other FOS in a reaction catalyzed by 1-FEH (fructan:fructan 1-exohydrolase).

The saccharides 1 and 2 isolated from both onion tissues and enzyme reaction were subjected to GLC and NMR analyses (Tables 1, 2 and Figure 4). The methanolyzates of saccharides 1 and 2 showed four peaks corresponding to methyl 1,3,4,6-tetra-o-methyl fructoside and methyl 3,4,6-tri-o-methylfructosideon on GLC chromatograms, respectively. From the findings above, saccharides 1 and 2 were

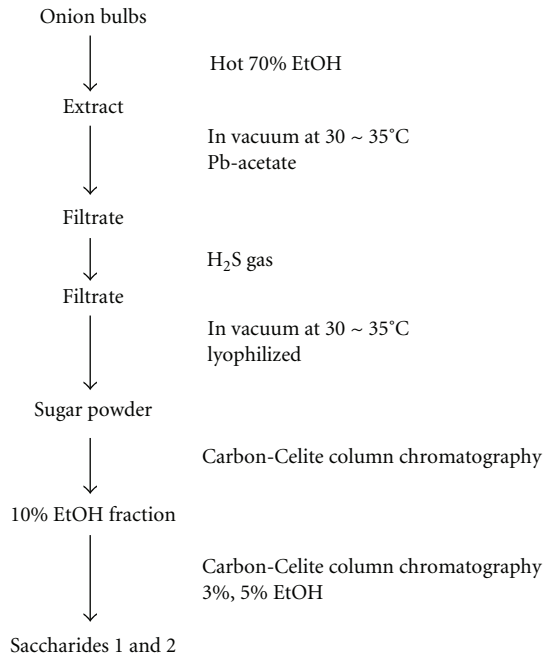

Figure 1: Isolation procedure of saccharides 1 and 2 from onion bulbs stored one month at 10°C.

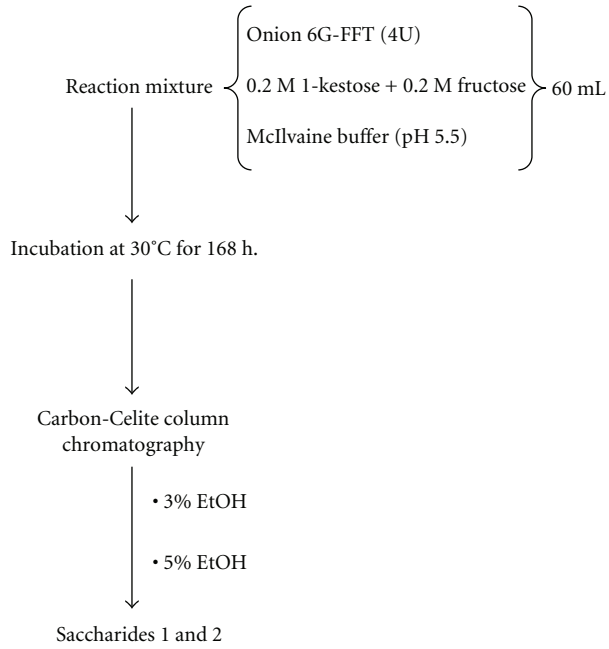

Figure 2: Isolation of saccharides 1 and 2 synthesized by purified onion 6G-FFT in vitro.

Table 1: GLC analysis of methanolyzates of permethylated saccharides 1 and 2 isolated from onion bulbs.

Methanolyzate from permethylated	Relative retention time*			
	Methyl 1,3,4,6-tetra-*O*-methyl-D-fructoside		Methyl 3,4,6-tri-*O*-methyl-D-fructoside	
Saccharide 1	1.03	1.25	2.60	3.89
Saccharide 2	1.03	1.26	2.67	3.96

*Retention time of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside = 1.

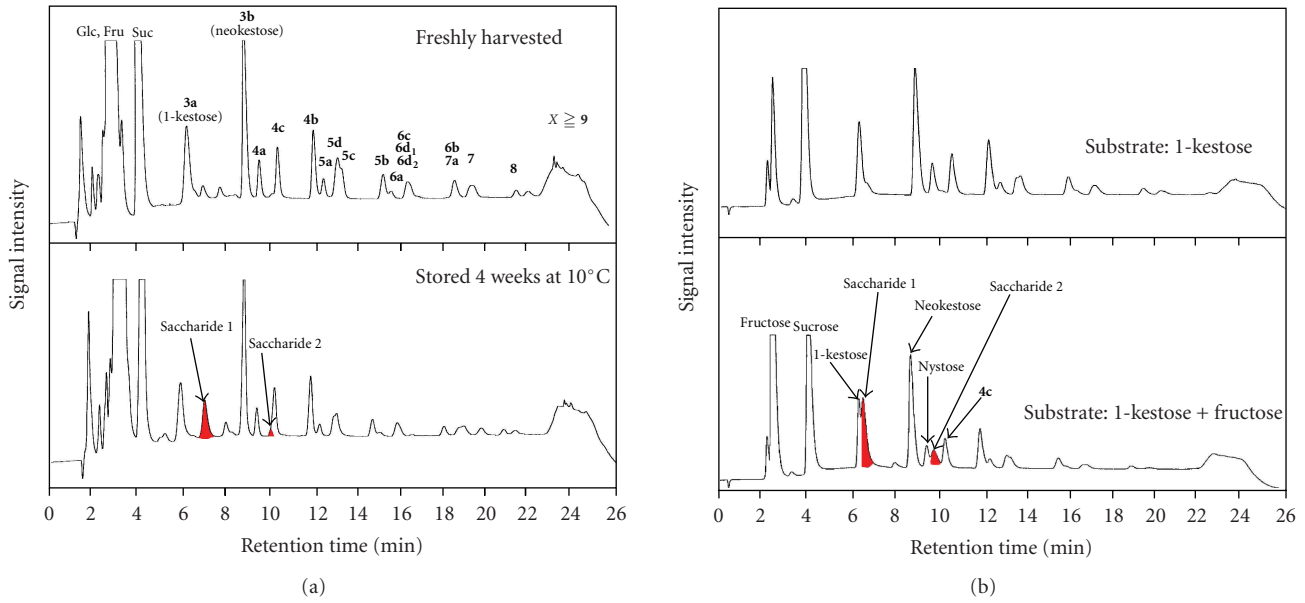

Figure 3: HPAEC charts of sacchaides 1 and 2; (a) chart of saccharides 1 and 2 extracted and purified from tissues of onion bulbs stored one month at 10°C, (b): saccharides 1 and 2 synthesized in vitro by purified 6G-FFT of onion.

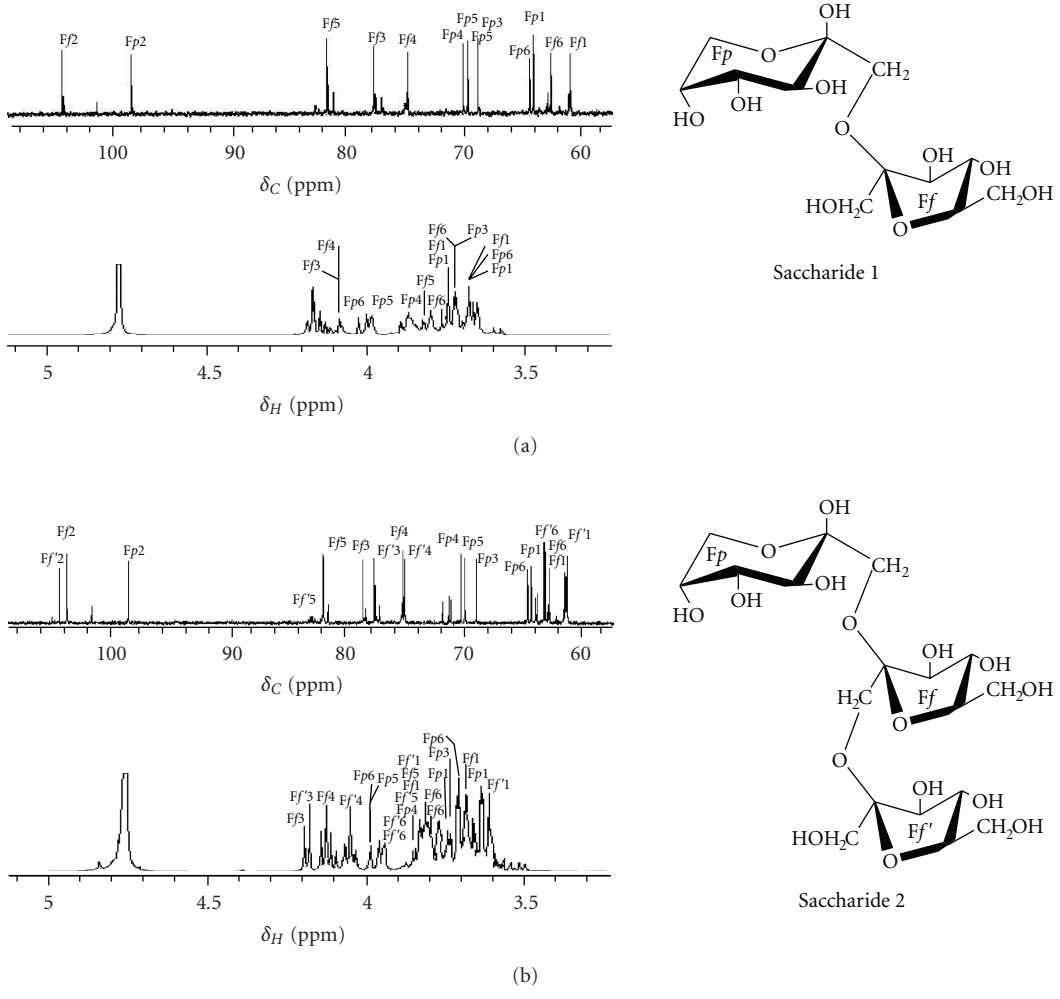

Figure 4: ^{13}C- and ^{1}H-NMR spectra of saccharides 1 and 2. F*f*; fructofuranosyl, F*p*; fructopyranosyl.

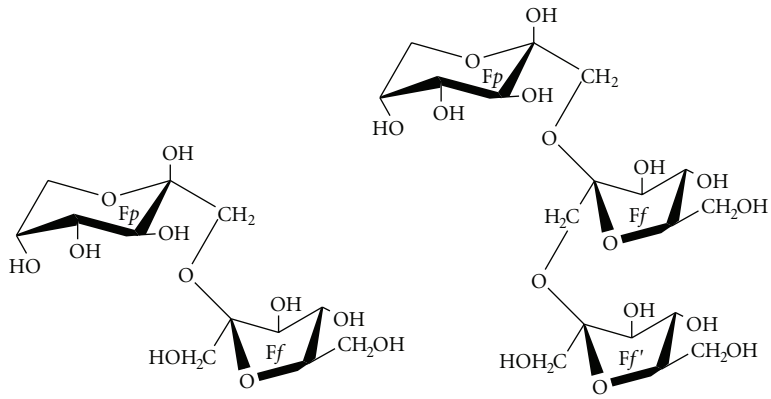

Figure 5: The structure of saccharides 1 and 2. F*f*; fructofuranosyl, F*p*; fructopyranosyl.

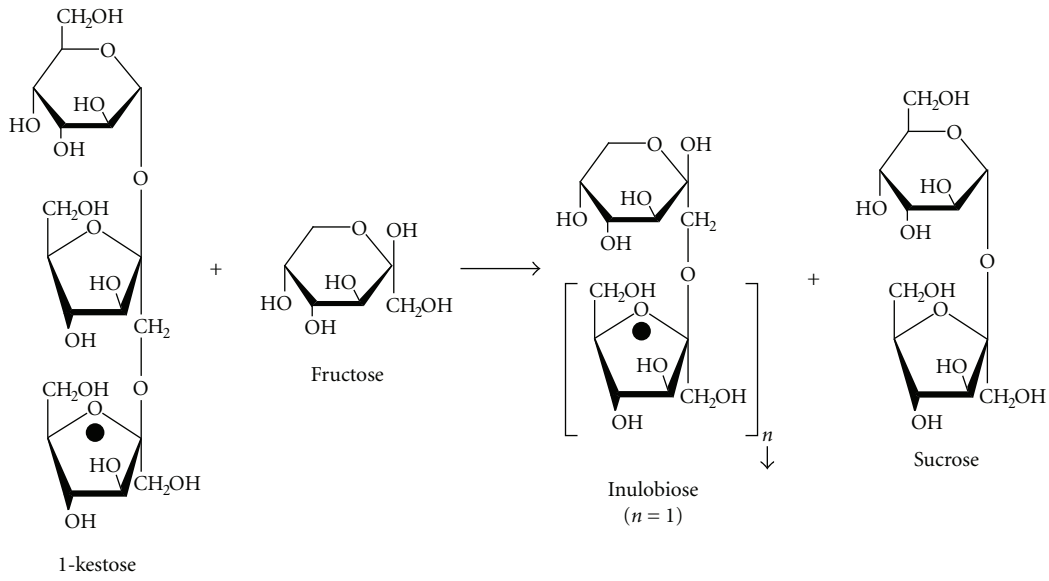

Figure 6: In vitro biosynthesis of inulobiose (saccharide 1) by purified onion 6G-FFT (full black circle indicate the transferred fructose moiety).

confirmed to be fructosyl-(2 → 1)-fructose and fructosyl-(2 → 1)-fructosyl-(2 → 1)-fructose.

From the chemical shift values and the relative intensities of the ^{13}C and ^{1}H NMR signals of the C2 atoms, it was concluded that saccharides 1 and 2 contain one reducing fructopyranosyl residue. The presence of typical signals for C2, C3, C4, and C5 atoms of fructopyranose residues shows the reducing end lacks 6-OH group. The spectra of the analyses confirmed the presence of two different structures: one structure composed by two fructose moieties and linked by $\beta(2 \rightarrow 1)$ linkage and identified as inulobiose [β-D-fructofuranosyl-(2 → 1)- β-D-fructopyranose], while saccharide 2 consists of three units of fructose linked by $\beta(2 \rightarrow 1)$ linkage and was inulotriose [β-D-frucofuranosyl-(2 → 1)-β-D-fructofuranosyl-(2 → 1)-β-D-fructopyranse] (Figure 5). The spectra also did show that 70–80% of the terminal (reducing) fructose residue of the two saccharides, inulobiose and inulotriose, have the pyranose form, while 20 to 30% have furanose form.

The two saccharides were subjected to some chemical property analyses as shown in Table 3. The purified saccharides 1 and 2 from onion bulb tissues had R-sucrose (retention time of the saccharide to retention time of sucrose) values of 1.55 and 2.15 by HPAEC, a reducing terminal, a reducing sugar-to-fructose ratio of 0.5 and 0.3, and a degree of polymerization determined (DP) by MALDI-TOF-MS of 2 and 3, respectively. Comparatively, the in vitro biosynthesized saccharides 1 and 2 showed similar chemical properties and exactly the same values for these parameters were noted (Table 3). However, due to the small amount of saccharides 1 and 2 obtained after purification (25 and 10 mg, resp.), it was not possible to determine the melting point of these two molecules. The suggested reaction of biosynthesis of these two saccharides is shown

Table 2: GLC analysis of methanolyzates of permethylated saccharides 1 and 2 synthesized by purified from G-FFT from onion bulbs.

Methanolyzate from permethylated	Relative retention time*			
	Methyl 1,3,4,6-tetra-*O*-methyl-D-fructoside		Methyl 3,4,6-tri-*O*-methyl-D-fructoside	
Saccharide 1	1.04	1.25	2.59	3.86
Saccharide 2	1.03	1.26	2.66	3.94

*Retention time of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside = 1.

Table 3: Some chemical properties of saccharides 1 and 2 (a) isolated from onion bulb tissues stored one month at 10°C, and (b) synthesized by purified 6G-FFT from onion.

(a) Saccharides isolated from onion bulns					
Saccharide	R-sucrose value	Reducing power	Molar ratio in the hydrolyzate: Red. Sugar/fructose	TOF-MS m/z	DP
1	1.55	+	0.5	365	2
2	2.15	+	0.3	527	3
(b) Saccharides synthesized by purified 6G-FFT from onion					
Saccharide	R-sucrose value*	Reducing power	Molar ratio in the hydrolyzate: Red. Sugar/fructose	TOF-MS m/z	DP
1	1.56	+	0.5	365	2
2	2.15	+	0.3	527	3

*R-sucrose = retention time of the saccharide to retention time of sucrose.

in Figure 6. 1-Kestose is the donor of the fructosyl unit, and the fructopyranosyl the acceptor. The analyses showed that the acceptor fructose is under the pyranosyl form because in aqueous solution, free fructose has almost pyranose form.

As plausible hypothesis, this biosynthesis might be induced by the quantity of free fructose in the form of fructopyranose found in the vacuoles resulting from the breakdown of FOS. Because of the continuous breakdown and release of fructose by the hydrolyzing activity of 1-FEH during storage of onion bulbs [28], this free fructose is used as a substrate for the synthesis of these new saccharides avoiding high accumulation of fructose which could be detrimental to the metabolic activities in the vacuoles by increasing the osmotic pressure of these organelles leading to the slowing down of the metabolic activities. This fact could be emphasized by the high water content of onion tissues (more than 85%), and the high 1-FEH activity observed preceding the onset of sprouting [28]. This high activity of 1-FEH reported previously [28] coincides well with the appearance of the two saccharides which are observed after at least one-moth storage. The synthesis carried out in vitro showed that the reaction is quite linear but faster during the first 100 hours slowing down after this (Figure 7). This pattern is regular and showed that reaction occurs without apparent inhibition, mainly caused by sucrose which is known to inhibit many enzymatic reactions when its concentration is high [16].

The formation of fructosyl-saccharides or fructo-inulinoses has been reported first in chicory during its forcing after a growth and storage [29]. The authors observed some changes which consisted of a significant increase in fructose concentration; an increase in the concentration of low degree of polymerization (DP) fructans; a decrease in high DP fructans; and the appearance new fructans without terminal glucose. Later, Van den Ende et al. [17] obtained three different fructose oligosaccharides, inulobiose (DP 2), inulotriose (DP 3), and inulotetraose (DP 4), by in vitro biosynthesis using purified chicory root fructan:fructan fructosyl transferase (FFT) with inulin as a donor and fructose as an acceptor. These newly produced fructooligosaccharides were also isolated from chicory roots [18]. Similar inulo-n-ose series of DP 2 and DP 3 of fructose polymers were identified in both sprouted and unsprouted onion bulbs after two-month storage [19]. However, although the degree of polymerization was determined, these authors did not report indeed the structural conformation of the fructosyl terminal of these isolated saccharides as reported in the present investigation.

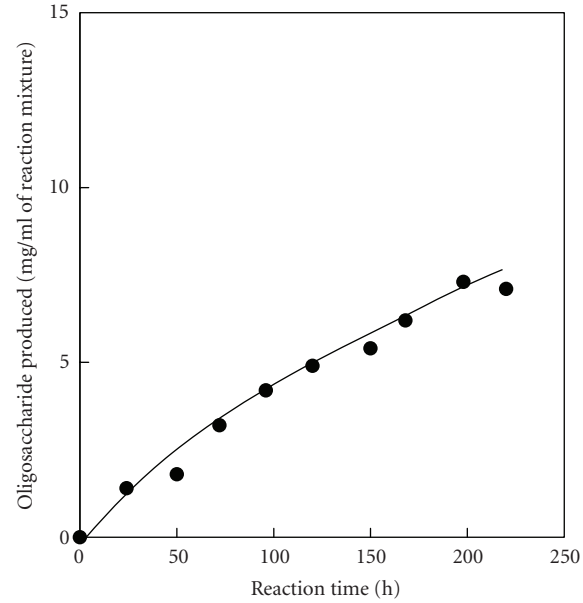

Figure 7: Time course of formation of saccharide 1 catalyzed by 6G-FFT in vitro.

Although from the chemical point of view we could suggest that the reaction would be triggered by the accumulation of fructose released from the hydrolysis of fructans and/or by the pyranosyl form of the free fructose in the vacuoles, the physiological process inducing this reaction is unclear and enigmatic. We can speculate on the fact that the formation of these short-chain inulibiose or inulintriose is initated by the high accumulation of fructose in the vacuoles and the possible unbalance and osmotic pressure created by this accumulation. Thus, the synthesis of these two new saccharides could play either a role of "balance" between the levels of the different high DP FOS (≥ 5) and/or a "buffer reaction" between the different tri- and tetrasaccharides, because of the low rate of fructose utilization in the metabolism. This speculation would be supported by further work on one hand the hydrolysis kinetic parameters of these saccharides and compare them with the parameters of other FOS studied previously, and on the other hand the rapid exhausting of high DP FOS ($5 \sim 12$), while low DP FOS ($3 \sim 4$) decrease less during the first two months of storage [11, 12].

In conclusion, the in vitro biosynthesis of new short saccharides composed by fructose molecules seem to occur under specific conditions such as forcing (chicory) or storage (chicory and onion). However, this reaction is unclear and the questions raised are: (i) what triggers this reaction?, (ii) which physiological is behind this reaction?, and (iii) what are the roles of these newly formed compounds? Thus, a lot remains to be investigated in order to answer these questions and understand better the complex metabolism of fructans in plants.

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