Changes in the Structure of Xyloglucan of Apple Fruit during Development

Seiko Ito^{1,4}, Yasushi Mitsuishi², Toshikatsu Okuno^{3,4} and Yoji Kato^{1*}

¹Laboratory of Food Science, Faculty of Education, Hirosaki University, Hirosaki 036–8560

²National Institute of Advanced Industrial Science and Technology, Higashi, Tsukuba 305–8566

³Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036–8561

⁴The United Graduate School of Agricultural Sciences, Iwate University, Morioka 020–8550

Summary

Cell-wall materials (80% methanol-insoluble fraction) of ‘Starking Delicious’ apple fruit obtained at different developmental stages were separated into water-soluble and water-insoluble polysaccharide fractions. Crude xyloglucans, prepared from both polysaccharide fractions, were hydrolyzed with *Geotrichum* sp. M128 xyloglucanase. The xyloglucanase-hydrolyzates were subjected to high-performance anion-exchange chromatography with pulsed amperometric detection before and after digestion with *Eupenicillium* sp. M9 isoprimeverose-producing oligoxyloglucan hydrolase. Both xyloglucan preparations were composed mainly of XXG, XXXG, XXLG, XLXG, XXFG, XLLG and XLFG [where each (1 → 4)-β-linked D-glucosyl residue in the backbone is given a one-letter code according to its substituents: G = β-D-Glc; X = α-D-Xyl-(1 → 6)-β-D-Glc; L = β-D-Gal-(1 → 2)-α-D-Xyl-(1 → 6)-β-D-Glc; F = α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 2)-α-D-Xyl-(1 → 6)-β-D-Glc]. The ratios of fucose-containing oligosaccharide units (XXFG and XLFG) to fucose-noncontaining oligosaccharide units (XXG, XXXG, XXLG, XLXG and XLLG) in the water-insoluble xyloglucan molecules decreased with fruit development. The molar ratio of a pentasaccharide, XXG, in the water-soluble and -insoluble xyloglucan molecules increased with fruit development.

Key Words: apple, ‘Starking Delicious’, structural modification of xyloglucan.

Introduction

Apple fruit softens at different rates according to cultivar, growth and storage conditions. Softening of apple tissues is believed to be caused by the degradation and/or modification of cell wall polysaccharides (Fischer et al., 1994; Nara et al., 2001; Wu et al., 1993; Yoshioka et al., 1992; Yoshioka et al., 1994). The cell wall polysaccharides, especially pectic substances of apple fruit have been extensively studied during growth, development and ripening. However, little work has been done on the changes in hemicelluloses (Percy et al., 1997). In addition to the changes in pectic polymers, changes in hemicellulose structure are likely to occur during fruit development.

We have been interested in the association of xyloglucan, one of the hemicellulosic polysaccharides, with fruit development. In a previous study of the basic structure of the xyloglucans, obtained from apple fruit cell-walls of four cultivars, ‘Starking Delicious’, ‘Fuji’, ‘Ohrin’ and ‘Ralls Janet (Kokko)’, we showed that the ratios of the oligosaccharide units (XXXG, XXLG,

XLXG, XXFG, XLLG and XLFG) of xyloglucan molecules differed among the four cultivars (Kato et al., 2001b).

The present work was conducted to study the changes in the structure of xyloglucan molecules in ‘Starking Delicious’ fruit cell-walls during development.

Materials and Methods

Apple samples

‘Starking Delicious’ apple was grown at Hirosaki University, Aomori Prefecture, and picked on Aug. 1, Oct. 10, Oct. 25 and Nov. 10, 1994. Normally, ‘Starking Delicious’ apple are harvested in mid-October. The unblemished fruit was peeled, cored and cut into small pieces and blended for 10 min with 4 volumes of methanol and centrifuged. After the supernatant was decanted, the precipitate was washed with 80% methanol and acetone, and dried. From the 80% methanol-insoluble materials, the water-soluble and water-insoluble polysaccharide fractions were prepared as described previously (Kato et al., 2001a). The 80% methanol-insoluble materials were treated with a mixture of isoamylase (*Pseudomonas*, Seikagaku corporation) and glucoamylase (*Rhizopus niveus*, Seikagaku

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* Corresponding author.

corporation). The enzyme-treated 80% methanol-insoluble materials were centrifuged. The supernatant was subjected to Bio-Gel P-2 chromatography to separate glucose derived from starch and water-soluble polysaccharides. The fractions eluted at void volume of the column were collected, concentrated and freeze-dried which resulted in the water-soluble polysaccharide fraction. The precipitate was freeze-dried to yield the water-insoluble polysaccharide fraction.

Sugar composition analysis

The water-soluble polysaccharide fractions (about 0.1 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 4 hr at 100 °C. The hydrolyzates were evaporated to dryness. For the water-insoluble polysaccharide fractions, each sample (about 2.0 mg) was first dissolved in 0.5 ml of 72% sulfuric acid. The solution was then diluted with water to a final concentration of 1 N sulfuric acid, and hydrolyzed for 4 hr at 100 °C. The hydrolyzate was neutralized with barium carbonate and filtered. The filtrate was deionized with Amberlite IR-120 resins (H^+ form) to remove residual barium ions and concentrated. Alditol trifluoroacetates derived from sugars were analyzed by gas liquid chromatography on a column (0.4×200 cm) packed with 1.5% QF-1 on Chromosorb W at 140 °C (Kato and Matsuda, 1980). Uronic acid was determined by the carbazole-sulfuric acid method (Bitter and Muir, 1962).

Extraction of xyloglucan from the water-insoluble polysaccharide fractions

The water-insoluble polysaccharide fractions were extracted twice with 24% KOH for 20 hr at room temperature. The extracts were neutralized with acetic acid and dialyzed against distilled water. The dialyzate was freeze-dried to give water-insoluble xyloglucan fraction.

Iodine staining of water-insoluble xyloglucan fractions

An aqueous solution of water-insoluble xyloglucan fraction (0.5 ml, containing about 0.2 mg) was combined with 0.5 ml of 0.5% iodine and 1% potassium iodide to which 2.5 ml of 20% sodium sulfate was added while shaking. After 1 hr in the dark, the absorption of the reaction mixture at 450–800 nm was recorded in a photometer against a blank (Kato and Matsuda, 1977).

DEAE-Sephadex A-25 chromatography of the water-soluble polysaccharide fractions

Each water-soluble polysaccharide fraction was dissolved in 3 ml of 20 mM Na-acetate buffer (pH 5.0), then centrifuged to remove insoluble material. The supernatant was applied to a column (2.5×11 cm) of DEAE-Sephadex A-25 equilibrated with 20 mM Na-acetate buffer (pH 5.0) and eluted stepwise with 200 ml of the same buffer, 200 ml of 0.5 M NaCl in the same buffer, 200 ml of 1.0 M NaCl in the same buffer, and

200 ml of 0.5 M NaOH. Fractions of 5.0 ml each were collected and assayed for carbohydrate by the phenol-sulfuric acid method (Dubois et al., 1956). Tubes 3–11 were combined, dialyzed against distilled water, and freeze-dried to give the water-soluble xyloglucan fractions.

Enzymic hydrolysis of the water-soluble and -insoluble xyloglucan fractions and analysis of the hydrolyzates

For the compositional analysis of the oligosaccharide units of xyloglucans, a xyloglucan-specific endo-1,4- β -D-glucanase (xyloglucanase) from *Geotrichum* sp. M128 (Mitsuishi, 1998) and an isoprimeverose-producing oligoxyloglucan hydrolase (IPase) from *Eupenicillium* sp. M9 (Mitsuishi et al., 1992), purified, respectively, to the electrophoretically pure state, were used. Xyloglucanase, a pure xyloglucan-specific endo-1,4- β -D-glucanase, hydrolyzes xyloglucan but does not hydrolyze cellulose, carboxymethyl cellulose, or hydroxyethyl cellulose. The IPase that is highly specific for xyloglucan oligosaccharides, splits off isoprimeverose (α -D-xylopyranosyl-D-glucopyranose) units from the non-reducing end of the backbone of the substrate and can not by-pass the glucosyl residues substituted with D-Gal-D-Xyl, L-Fuc-D-Gal-D-Xyl side chains.

Each xyloglucan fraction ($1 \text{ mg} \cdot \text{ml}^{-1}$ 20 mM Na-acetate buffer, pH 5.5) was hydrolyzed at 40 °C for 24 hr with M128 xyloglucanase. A portion of xyloglucanase-treated xyloglucan fractions was subjected to high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis (Konishi et al., 1998a) and to matrix-assisted laser-desorption ionization time of the flight mass (MALDI-TOF-MS) spectrum analysis (Watanabe et al., 1999). The residual xyloglucanase-treated xyloglucan fractions ($0.5 \text{ mg} \cdot 0.5 \text{ ml}^{-1}$ 20 mM Na-acetate buffer, pH 5.5) were hydrolyzed at 40 °C for 24 hr with M9 IPase, and the resultant reaction mixture was subjected to HPAEC-PAD analysis.

HPAEC-PAD analysis

HPAEC-PAD analysis of oligosaccharides was performed on a Dionex ion chromatography system DX-300 (Dionex gradient pump, Dionex pulsed electrochemical detector with a gold working electrode and an Ag/AgCl reference electrode) interfaced to an AI-450 workstation. Separations were performed at 20 °C on a column (4×250 mm) of Dionex CarboPac PA 1 anion exchange resin with a CarboPack PA 1 guard column, using a flow rate of $1 \text{ ml} \cdot \text{min}^{-1}$. Oligosaccharides were eluted with the following NaOAc gradient profile in 100 mM NaOH, 0–50 min, 0–100 mM; 50–60 min, 100–500 mM (Konishi et al., 1998a).

MALDI-TOF-MS spectrum

An aqueous solution ($0.5 \mu\text{l}$, about $100 \mu\text{g} \cdot \text{ml}^{-1}$) of

each sample was mixed with an equal volume of 1% 2,5-dihydroxybenzoic acid in 40% acetonitrile aqueous solution. This mixture was applied to the MALDI probe and dried. MALDI-TOF-MS was recorded with a Voyager RP (PerSeptive Biosystems, USA) operating at a 20 kV accelerating voltage. Ion desorption was accomplished with a nitrogen laser ($\lambda = 337$ nm) (Watanabe et al., 1999).

Results and Discussion

The yield and sugar composition of water-soluble and -insoluble polysaccharide fractions derived from the 'Starking Delicious' apple fruit obtained at various developmental stages revealed a significant decrease in the content of water-insoluble polysaccharides (Table 1).

The water-soluble polysaccharide fractions that were individually subjected to DEAE-Sephadex A-25 (acetate form) chromatography (Fig. 1). The tracing shows the elution pattern of the water-soluble polysaccharide fraction prepared from apple fruit of Oct. 10. The unbound fraction was used as water-soluble xyloglucan fraction. The dialyzate of the water-insoluble polysaccharide fractions that were extracted with 24% KOH and analyzed by the iodine method yielded xyloglucan / 100 g FW edible portion of apple fruit of 260 mg for Aug. 1, 137 mg for Oct. 10, 116 mg for Oct. 25 and 108 mg for Nov. 10. The xyloglucan content in water-soluble xyloglucan fraction was not determined because of low yield.

The detailed structures of water-soluble and -insoluble xyloglucans isolated from the 80% methanol-insoluble fraction of apple fruit at different developmental stages were investigated by the compositional analysis of oligosaccharide units of xyloglucans. Hydrolyzates derived from the M128 xyloglucanase and M9-IPase subjected to HPAEC-PAD analysis revealed that the

retention time of peaks of the two hydrolyzates (Fig. 2). The tracings show the products of xyloglucanase-treated water-insoluble xyloglucan fraction prepared from apple fruit of Oct. 10. The molar ratio of oligosaccharide units of each xyloglucan fraction (Table 2) shows that both xyloglucan fractions are composed mainly of XXG, XXXG, XXLG, XLXG, XXFG, XLLG and XLFG. The presence of structural oligosaccharide units, XXG, XXXG, XLXG and XXLG, XXFG, XLLG and XLFG, was confirmed by the matrix-assisted laser-desorption ionization time of the flight mass (MALDI-TOF-MS) spectrum of each xyloglucanase-treated xyloglucan fraction (Fig. 3) prepared from apple fruit of Oct. 10. The results of HPAEC-PAD analysis, indicate that the signals at m/z 792.34, 1086.02, 1248.22, 1394.08, 1410.38 and 1555.97 are due to $[M + Na]^+$

Fig. 1. DEAE-Sephadex A-25 chromatography of water-soluble polysaccharide fraction from apple fruit of Oct. 10. Tubes 3–11 were combined, dialyzed against distilled water, and freeze-dried to give the water-soluble xyloglucan fractions.

Table 1. Yields and sugar composition of water-soluble and -insoluble polysaccharide fractions of 'Starking Delicious' apple fruit obtained at various developmental stages.

Harvested date	Yield (mg) ^z	Sugar composition (wt%)							
		U.A.	Rha	Fuc	Ara	Xyl	Man	Glc	Gal
<i>Water-soluble</i>									
Aug. 1	329	88.3	0.6	0.1	4.7	0.8	0.3	0.8	4.3
Oct. 1	333	90.0	0.7	0.1	3.2	1.0	0.0	1.9	3.1
Oct. 25	300	88.2	1.1	0.1	3.4	1.6	—	1.6	4.0
Nov. 10	276	86.5	1.2	0.2	4.4	1.3	—	2.6	3.8
<i>Water-insoluble</i>									
Aug. 1	1860	12.1	3.1	4.9	23.9	8.4	6.6	28.5	12.5
Oct. 10	979	15.1	1.7	2.4	18.3	9.6	3.0	39.6	10.3
Oct. 25	831	10.8	2.1	3.5	18.8	13.9	5.1	34.1	11.7
Nov. 10	771	11.2	1.0	1.7	19.1	9.5	2.2	40.9	10.3

^z /100g edible portion.

pseudomolecular ions of XXG, XXXG, XLXG, XXLG and XXFG, XLLG and XLFG, respectively.

It is known that subtle structural modifications of constituent cell-wall polysaccharides are often observed during softening (Fischer and Bennett, 1991). The hemicellulosic xyloglucan forms hydrogen bonds with cellulose microfibrils and hydrolysis of xyloglucan by endo- β -1,4-glucanase and/or xyloglucan endotrans-

glycosylase, which is responsible for cutting and re-joining intermicrofibrillar xyloglucan chains, could allow for wall-expansion (Hayashi, 1989; Nishitani and Tominaga, 1992). In addition, the removal of terminal fucose from side chains of xyloglucans in azuki bean epicotyls is thought to contribute to cell elongation through various mechanisms (Hoson, 1995). Hayashi (1989) reported that plant cell walls contain xyloglucan-hydrolyzing glycosidases, α -xylosidase, β -glucosidase, α -fucosidase and β -galactosidase.

Fucose, which is a constituent monosaccharide of xyloglucan, in the water-insoluble polysaccharide fraction decreased during fruit development (Table 1). In addition, the ratios of fucose-containing oligosaccharide units (XXFG and XLFG) to fucose-noncontaining oligosaccharide units (XXG, XXXG, XXLG, XLXG and XLLG) in the water-insoluble xyloglucan

Fig. 2. HPAEC-PAD chromatograph of enzyme-treated water-insoluble xyloglucan fraction from apple fruit of Oct. 10. A: xyloglucanase-hydrolyzate of water-insoluble xyloglucan fraction. (1)XXG, (2)XXXG, (3)XXFG, (4)XLXG, (5)XLFG and XXLG and (6)XLLG. B: IPase-hydrolyzate of xyloglucanase-treated water-insoluble xyloglucan fraction. (1)glucose, (2)isoprimeverose, (3)FG, (4)LG, (5)LXG, (6)LFG and (7)LLG.

Fig. 3. MALDI-TOF-MS spectrum of xyloglucanase-hydrolyzate of water-insoluble xyloglucan fraction from apple fruit of Oct. 10.

Table 2. Structural oligosaccharide units of xyloglucans of 'Starking Delicious' apple fruit obtained at various developmental stages.

Harvested date	Oligosaccharide units (mol %)							Ratio of fucose containing unit (%) ^z
	XXG (a)	XXXG (b)	XXLG (c)	XLXG (d)	XXFG (e)	XLLG (f)	XLFG (g)	
<i>Water-soluble</i>								
Aug. 1	8	34	11	6	17	6	18	35
Oct. 10	22	29	14	2	19	7	7	26
Oct. 25	20	29	16	4	12	8	11	23
Nov. 10	23	30	12	1	20	4	10	30
<i>Water-insoluble</i>								
Aug. 1	3	29	5	8	18	3	34	52
Oct. 10	8	33	10	9	18	3	19	37
Oct. 25	12	33	12	7	16	3	17	33
Nov. 10	10	33	11	7	18	3	18	36

^z(e + g) / (a + b + c + d + e + f + g).

decreased as the apple matured (Table 2). These results suggest that the removal of terminal fucose from side chains of xyloglucans occurs in apple fruit during development.

We have reported that most fucose-containing xyloglucans are composed of XXXG, XXLG, XLXG, XXFG, XLLG and XLFG (Konishi et al., 1998a, b; Kato et al., 1999). Present results show that the water-soluble xyloglucans contain a pentasaccharide, XXG as a major structural oligosaccharide unit, and that its molar ratio in the water-soluble and -insoluble xyloglucan molecules, especially in the water-soluble xyloglucan, increases during development, indicating that structural modifications of cell-wall xyloglucan occur during development of 'Starking Delicious' apple fruit. However, it is not certain whether the increase of XXG in xyloglucan molecules is due to hydrolysis with glycosidases, and subsequent cutting and re-joining of the intermicrofibrillar xyloglucan chains by endo- β -1,4-glucanase and/or xyloglucan endotransglycosylase. Further studies on the presence of XXG in xyloglucan polymer are required.

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リンゴ果実の生育中における xyloglucan の構造変化

伊藤聖子^{1,4}・三石 安²・奥野智旦^{3,4}・加藤陽治^{1*}¹弘前大学教育学部食物学研究室 036-8560 弘前市文京町²産業技術総合研究所 305-8566 つくば市東³弘前大学農学生命科学部 036-8561 弘前市文京町⁴岩手大学大学院連合農学研究科 020-8550 盛岡市上田

摘 要

生育時期の異なるリンゴ果実‘スターキング・デリシャス’の細胞壁を水可溶性と水不溶性多糖画分に分画した。これらの多糖画分に含まれる xyloglucan を *Geotrichum* sp. M128 由来精製 xyloglucanase を用いて分解した。xyloglucanase 分解物は、さらに、*Eupenicillium* sp. M9 由来精製 isoprimeverose-producing oligoxyloglucan hydrolase による分解前後でパルスドアンペロメトリー検出器を用いた HPLC 分析に供した。両画分の xyloglucan はいずれも, XXG, XXXG, XXLG, XLXG, XXFG, XLLG および XLFG の主要オリゴ糖単位から構成されていた (XXXG 等は Fry らによる xyloglucan オリ

ゴ糖の表示法で、主鎖の各 (1→4)-β 結合のグルコース残基の分岐様式により一文字コードで示される。G= β-D-Glc; X= α-D-Xyl-(1→6)-β-D-Glc; L= β-D-Gal-(1→2)-α-D-Xyl-(1→6)-β-D-Glc; F= α-L-Fuc-(1→2)-β-D-Gal-(1→2)-α-D-Xyl-(1→6)-β-D-Glc)。果実の発育が進むにつれて、水不溶性多糖画分の xyloglucan では、フコース含有オリゴ糖単位 (XXFG と XLFG) の減少がみられた。また、水可溶性および不溶性 xyloglucan 多糖画分構成オリゴ糖単位中で 5 糖 (XXG) が増加していた。