Isolation of Galactoglucomannan from Apple Hemicellulosic Polysaccharides with Binding Capacity to Cellulose

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Abstract: The hemicellulose fraction which is soluble in 24% KOH solution (HC-II) from the cell-walls of apple flesh contained a small amount of galactoglucomannan in addition to a large amount of xylan. A crude galactoglucomannan was isolated from the HC-II fraction with binding capacity to cellulose microfibrils. The crude galactoglucomannan fraction was treated with xylanoglucanase to remove concomitant xylan, followed by DEAE-Sephadex A-25 chromatography to give a purified galactoglucomannan fraction. Sugar composition analysis showed that the purified galactoglucomannan fraction consisted of Man, Glc and Gal in the molar ratio of 5.7 : 3.0 : 1.1. Methylation analysis suggested that a galactoglucomannan present in apple flesh had β-(1→4)-linked Glc and Man residues, some of which were branched at the O-6 position with Gal residues.

Key words: apple, galactoglucomannan, hemicellulose, binding capacity to cellulose

The plant cell wall consists of polysaccharides such as pectin, hemicelluloses and cellulose. Hemicellulose components are covalently linked to pectin and hydrogen-bonded to cellulose.¹² Xyloglucan is a hemicellulosic polysaccharide that is present in the primary cell walls of all higher plants, and is known to interact specifically with cellulose.³⁴ The interaction between cellulose and xyloglucan is thought to be involved in hydrogen bonding.

In our previous works on the cell-wall polysaccharides of apple flesh, xyloglucan was isolated from 24% KOH soluble fraction, and then the xyloglucan oligosaccharides were obtained by hydrolysis with Penicillium sp. M451 xyloglucanase.⁷⁻⁹ The constituent sugars of the xyloglucan oligosaccharides were Glc, Xyl, Gal and Fuc. Although Man was found in the 24% KOH soluble fraction from apple flesh cell walls,¹⁰⁻¹² the Man residue was not recovered in xyloglucan oligosaccharides. In addition, Vinken et al.¹⁰ showed that the neutral fraction of the 4 M KOH extract, which was mainly xyloglucan, was contaminated with Man residues. From these findings, it is likely that a mannose-containing polysaccharide is present in 24% KOH soluble polysaccharide fraction from apple flesh cell walls. In general, the mannose-containing polysaccharides require relatively strong alkali, typically 24%, for their extraction from cell walls. Therefore, it is conceivable that the mannose-containing polysaccharides also bind to cellulose with hydrogen bonds in cell walls.

Glucanmannan acts as a preventative of chronic disease¹⁰ and as a weight control agent.¹²¹³ In softwoods, glucanmannan has β-(1→4) linked Glc and Man residues as main chain with branches through β-(1→6)-Glc unit.¹⁴¹⁵ In konjac, it was reported that β-Glc-(1→4)-β-Glc residues, cellobiose, Man and mannitol units compose the main chain structure.¹⁶¹⁷ Recently, galactoglucomannan was extracted from kiwifruit cell walls, and its chemical structure was characterized.¹⁸¹⁹ In addition, the presence of glucanmannan has been described during xylanoglucan purification in persimmon.²⁰ However, in apple, there is no detailed information for glucanmannan or galactoglucomannan from apple fruit.

The polysaccharides extracted with 24% KOH from apple fruit cell wall polysaccharides contained mainly xyloglucan. Therefore, in this paper, xyloglucan was removed from the 24% KOH extracts by treatment with xylanogluca-

MATERIALS AND METHODS

Plant materials. Methanol-insoluble materials of apple flesh were fractionated into five fractions, WS (water-soluble polysaccharide), PS (pectic substance), HC (hemicellulose)-I, HC-II and CL (cellulose) fractions, by using successive extraction with water, 0.25% ammonium oxalate, 4% KOH and 24% KOH, and subsequent dialysis of the individual extracts as described previously.²¹ In this study, we used the HC-II fraction, which was composed mainly of Xyl, Glc, Ara, Gal and Man residues.

Enzymes. Cellulase was purified from a commercial enzyme preparation from Trichoderma viride as follows: Meicelase (Meiji Seika Kaisha, Ltd.) was dissolved in a 20 mM McIlvaine buffer (pH 3.0) and centrifuged. The supernatant was applied to a cellulose column (5.0×25 cm) pre-equilibrated with the same buffer. The column was washed with the same buffer (1000 mL), and adsorbed materials were eluted with distilled water. Fractions (10.0 mL) were collected and assayed for cellulase activity.

Abbreviations: Fuc, l-fucose; Ara, l-arabinose; Rha, l-rhamnose; Gal, d-galactose; Glc, d-glucose; Xyl, d-xylene; Man, d-mannose.

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activity using xyloglucan as a substrate. Cellulase activity was determined by measuring the decrease in the color of iodine-xyloglucan complex. Absorbance at 280 nm was used to monitor protein in column chromatography. The active fraction was collected and freeze-dried. The fraction, in distilled water, was chromatographed on Bio-Gel P2 (2.5×43 cm) preequilibrated with distilled water. Fractions (3.0 mL) were collected and assayed for cellulase activity. The active fraction was combined, and designated partially purified cellulase. The partially purified cellulase could hydrolyze both xyloglucan and carboxymethyl cellulose (CMC).

*Geotrichum* sp. M128 xyloglucanase (xyloglucan-specific endo-1,4-β-d-glucanase, M128XGase) was kindly provided by Dr. Y. Mitsuiishi of the National Institute of Bioscience and Human Technology, AIST. The M128XGase hydrolyzes xyloglucan but does not hydrolyze cellulose, CMC, or carboxymethyl cellulose.

**Assay for binding capacity of polysaccharide fractions to cellulose.** Assay for binding capacity was performed as follows: Each polysaccharide fraction (200 µg) was mixed with 20 mg microcrystalline cellulose in 20 mM sodium acetate buffer (pH 5.5) and incubated for 3 h at 25°C. After incubation the mixture was centrifuged and the amount of unbound total sugar present in the supernatant was determined by the phenol-sulfuric acid method. The rate of binding capacity was calculated from the difference between the amount of total sugar in the supernatant before and after incubation.

**Neutral sugar composition analysis.** Samples were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 3 h at 100°C. The hydrolyzates were evaporated to dryness to remove TFA. The sugar composition was determined by high-performance anion-exchange chromatography (HPAEC). HPAEC was done with a Dionex DX-500 system (Dionex, Sunnyvale, CA) with a pulsed amperometric detector. The column was CarboPack PA-1 (4×250 mm) with a CarboPack guard column, using a flow rate of 1 mL/min.

**Methylation analysis.** The purified polysaccharide was methylated according to the Hakomori method. The methylated polysaccharide was hydrolyzed with 2 M TFA at 120°C for 3 h, then the hydrolyzate was evaporated to remove the acid. Sugars were reduced, and converted to alditol acetates. The partially methylated alditol acetates were analyzed by GLC on a DB-225 fused silica capillary column (30 m×0.32 mm: J&W Scientific, Folsom, CA) as in a previous paper.

**RESULTS AND DISCUSSION**

**Presence of galactoglucomannan in the cell-walls of apple flesh.**

Methanol-insoluble materials of apple flesh were fractionated into five fractions, WS, PS, HC-I, HC-II and CL fractions, by using successive extraction with water, 0.25% ammonium oxalate, 4% KOH and 24% KOH, and subsequent dialysis of the individual extracts as described previously. Neutral sugar composition analysis suggested that the HC-II fraction was composed of Fuc, Ara, Rha, Gal, Glc, Xyl and Man in the molar ratio of 3.1 : 16.4 : 1.9 : 13.8 : 33.9 : 25.8 : 5.0, respectively. The results indicated that the major polysaccharide in HC-II was xyloglucan as described previously. In addition, it is conceivable that the HC-II fraction involved a mannose-containing polysaccharide because the fraction has a relatively higher amount of Man residue (5.0 mol%) as a constituent monosaccharide.

Isolation of mannose-containing polysaccharide from a mixture of hemicellulosic polysaccharides in HC-II fraction is performed conventionally by graded ethanol precipitation, or by complexing with Fehling’s solution. However, attempts to isolate a mannose-containing polysaccharide from HC-II fraction by these methods were unsuccessful. In previous papers, it was shown that HC-II fraction obtained from the cell-walls of apple flesh was mainly composed of xyloglucan which consisted of Fuc, Gal, Xyl and Glc. Xyloglucan polymer is known specifically to bind cellulose.

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![Fig. 1](image-url) The binding capacity to microcrystalline cellulose of HC-II and the enzymic digest of HC-II.

The binding assays were carried out under the conditions described in MATERIALS AND METHODS. The bars represent the standard deviation. **HC-II was treated by *Geotrichum* sp. M128 xyloglucanase. **HC-II was treated by the partially purified cellulase from *Trichoderma viride.*
Isolation and preliminary characterization of galactoglucomannan.

On the basis of the above results, the isolation of galactoglucomannan from the HC-II was carried out. The HC-II fraction (100 mg) obtained from the cell-walls of apple flesh was suspended in 20 mL of 20 mM Na-acetate buffer (pH 5.5) and treated with M128XGase. The M128 XGase-treated HC-II was resolved into supernatant (hydrolyzed materials: low molecular weight, yield 62.4 mg) and precipitate (unhydrolyzed materials: high molecular weight, yield 31.7 mg) fractions by the methanol fractional precipitation method. Both fractions were dried and subjected to the binding assay. The binding activity was detected in the precipitate (unhydrolyzed materials) and not in the supernatant (hydrolyzed materials) [Fig. 2-(A)]. Therefore the precipitate fraction (25.0 mg) was then subjected to a chromatography on a column (2.5×20 cm) of DEAE-Sephadex A-25 preequilibrated with 20 mM Na-acetates buffer (pH 5.5). After the column was eluted with the same buffer (300 mL), the bound fraction was eluted using 300 mL of 0.5 M NaCl in the same buffer. The unbound and bound fraction containing the polysaccharides were designated as HC-II-1 and HC-II-2, respectively. The HC-II-1 and HC-II-2 fractions were obtained in the ratio of 1.2 : 1.0. Neutral sugar composition analysis showed that HC-II-1 and HC-II-2 were composed of Fuc, Ara, Rha, Gal, Glc, Xyl and Man in the molar ratio of 0.9 : 0.9 : 0.2 : 10.4 : 33.3 : 6.7 : 47.5 and of 0.3 : 7.4 : 19.5 : 19.1 : 17.1 : 0.7, respectively (Table 2). Furthermore the high binding capacity to microcrystalline cellulose was compared between HC-II-1 and HC-II-2. The former was about 70% whereas the latter was about 7% [Fig. 2-(B)]. It is clear that the high binding capacity of HC-II-1 is due to a mannose-containing polysaccharide. However HC-II-1 still contained a small amount of Fuc, Ara, Rha and Xyl in addition to Gal, Glc and Man. These monosaccharides may be derived from xyloglucan and acidic polysaccharide contaminant with a mannose-containing polysaccharide. To remove additional xyloglucan polysaccharide, therefore, the HC-II-1 (11.4 mg) was treated with M128XGase again. The M128XGase-treated HC-II-1 was separated into supernatant (oligoxyloglucans) and precipitate (polysaccharides unhydrolyzed with M128XGase) fractions by the methanol fractional precipitation method. The precipitate was resuspended in distilled water, dialyzed and freeze-dried to give purified HC-II-1. The purified HC-II-1 corresponds to about 8% of HC-II. Neutral sugar composition analysis of the purified HC-II-1 showed that it consisted of Man, Glc, Gal, Ara and Xyl in the molar ratio of 57.0 : 30.1 : 11.1 : 0.9 : 0.9. Ara and Xyl are considered to be due to concomitance with a polysaccharide like arabinoxylan. The ratio of Man and Glc of glucomannan obtained from the bulbs of daffodil is reported to be about 3 : 2.29 In addition, galactoglucomannan of kiwifruit is known to be composed of Man, Glc and Gal in the ratio of 2 : 2 : 1.30 So it could be concluded that the purified HC-II-1 is a galactoglucomannan (GGM).

### Table 1. Total sugar content and neutral sugar composition of the unbound material obtained in the binding assay.

<table>
<thead>
<tr>
<th>No</th>
<th>Sample</th>
<th>Total sugar content (mg)</th>
<th>Neutral sugar composition (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fuc</td>
</tr>
<tr>
<td>1</td>
<td>HC-II</td>
<td>200.0</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>HC-II + cellulose</td>
<td>135.3</td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>HC-II (XGase)</td>
<td>169.5</td>
<td>3.1</td>
</tr>
</tbody>
</table>

*HC-II was treated with Geotrichum sp. M128 xyloglucanase. *HC-II was treated with the partially purified cellulase from Trichoderma viride. *After incubation of a mixture of cellulose microfibrils and each polysaccharide, the mixture was centrifuged and the total sugar present in the supernatant was determined by the phenol-sulfuric acid method.

### Table 2. Neutral sugar composition of HC-II-1 and HC-II-2 obtained by DEAE-Sephadex A-25 chromatography.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Neutral sugar composition (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fuc</td>
</tr>
<tr>
<td>HC-II-1</td>
<td>0.9</td>
</tr>
<tr>
<td>HC-II-2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Fig. 2. The binding capacity to microcrystalline cellulose.

(A) The precipitate (ppt.) and supernatant (sup.) fractions were separated from the digest of M128XGase by the methanol fractional precipitation method. (B) The precipitate fraction was separated into HC-II-1 and HC-II-2 fractions by DEAE-Sephadex A-25. The binding assays were carried out under the conditions described in MATERIALS AND METHODS. The bars represent the standard deviation.
Table 3. Glycosidic linkage analysis of the purified galactoglucomannan (GGM) from HC-II in apple flesh cell walls.

<table>
<thead>
<tr>
<th>Linkage*</th>
<th>Amount % total area</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Glc and/or T-Man</td>
<td>4.9</td>
</tr>
<tr>
<td>4-Man</td>
<td>46.3</td>
</tr>
<tr>
<td>4,6-Man</td>
<td>7.2</td>
</tr>
<tr>
<td>4-Glc</td>
<td>27.5</td>
</tr>
<tr>
<td>4,6-Glc</td>
<td>3.3</td>
</tr>
<tr>
<td>T-Gal</td>
<td>4.9</td>
</tr>
<tr>
<td>2-Gal, 4-Gal and/or 6-Glc</td>
<td>1.6</td>
</tr>
<tr>
<td>6-Gal</td>
<td>4.3</td>
</tr>
</tbody>
</table>

*The numerical prefixes represent the carbon atoms involved in glycosidic linkages in the original polysaccharides. Prefix T indicates sugars linked through C(O)-1 only.

The methylation analysis was carried out to investigate the sugar linkage composition of apple GGM. A summary of the identified derivatives present in apple GGM is shown in Table 3. The result shows that 4-linked Man and 4-linked Glc form an important part of this polysaccharide, suggesting that apple GGM contains a backbone chain consisting of 4-linked Man and 4-linked Glc. Additionally, apple GGM contains 4,6-linked Man, 4,6-linked Glc, and terminal-, 6-linked and 2-linked Gal residues. This suggests that a galactoglucomanann present in apple flesh has a backbone chain consisting of \( \beta-(1\rightarrow4) \)-linked Glc and Man residues, some of which were branched at the O-6 position with Gal residues. In kiwifruit galactoglucomannan,\(^6\) the structural characterization of side chains obtained by enzymatic hydrolysis showed the presence of single Gal residues and the disaccharide \( \beta \)-Gal-(1\ \rightarrow\ 2)-\beta-Gal(-). Therefore, we consider that several potential side chains [for example, single Gal residues, \( \beta \)-Gal-(1\ \rightarrow\ 2)-\beta-Gal(-) residues and \( \beta \)-Gal-(1\ \rightarrow\ 6)-\beta-Gal(-)residues] are present in apple GGM.

It is well known that xyloglucan polysaccharide is highly associated with the cellulose microfibrils forming a rigid network structure.\(^9\) In addition, xyloglucan is one of the load-bearing components in the primary cell walls where it is involved in cross-linking with cellulose microfibrils. And this cross-linking is considered to be the major factor controlling the rate of cell expansion.\(^10\) In our study, GGM was isolated from the apple cell-walls by extraction with a high concentration of alkaline solution, 24% KOH. This means that in apple flesh GGM is highly associated with the cellulose microfibrils. In fact, apple GGM is shown to have binding capacity to cellulose microfibrils. Based on these facts, it is expected that GGM has an important role in the controlling of cell expansion, such as xyloglucan.

Moreover, it was considered that the method using the affinity to cellulose in this study was effective for the isolation of galactoglucomannan from HC-II. Therefore, it seems to be possible to examine the detailed structure of apple GGM in future.

We thank Dr. Y. Mitsuishi of the National Institute of Bioscience and Human Technology, AIST, for supplying purified xyloglucanase.

REFERENCES


