JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Identification of Fructo- and Malto-oligosaccharides in Cured Tobacco Leaves (*Nicotiana tabacum*)

Atsushi Nagai,*[,†] Takeshi Yamamoto,[‡,⊥] and Hiroyuki Wariishi[§]

[†]Tobacco Science Research Center, Japan Tobacco Inc., 6-2, Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan

[‡]Plant Innovation Center, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan

[§]Department of Forest and Forest Products Sciences, Graduate School of Bioresource and Bioenvironmental Sciences, Kyusyu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka, Fukuoka 812-8581, Japan

ABSTRACT: Fructo-oligosaccharides (FOSs) and malto-oligosaccharides (MOSs) in cured tobacco leaves (*Nicotiana tabacum*) were detected and quantified using high-performance liquid chromatography (HPLC) coupled with electrospray ionization (ESI) tandem mass spectrometry (MS/MS). These oligosaccharides were present in several tobacco materials including flue-cured tobacco, sun/air-cured tobacco, and cut filler of commercially available tobacco products, but were not detected in air-cured tobacco. The changes in these oligosaccharides during storage were also investigated. The results revealed that MOSs simply decreased under a warm atmosphere and FOSs increased. In addition, the formation of FOSs in cured tobacco leaves occurred in the presence of sucrose. With regard to FOSs, it has been reported that green tobacco leaves do not contain FOSs such as kestose, nystose, and fructosyl-nystose. The results of a model test suggested that the changes in FOS amount were caused by enzymatic reactions.

KEYWORDS: *fructo-oligosaccharide, malto-oligosaccharide, cured tobacco, storage, enzyme, LC-MS/MS*

INTRODUCTION

Sugars are one of the main components of tobacco leaves. In addition, the sugar composition in tobacco leaves is directly related to the taste and aroma of cigarette smoke.[1−4] Therefore, investigating the composition is the first step toward a comprehensive understanding of the relationship between the sugars in tobacco leaves and the taste/aroma of cigarette smoke. To date, studies have revealed that certain types of cured tobacco leaves, such as flue-cured tobacco and sun/air-cured tobacco, contain abundant fructose, glucose, and sucrose. Furthermore, studies have also been carried out to identify the sugars in green tobacco leaves, cured tobacco leaves, and tobacco products.[5−12] However, these studies have predominantly focused on mono-, di-, and polysaccharides. There is a limited amount of information on oligosaccharides; planteose in tobacco seeds, raffinose and stachyose in green tobacco leaves, and erlose and theanderose in cured tobacco leaves have been reported.[6,13,14]

Many different oligosaccharides are widely distributed in plants, algae, and bacteria. Among them, fructo-oligosaccharides (FOSs) are a condensate of fructose in which fructose residues are bound by β-linkage with or without a single glucose unit in each molecule. FOSs are divided into three main types: inulin, levan, and graminan, on the basis of the glycosidic linkage between fructose residues.[15] Highly polymerized FOSs, which are fructans, have also been found in plants, algae, and bacteria. Approximately 15% of flowering plants accumulate fructan. Inulin-type (β2-1 linkage) fructans primarily occur in dicotyledon plants as typified by Asterales, whereas levan-type (β2-6 linkage) and graminan-type (mixed linkage, branched structure) fructans typically occur in monocotyledon plants.[15,16] Although fructans are widely distributed in plants, tobacco is classified as a non-fructan-storing plant, and several studies have reported that tobacco did not produce fructan in the absence of transformation.[17,18] On the other hand, MOSs are a condensate of glucose in which glucose residues are bound by α1-4 linkage. Maltose, which consists of two glucose units and is regarded as an MOS (degree of polymerization (DP) 2), is one of the sugars contained in tobacco leaves.[5,6,9] However, there are few reports about MOSs measuring more than DP 2.[19] In cured tobacco leaves, the presence of MOSs resulting from the hydrolysis of starch during the curing stage was expected at the beginning of this research.[1] The structures of FOSs and MOSs are shown in Figure 1.

Various methods for analyzing the oligosaccharides mentioned above have been reported. Today, the most commonly used technique for analysis of oligosaccharides is high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).[20−22] Although HPAEC-PAD is very helpful for the separation and quantification of oligosaccharides, it does not provide much structural information and often requires extensive sample purification. Several methods based on MS detection (MSD) have recently been reported.[23−26] MSD has some advantages compared to PAD, particularly in terms of providing information on molecular weight, peak purity, and partial structure. Furthermore, matrix-assisted laser desorption/ionization (MALDI) coupled with time-of-flight mass spectrometry (TOF-MS) or Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) often provides information about DP, composition, and concentration without chromatographic separation.[27,28]

Received: April 3, 2012
Revised: June 6, 2012
Accepted: June 11, 2012
Published: June 11, 2012

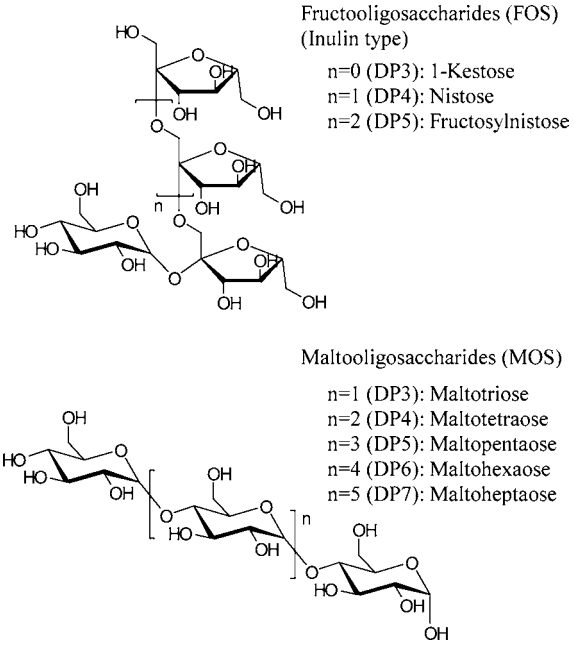

Figure 1. Structures of fructo-oligosaccharides (FOSs) and malto-oligosaccharides (MOSs).

In this paper, we focus on oligosaccharides in which the constituent sugars are glucose or fructose and report (i) a new technique that enables the simultaneous analysis of MOSs and FOSs in cured tobacco leaves; (ii) the content of FOSs and MOSs in typical tobacco materials including green tobacco leaves and cut filler of commercially available cigarettes; and (iii) changes in the content of these oligosaccharides during storage.

MATERIALS AND METHODS

Materials. Green tobacco leaves were harvested from a field managed by Japan Tobacco Inc. and were immediately lyophilized. Cured tobacco leaves were stored in a warehouse of Japan Tobacco Inc. Commercial cigarettes were purchased at a Japanese market. Acetonitrile was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). D-Glucose, D-fructose, sucrose, kestose, nystose, fructosyl-nystose, and ammonium acetate were also purchased from Wako Pure Chemical Industries, Ltd. Maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). All solvents for sample preparation, standard solutions, and mobile phase in liquid chromatography were prepared using 18.2 MΩ·cm deionized water produced by a Milli-Q system (Millipore Co., Bedford, MA, USA).

Moisture Measurement. Approximately 1 g of cut tobacco filler was weighed and then dried using a rotary oven (Tsukasa Co., Ltd., Tokyo, Japan) at 100 °C for 1 h. After drying, the sample was cooled to room temperature in a desiccator for 1 h and then weighed. The reduction in weight was taken to be the moisture content, which was used to evaluate the oligosaccharide content on a dry weight basis (DB).

Effects of Storage on Tobacco Materials. The effects of storage on the tobacco materials were investigated under the following conditions: temperature, 7, 22, 30, and 40 °C; storage period, 12 weeks; tobacco materials, cut filler of commercial cigarettes.

Sample Preparation. Approximately 20 g of cured tobacco leaves was pulverized into 1−2 mm mesh powder using a MiniBlender (Melitta Japan Ltd., Tokyo, Japan). The 1.000 ± 0.001 g of powder was placed in 50 mL vials, extracted with 40 mL of 50 v/v % acetonitrile at room temperature for 30 min using a shaker (200 rpm), and then further extracted by ultrasonication using Bransonic cleaner (Branson Ultrasonic Co., Danbury, CT, USA), keeping extract below room temperature for 30 min. The subsequent supernatant was decanted and centrifugally filtered using Amicon Ultra 10000 Da cutoff filters (Millipore Co.). Filtrate of 20 mL was evaporated for complete removal of acetonitrile (to approximately 5 mL). The total volume of collected solution was adjusted to 10 mL and filtered with a Whatman PVDF membrane with a pore size of 0.20 μm (GE Healthcare U.K. Ltd., Buckinghamshire, U.K.) to remove the insoluble components in deionized water.

With regard to the measurement of monosaccharides and disaccharides, the ultrafiltrate was directly injected into the device.

When acquiring product ion chromatography for identification of oligosaccharides, the ultrafiltrate was passed through an Oasis MCX cartridge (6 cm^3/150 mg, Waters Co., Milford, MA, USA) after evaporation of the acetonitrile. Then, the liquid that passed through the cartridge was concentrated to 2 mL and filtered with a Whatman PVDF membrane with a pore size of 0.20 μm (GE Healthcare).

Measurement of Monosaccharide and Disaccharides. The analyses of glucose, fructose, and sucrose were performed using an Agilent 1200 HPLC system coupled with a G1362A refractive index detector (Agilent Technology, Santa Clara, CA, USA) set at 35 °C. Chromatographic separations were carried out using a high performance carbohydrate column (250 × 4.6 mm i.d., 4 μm, Waters Co.). Acetonitrile (75 v/v %) was used as the mobile phase at a flow rate of 1.0 mL/min. The injection volume was 20 μL. The determination of concentration was performed using the absolute calibration method. The calibration range was from 0.1 to 10 g/L. Standard solutions were prepared with 50 v/v % acetonitrile.

Measurement of Oligosaccharides. The analyses of MOSs and FOSs were performed using an Agilent 1200 HPLC system coupled with a 6410 triple-quadrupole MSD (Agilent Technology). Chromatographic separations were carried out using a Develosil RP Aqueous column (150 × 1.5 mm i.d., Nomura Chemical Co., Ltd., Japan). Mobile phase A (5 mM ammonium acetate) and mobile phase B (acetonitrile) were used for elution under the following conditions: flow rate, 0.1 mL/min; A, 100% at 0 min; A, 100% holding until 15 min; A, 85% at 30 min (linear gradient); and A, 85% holding until 40 min; post run time, A, 100% 20 min. The column temperature was set at 30 °C. The injection volume was 5 μL. Ionization using ESI/MS was performed under the following conditions: negative ion mode; capillary voltage, 4000 V; drying gas temperature, 350 °C; gas flow, 11 L/min; nebulizer pressure, 35 psi. The other MS parameters of fragmentor voltage, precursor ion (m/z), collision energy (CE), product ion (m/z), and

Table 1. MS/MS Paramaters and Retention Time of Each Analyte

compd	DP[a]	fragmentor (V)	precursor ion (m/z)	CE[b] (V)	product ion (m/z)	dwell time (ms)	RT[c] (min)
FOS	3	140	503	20	323	50	5.6
FOS	4	160	665	25	485	100	13.2
FOS	5	160	827	30	647	200	31 ± 0.3
MOS	4	140	665	10	161	200	4.8−6.2
MOS	5	140	827	10	665	200	7.0−8.6
MOS	6	140	989	10	827	200	9.5−11.1
MOS	7	140	989	10	827	200	12.0−13.6

[a]DP, degree of polymerization. [b]CE, collision energy. [c]RT, retention time.

dx.doi.org/10.1021/jf301395v | J. Agric. Food Chem. 2012, 60, 6606−6612

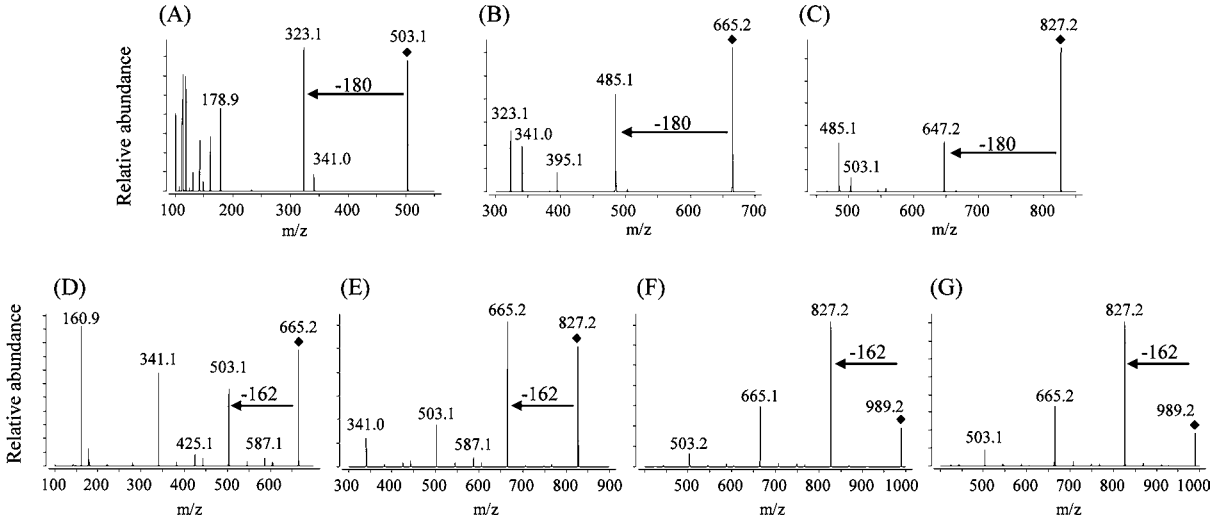

Figure 2. MS spectra of the standard oligosaccharides used for ESI/MS/MS in negative-ion mode: (A) FOS DP3; (B) FOS DP4; (C) FOS DP5; (D) MOS DP4; (E) MOS DP5; (F) MOS DP6; (G)MOS DP7. ◆ indicates precursor ions.

dwell time were switched at time segments corresponding to the retention time of each compound. Tandem mass spectra on product ion chromatography were obtained under various conditions: injection volume, 2−10 μL; collision energy, between 5 and 35 V; and scan time, 500−1000 ms. The other conditions were the same as those for the quantification method.

RESULTS AND DISCUSSION

Detection and Quantification of Oligosaccharides in Cured Tobacco Leaves. We expected it to be difficult to identify the oligosaccharides by LC-MS due to the possible presence of several oligosaccharides with the same molecular weight in the analyte. It was also expected that various impurities as typified in the brown pigment of cured tobacco leaves might prevent the analysis of oligosaccharides. Thus, analysis of oligosaccharides by a LC-MS/MS system was selected on the basis of its high selectivity, possibility of fragmentation analysis, and non-necessity of perfect chromatographic separation compared to a LC-MS system. The MS/MS parameters and retention time of each standard oligosaccharide used in this study are shown in Table 1.

The preliminary ionization experiment was carried out using the standard and a sample in which the standards were spiked. As a result, the ionization of FOSs and MOSs was achieved in the negative-ion mode. Ionization of underivatized oligosaccharides using ESI in the positive-ion mode has achieved significant results through the formation of alkali-cationized ions.[25,26,29] However, when the tobacco extract was analyzed, the negative-ion mode gave a better signal-to-noise ratio and higher reproducibility. Deprotonated ion $[M - H]^-$ could basically be monitored as precursor ion. With regard to maltoheptaose, fragmentation occurred concurrently with ionization; therefore, the fragment ion (m/z 989), with good ionic yield, was selected as the precursor ion. Although maltotriose was able to be ionized through this method, quantitative results with scarce reproducibility were obtained for the tobacco extract (data not shown). Subsequently, MS/MS conditions were investigated to raise the peak purity and to differentiate FOSs from MOSs eluting at the same time. The precursor ion of each oligosaccharide tended to fragment with regularity (shown in Figure 2). MS spectra on product ion chromatography (PIC) of MOS standards commonly showed some dominant losses appearing at intervals of 162 amu. The fragmentation of deprotonated ions of MOSs

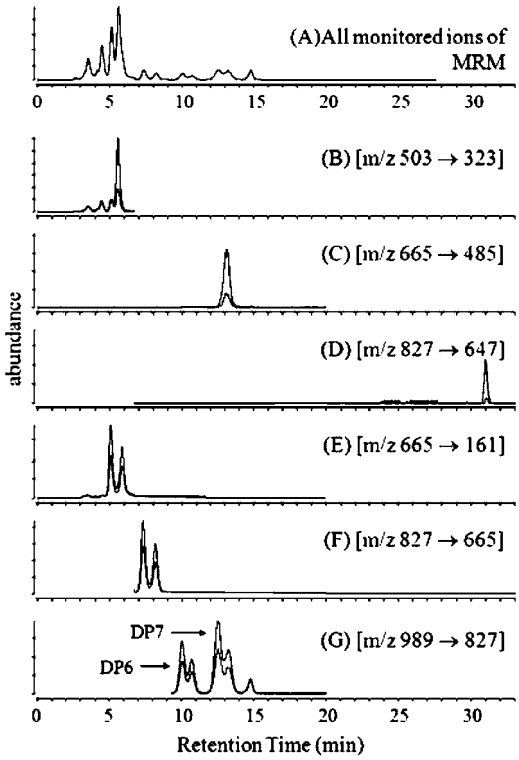

Figure 3. Chromatograms of multiple-reaction monitoring (MRM). Tobacco extract sample and sample with spiked standard were overlaid: (A) all monitored ions of MRM; (B) FOS DP3; (C) FOS DP4; (D) FOS DP5; (E) MOS DP4; (F) MOS DP5; (G) MOS DP6 and MOS DP7. The peak in which the intensity increased in each extracted chromatogram was based on the latter sample.

may be the result of preferential cleavage of glycosidic linkage. This behavior coincided with the results of fragmentation patterns by negative ion fast atom bombardment found in the literature.[30] On the other hand, MS spectra of FOSs exhibited different rules from MOSs. That is, FOSs consistently showed dominant losses of 180 amu. Even if the collision energy were changed between 5 and 30 V, typical losses of other glycosidic ring cleavages, such as 18, 30, 60, and 90 amu, were not observed.[29,30] The trace of a loss of 162 amu was marginally

dx.doi.org/10.1021/jf301395v | J. Agric. Food Chem. 2012, 60, 6606−6612

Table 2. Quantitative Value of Oligosaccharides in Tobacco Leaves

	leaf type:	flue-cured Virginia		burley	Orient		green leaf		cut filler[b]	
compd/DP[a]	growing district:	USA	Brazil	USA, Brazil	Greece	Turkey	Japan	Japan	sample A	sample B
FOS DP3	av (ppm)[c]	31.45	41.31	ND	51.55	224.4	ND	ND	283.3	70.89
	SD (ppm)	±0.96	±2.84		±3.47	±5.15			±16.57	±3.52
	RSD (%)	3.0	6.9		6.7	2.3			5.8	5.0
FOS DP4	av (ppm)	trace	trace	ND	2.16	10.00	ND	ND	19.09	2.77
	SD (ppm)				±0.07	±0.63			±0.58	±0.29
	RSD (%)				3.3	6.3			3.0	10.3
FOS DP5	av (ppm)	ND	ND	ND	trace	1.18	ND	ND	4.97	trace
	SD (ppm)					±0.14			±0.27	
	RSD (%)					11.7			5.5	
MOS DP4	av (ppm)	4.63	6.74	ND	7.41	46.62	11.79	1.84	72.21	10.23
	SD (ppm)	±0.64	±0.78		±0.09	±1.43	±1.01	±0.10	±3.38	±0.16
	RSD (%)	13.7	11.6		1.2	3.1	8.6	5.3	4.7	1.5
MOS DP5	av (ppm)	2.46	3.75	ND	3.08	26.12	12.66	1.73	53.58	5.34
	SD (ppm)	±0.04	±0.23		±0.35	±0.46	±0.82	±0.16	±3.15	±0.41
	RSD (%)	1.8	6.1		11.4	1.8	6.5	9.2	5.9	7.7
MOS DP6	av (ppm)	6.11	8.07	ND	3.36	31.83	29.8	3.64	58.55	10.44
	SD (ppm)	±0.58	±0.21		±0.17	±3.22	±1.52	±0.32	±4.27	±0.19
	RSD (%)	9.5	2.5		5.1	10.1	5.1	8.9	7.3	1.8
MOS DP7	av (ppm)	7.89	7.04	ND	4.63	37.11	31.24	5.58	45.55	8.51
	SD (ppm)	±0.38	±0.24		±0.14	±3.57	±0.87	±0.33	±0.96	±0.56
	RSD (%)	4.8	3.4		3.0	9.6	2.8	5.9	2.1	6.6

[a]Degree of polymerization. [b]Cut filler of the product was taken from commercial cigarettes. Samples A and B were purchased at Japanese markets. [c]Amounts of analytes in extract were converted to the amounts in tobacco leaves. Average (ppm) was evaluated on a dry weight base (method described under Materials and Methods). Analyses including preparation of tobacco extract were repeated three times.

detected. For this reason, the dominant loss of 180 amu may be caused through dehydration occurring almost simultaneously with a loss of 162 amu. Moreover, oligosaccharides with the same DP (i.e., in this case, the molecular weight is the same) were able to be separated on chromatography. Fragmentation was accelerated by increasing the collision energy (CE). CE was adjusted to the most efficient voltage for generating the objective ions (conditions are shown in Table 1) in quantitative analysis. Consequently, all FOSs and MOSs were able to be measured independently by monitoring specific precursor ions and product ions at each retention time. The chromatograms of multiple-reaction monitoring (MRM) of cured tobacco extract are shown in Figure 3. Identification of each oligosaccharide in the sample solutions was based on the increase in peak intensity by spiking the standards and the consistency of precursor ions and product ions using the standards. Furthermore, each FOS was identified by comparison of MS spectra on PIC as described below.

To evaluate the difference between the samples, quantitative analysis was carried out. Signal abundance of some oligosaccharides that appeared at a short retention time on the chromatogram, such as kestose and maltotetraose, did not sufficiently recover toward the spiked amount of standard. Instead, the signal intensity increased linearly in proportion to the added quantity. The determination of concentration of each analyte was performed using the standard addition method. The concentration range of oligosaccharides in prepared sample solution was as follows: FOS DP3 (kestose), 1.0−50 μg/mL; the other oligosaccharides, 0.05−5.0 μg/mL. The linearity of all oligosaccharides exhibited favorable values with coefficients of determination >0.999 in the range of sample intensity from 5 to 10 times the intensity. The recovery tests were performed by comparing the peak intensity between addition of the standards before extraction and addition of the corresponding amounts of standards after sample preparation. All oligosaccharides exhibited favorable recoveries between 80 and 120%.

Quantification of oligosaccharides was conducted for typical tobacco leaves and cut filler of commercial cigarettes. Oligosaccharides in green tobacco leaves were also measured. Quantitative results are shown in Table 2. The composition of oligosaccharides was characterized by leaf type and growing district. FOSs and MOSs could not be observed in burley-type leaves. Burley-type leaves are usually dried through a lengthy air-curing process. Thus, it was considered that oligosaccharides in burley-type leaves were distinguished during air-curing like other sugars.[1] On the other hand, FOSs were observed in some cured tobacco leaves despite the fact that no FOSs were detected in green tobacco leaves. Because the tobacco plant (*Nicotiana tabacum*) is classified as a non-fructan-storing plant, the absence of FOSs in green tobacco leaves is expected.[17,18] In light of these facts, FOSs in cured tobacco leaves and cut filler were expected to increase during the curing process or storage period.

Change of Oligosaccharides in Cured Tobacco Leaves during Storage. According to the methods described under Materials and Methods, the effects of storage were investigated to confirm the change in oligosaccharide composition in tobacco materials. Figure 4 shows variations in the amount of

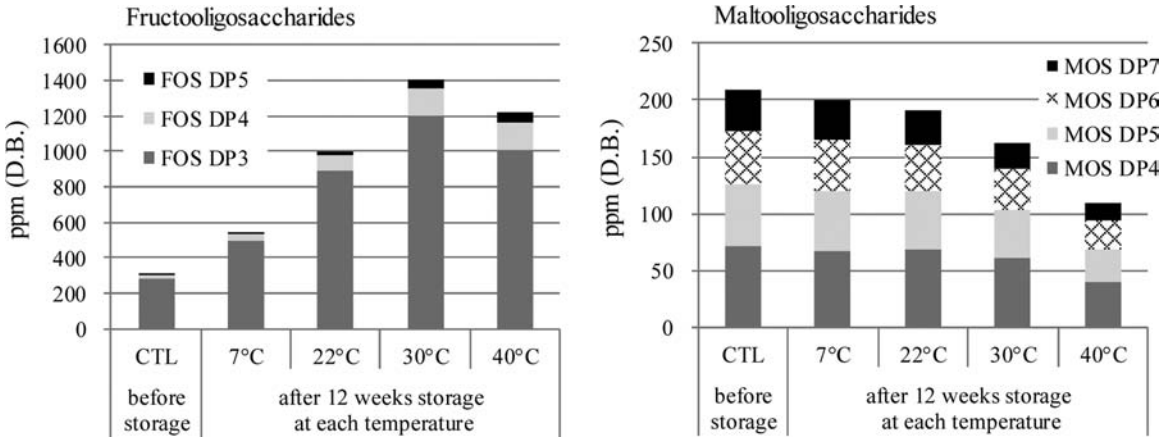

Figure 4. Changes in oligosaccharides in tobacco material before and after storage.

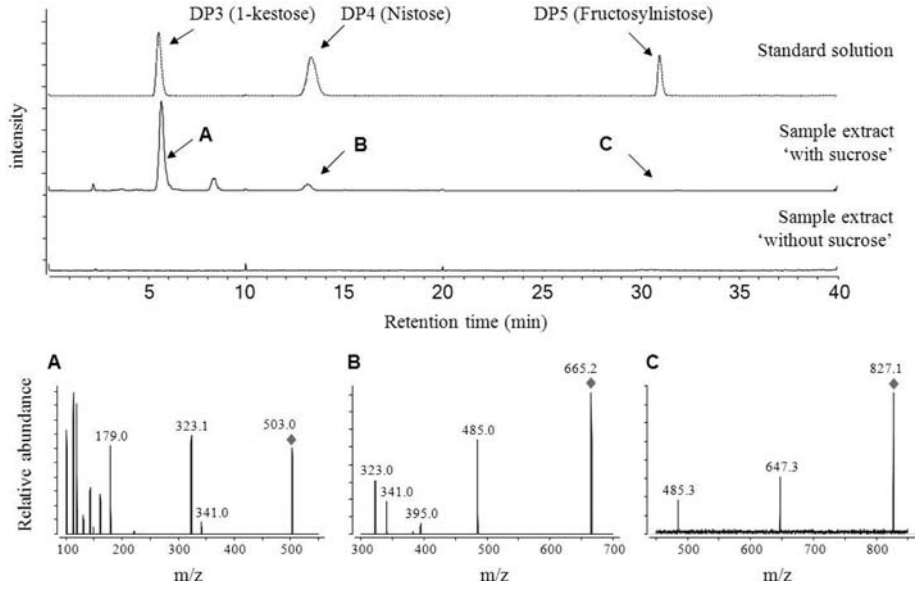

Figure 5. Product ion chromatogram and MS spectra of sample extract. The sample was a burley-type tobacco leaf spiked with or without sucrose and stored for 4 weeks at 40 °C. Peaks (A–C) on PIC correspond to the same letter indicated on the MS spectra.

oligosaccharides in the tobacco materials during storage examination. MOSs simply decreased at warm temperatures. Because MOSs have a reducing end, they might decompose chemically or react with nitrogen compounds, causing amino carbonyl reactions.[31] On the contrary, FOSs increased depending on the temperature of the storage environment. The generation of FOSs would not be based on the hydrolysis of carbohydrate, because tobacco does not accumulate fructan as previously described. It was also considered unlikely that other new kinds of sugars would be generated by chemical condensation reaction of sugar under this mild environment. FOSs did not necessarily increase in some of the materials tested for the same examination. Thus, we conjectured that FOSs had been produced by an enzyme reaction with sucrose as the substrate.

Origin of Fructo-oligosaccharides. An additional examination to confirm our hypothesis was carried out using three samples prepared by the method described below. The first sample was a burley-type leaf that hardly contains any sugars. The second sample was a burley-type leaf to which approximately 5 wt % sucrose was added. The sample was dried as quickly as possible without the application of heat, after the sucrose solution was sprayed. The third sample was a burley-type leaf heat-treated before sucrose was added. Heat treatment was performed at 120 °C for 10 min. Sucrose was added according to the same method as for the second sample. The three samples were stored at 40 °C concurrently and then the change of components was analyzed at fixed intervals.

Figure 5 shows PICs and MS spectra of the standard and sample extract. The phenomenon of FOS formation was confirmed in a burley-type leaf spiked with sucrose, although burley leaves naturally do not have and do not generate FOSs. MS spectra of each peak on PIC coincide with the pattern of the standard (shown in Figure 2). These results strongly suggest that FOSs were generated in tobacco leaves by enzymatic reaction under the conditions used in this study. In addition, the enzyme associated with the increase in FOSs was thought to be included in the leaves.

Figure 6 shows the behavior of fructose, glucose, sucrose, and FOSs during storage. When burley-type leaves without sucrose were stored, FOSs were not produced, and no other sugars were generated (data not shown). As described above, the enzyme related to FOSs increasing might exist in burley-type leaves; however, the leaves did not contain sucrose, the substrate of the

dx.doi.org/10.1021/jf301395v | J. Agric. Food Chem. 2012, 60, 6606–6612

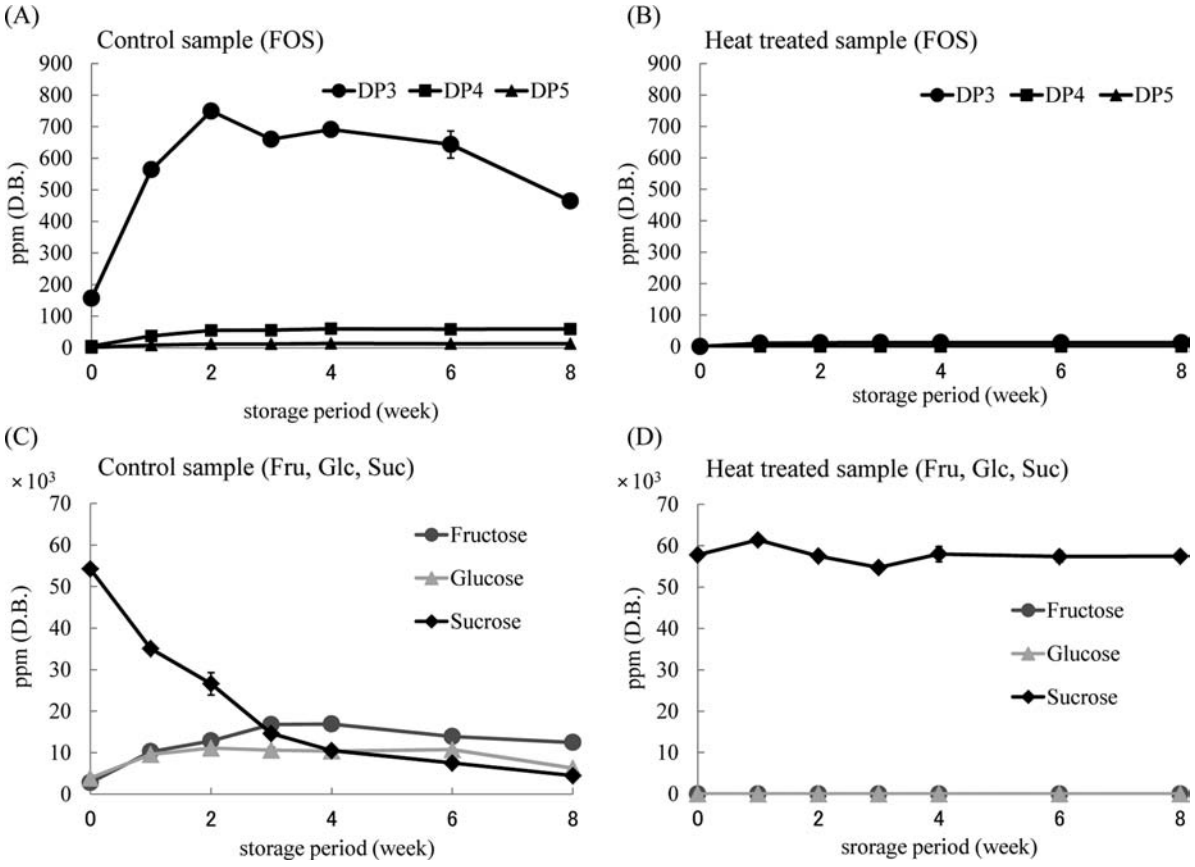

Figure 6. Temporal changes in glucose, fructose, sucrose, and FOSs in two burley-type tobacco samples with added sucrose. The control sample was not heat-treated before sucrose was added (A, C). The other sample was heat-treated (B, D). Samples were stored at 40 °C. DP means degree of polymerization.

enzyme. Thus, in the case of sugar-free burley-type leaves, the formation of FOSs was not observed. On the other hand, in the case of the leaf with added sucrose, various changes were observed. Whereas sucrose decreased according to the storage time, fructose and glucose increased simultaneously (Figure 6C). However, increased amounts of fructose and glucose were not equivalent. We had expected that these sugars could be reacted by another enzymatic reaction or chemical reaction in cured tobacco leaves. Then, an increase in FOSs could also be observed (Figure 6A). These results indicated that sucrose was the origin of FOSs. It should be noted that this series of changes did not occur on the heat-treated sample despite the presence of sucrose (Figure 6B,D). This difference was ascribable to the deactivation of the related enzymes in sucrose.

From the results obtained in this study, the reaction is considered to a transfructosylation reaction by the enzyme. Sucrose:sucrose 1-fructosyltransferase (1-SST) is known as an enzyme that produces 1-kestose (FOS DP3) from sucrose.[32] SST is an important enzyme for accumulating fructan. Certain plants such as Asteraceae and Gramineae contain SST, but this type of enzyme was not contained in the tobacco plant.[33−35] However, the tobacco plant (*N. tabacum*: Solanaceae) contains invertase, which converts sucrose into fructose and glucose, and the survival of invertase activity in cured tobacco leaves was obvious from this examination. SST-like activity found in the present study was possibly caused by the side reaction activity of invertase. Several studies have already clarified that invertase partially acts like SST.[36−38] In the low-moisture environment of cured tobacco leaves, the side reaction may be predominant rather than hydrolysis.[39] In summary, the potential change in FOSs during storage will be dependent on the concentration of sucrose and the remaining enzyme activity in the cured tobacco leaves.

In this paper, we demonstrated that oligosaccharide analysis using LC-MS/MS can be used to determine the composition of FOSs and MOSs in various tobacco materials. We also reported the variability and one of the factors for the variability of sugars including FOSs and MOSs. We are presently attempting to purify the enzyme responsible for generating FOSs from cured tobacco leaves to better understand the details of the mechanism. This type of knowledge is indispensable for advancement of quality control in food science.

AUTHOR INFORMATION

Corresponding Author

*Phone: +81 45 345 5165. Fax: +81 45 973 6781. E-mail: atsushi.nagai@jt.com.

Present Address

⊥Product Science Division, Japan Tobacco Inc., 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Mari Kawase for technical assistance and valuable discussions.

REFERENCES

(1) Leffingwell, J. C. Leaf chemistry: basic chemical constituents of tobacco leaf and differences among tobacco types. In *Tobacco: Production Chemistry and Technology*; Davis, D. L., Nielsen, M. T., Eds.; Blackwell Science: Oxford, U.K., 1999; pp 265−284.

(2) Weeks, W. W. Leaf chemistry: relationship between leaf chemistry and organoleptic properties of tobacco smoke. In *Tobacco: Production, Chemistry and Technology*; Davis, D. L., Nielsen, M. T., Eds.; Blackwell Science: Oxford, U.K., 1999; pp 304−312.

(3) Baker, R. R.; Pereira da Silva, J. R.; Smith, G. The effect of tobacco ingredients on smoke chemistry. Part I: Flavourings and additives. *Food Chem. Toxicol.* **2004**, *42S*, S3−S37.

(4) Baker, R. R.; Pereira da Silva, J. R.; Smith, G. The effect of tobacco ingredients on smoke chemistry. Part II: Casing ingredients. *Food Chem. Toxicol.* **2004**, *42S*, S39−S52.

(5) Stedman, R. L. The chemical composition of tobacco and tobacco smoke. *Chem. Rev.* **1968**, *68* (2), 153−207.

(6) Siddiqui, I. R.; Rosa, N. Low molecular weight carbohydrates of tobacco. *Tob. Sci.* **1983**, *27*, 130−134.

(7) Clarke, M. B.; Bezabeh, D. Z.; Howard, C. T. Determination of carbohydrates in tobacco products by liquid chromatography−mass spectrometry/mass spectrometry: a comparison with ion chromatography and application to product discrimination. *J. Agric. Food Chem.* **2006**, *54*, 1975−1981.

(8) Tang, K.; Liang, L.; Cai, Y.; Mou, S. Determination of sugars and alditols in tobacco with high performance anion-exchange chromatography. *J. Sep. Sci.* **2007**, *30*, 2160−2166.

(9) Švob Troje, Z.; Fröbe, Z.; Perović, Đ. Analysis of selected alkaloids and sugars in tobacco extract. *J. Chromatogr., A* **1997**, *775*, 101−107.

(10) Bourne, E. J.; Pridham, J. B.; Worth, H. G. J. Pectic substances in cured and uncured tobacco. *Phytochemistry* **1967**, *6*, 423−431.

(11) Oakley, E. T. Enzymatic determination of starch in fresh green, lyophilized green, and cured tobacco. *J. Agric. Food Chem.* **1983**, *31*, 902−905.

(12) Hall, R. A.; Wooten, J. B. Quantitative analysis of cellulose in tobacco by ^{13}C CPMAS NMR. *J. Agric. Food Chem.* **1998**, *46*, 1423−1427.

(13) French, D. Isolation and identification of planteose from tobacco seeds. *J. Am. Chem. Soc.* **1955**, *77* (4), 1024−1025.

(14) Mizuno, T.; Kinpyo, T. Studies on the carbohydrates on tobacco. Part I: Free sugars and the constructive sugars of glycosides in green leaves of Japanese bright yellow tobacco. *J. Agric. Chem. Soc. Jpn.* **1957**, *31*, 297−299.

(15) Ritsema, T.; Smeekens, S. Fructans: beneficial for plants and humans. *Curr. Opin. Plant Biol.* **2003**, *6*, 223−230.

(16) Hendry, G. A. F. Evolutionary origins and natural functions of fructans − a climatological, biogeographic and mechanistic appraisal. *New Phytol.* **1993**, *123*, 3−14.

(17) Pilon-Smits, E. A. H.; Ebskamp, M. J. M.; Paul, M. J.; Jeuken, M. J. W.; Weisbeek, P. J.; Smeekens, S. C. M. Improved performance of transgenic fructan-accumulating tobacco under drought stress. *Plant Physiol.* **1995**, *107*, 125−130.

(18) Sprenger, N.; Schellenbaum, L.; Dun, K. V.; Boller, T.; Wiemken, A. Fructan synthesis in transgenic tobacco and chicory plants expressing barley sucrose:fructan 6-fructosyltransferase. *FEBS Lett.* **1997**, *400*, 355−358.

(19) Abbott, I. R.; Matheson, N. K. Starch depletion in germinating wheat, wrinkled-seeded peas and senescing tobacco leaves. *Phytochemistry* **1972**, *11*, 1261−1272.

(20) Tabata, S.; Dohi, Y. An assay for oligo-(1→4)→(1→4)-glucantransferase activity in the glycogen debranching enzyme system by using HPLC with a pulsed amperometric detector. *Carbohydr. Res.* **1992**, *230*, 179−183.

(21) Campbell, J. M.; Bauer, L. L.; Fahey, G. C., Jr.; Hogarth, A. J. C. L.; Wolf, B. W.; Hunter, D. E. Selected fructooligosaccharide (1-kestose, nystose, and 1^F-β-fructofuranosylnystose) composition of foods and feeds. *J. Agric. Food Chem.* **1997**, *45*, 3076−3082.

(22) Hogarth, A. J. C. L.; Hunter, D. E.; Jacobs, W. A.; Garleb, K. A.; Wolf, B. W. Ion chromatographic determination of three fructooligosaccharide oligomers in prepared and preserved foods. *J. Agric. Food Chem.* **2000**, *48*, 5326−5330.

(23) Wang, J.; Sporns, P.; Low, N. H. Analysis of food oligosaccharides using MALDI-MS: quantification of fructooligosaccharides. *J. Agric. Food Chem.* **1999**, *47*, 1549−1557.

(24) Mauri, P.; Minoggio, M.; Simonetti, P.; Gardana, C.; Pietta, P. Analysis of saccharides in beer samples by flow injection with electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 743−748.

(25) Liu, Y.; Urgaonkar, S.; Verkade, J. G.; Armstrong, D. W. Separation and characterization of underivatized oligosaccharides using liquid chromatography and liquid chromatography−electrospray ionization mass spectrometry. *J. Chromatogr., A* **2005**, *1079*, 146−152.

(26) Penna, N.; Capellacci, S.; Ricci, F.; Giorgi, M.; Penna, A.; Famiglini, G.; Pierini, E.; Trufelli, H.; Cappiello, A. Study on the maltooligosaccharide composition mucilage samples collected along the northern Adriatic coast. *Carbohydr. Res.* **2009**, *344*, 120−126.

(27) Kazmaier, T.; Roth, S.; Zapp, J.; Harding, M.; Kuhn, R. Quantitative analysis of malto-oligosaccharides by MALDI-TOF mass spectrometry, capillary electrophoresis and anion exchange chromatography. *Fresenius' J. Anal. Chem.* **1998**, *361*, 473−478.

(28) Seipert, R. R.; Barboza, M.; Niñonuevo, M. R.; LoGascio, R. G.; Mills, D. A.; Freeman, S. L.; German, J. B.; Lebrilla, C. B. Analysis and quantitation of fructooligosaccharides using matrix-assusted laser desorption/ionization fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem.* **2008**, *80*, 159−165.

(29) Bahr, U.; Pfenninger, A.; Karas, M. High-sensitivity analysis of neutral underivatized oligosaccharides by nanoelectrospray mass spectrometry. *Anal. Chem.* **1997**, *69*, 4530−4535.

(30) Garozzo, D.; Giuffrida, M.; Impallomeni, G.; Ballistreri, A.; Montaudo, G. Determination of linkage position and identification of the reducing end in linear oligosaccharides by negative ion fast atom bombardment mass spectrometry. *Anal. Chem.* **1990**, *62*, 279−286.

(31) Friedmann, M. Food browning and its prevention: an overview. *J. Agric. Food Chem.* **1996**, *44*, 631−653.

(32) Shiomi, N. Food biochemical study on fructans and related synthesis enzymes. *J. Appl. Glycosci.* **2008**, *55*, 25−33.

(33) Pranznik, W.; Beck, R. H. F.; Spies, T. Isolation and characterization of sucrose:sucrose 1^F-β-D-fructosyltransferase tubers of *Helianthus tuberosus* L. *Agric. Biol. Chem.* **1990**, *54*, 2429−2431.

(34) Lüscher, M.; Hochstrasser, U.; Boller, T.; Wiemken, A. Isolation of sucrose:sucrose 1-fructosyltransferase (1-SST) from barley (*Hordeum vulgare*). *New Phytol.* **2000**, *145*, 225−232.

(35) Ghazi, I.; Fernández-Arrojo, L.; Garcia-Arellano, H.; Ferrer, M.; Ballesteros, A.; Plou, F. J. Purification and kinetic characterization of a fructosyltransferase form *Aspergillus aculeatus*. *J. Biotechnol.* **2007**, *128*, 204−211.

(36) Cairns, A. J.; Ashton, J. E. The interpretation of in vitro measurements of fructosyl transferase activity: an analysis of patterns of fructosyl transfer by fungal invertase. *New Phytol.* **1991**, *118*, 23−34.

(37) Ende, W. V. D.; Laere, A. V. Purification and properties of an invertase with surose:sucrose fructosyltransferase (SST) activity from the roots of *Cichorium intybus* L. *New Phytol.* **1993**, *123*, 31−37.

(38) Agopian, R. G. D.; Purgatto, E.; Cordenunsi, B. R.; Lajolo, F. M. Synthesis of fructooligosaccharides in banana 'Prata' ana its relation to invertase activity and sucrose accumulation. *J. Agric. Food Chem.* **2009**, *57*, 10765−10771.

(39) Straathof, A. J. J.; Kieboom, A. P. G.; Bekkum, H. V. Invertase-catalysed fructosyl transfer in concentrated solutions of sucrose. *Carbohydr. Res.* **1986**, *146*, 154−159.