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Structural features of arabinogalactan–proteins from the fruit of *Lycium chinense* Mill.

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Abstract

In addition to the other arabinogalactan–proteins (AGPs) (Cp-1-C and -D) already reported, two kinds of AGP (Cp-2-B and Hp-2-C) were obtained from the fruit of *Lycium chinense* Mill. The ratio of arabinose to galactose was ~1:1 in both samples, and the carbohydrate was linked O-glycosidically to serine in Cp-2-B, and to both serine and threonine residues of the protein in Hp-2-C. The weight-average molecular weight was 71,000 for Cp-2-B and 120,000 for Hp-2-C. Both samples also contained non-reducing terminal 3-*O*- and 4-*O*-substituted galacturonic acids. The ratio of 6-*O*-substituted galactose (linear part) and 3,6-di-*O*-substituted galactose (branching point) was almost unity in both samples, being obviously different from the case of Cp-1-C (predominant in the branching domain) and Cp-1-D (predominant in the linear domain). These results offer fresh insight into the grouping of the AGPs, based on the ratio of 6-*O*- and 3,6-di-*O*-substituted galactosyl residues.

Keywords: Arabinogalactan–protein; *Lycium chinense* Mill.

1. Introduction

We previously reported[1] that the polysaccharide extracted with cold water from the fruit of *Lycium chinense* Mill. was separated into three main fractions (Cp-1, -2, and -3) by DEAE-cellulose column chromatography (HCO_3^- form), and Cp-1 and Cp-2 were presumed to be composed mainly of arabinogalactan–protein (AGP), and Cp-3 pectin. A hot-water soluble polysaccharide fraction obtained from the residue after cold-water extraction, was also separated into three main fractions (Hp-2, -3, and -4) by the same ion-exchange chromatography, and Hp-2 was also presumed to contain mainly AGP, and Hp-3 and -4 pectin.[1] In a subsequent paper,[2] Cp-1 was reported to be a mixture of four kinds of polysaccharide, two of which were tentatively identified as arabinoxylan (1:1 Ara:Xyl, Cp-1-A) and arabinan (Cp-1-B), and the others (Cp-1-C and -D) were clarified as being AGP. Here we report characterization of the AGPs (Cp-2-B from Cp-2 and Hp-2-C from Hp-2), and offer a fresh insight into a grouping of the AGP (type II) through the discussion that includes Cp-1-C and -D, which were reported previously.[2]

2. Experimental

Materials.—A cold-water-soluble polysaccharide from the fruit was fractionated as

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shown in Fig. 1 by DEAE-cellulose column chromatography (HCO_3^- form)[1] and the fraction (Cp-2) eluted with 0.2 M NH_4HCO_3 was collected. The residue after cold-water extraction was treated with water in an autoclave (120 °C) for 1 h. A hot-water-soluble polysaccharide was also fractionated on the same column as just mentioned to yield the elution profile shown in Fig. 1.[1] The fraction (Hp-2) eluted with 0.2 M NH_4HCO_3 was collected.

General methods.—Total sugar was determined by the phenol–H_2SO_4 method,[3] and galacturonic acid by the carbazole–H_2SO_4 method.[4] A standard curve was calibrated with a 2:2:1 mixture of arabinose–galactose–galacturonic acid, and galacturonic acid. Protein content was estimated by a modified Lowry method.[5] Commercially available bovine serum albumin was used as a reference protein. Glycosyl residues (neutral sugar) of the polysaccharide were analyzed by GLC as alditol acetates after acid hydrolysis.[6] The ratio of neutral sugars was calculated from the GLC peak areas. Specific rotations were measured at rt with a Horiba polarimeter SEPA-300.

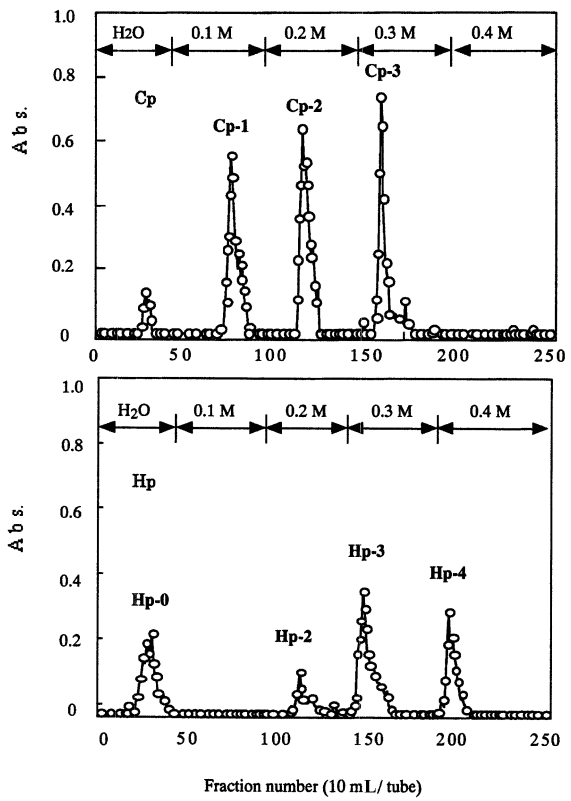

Fig. 1. Elution profiles of the polysaccharides from *L. chinense* Mill. On a DEAE–cellulose column (HCO_3^- form). The crude polysaccharides (300 mg) were subjected to chromatography on a DEAE–cellulose column (4.0 × 50 cm, HCO_3^- form). After washing with distilled water, the column was eluted with 0.1–0.4 M NH_4HCO_3 solution. The eluate was collected in 10-mL fractions. Cp, the polysaccharide extracted with cold water. Hp, the polysaccharide extracted with hot water.

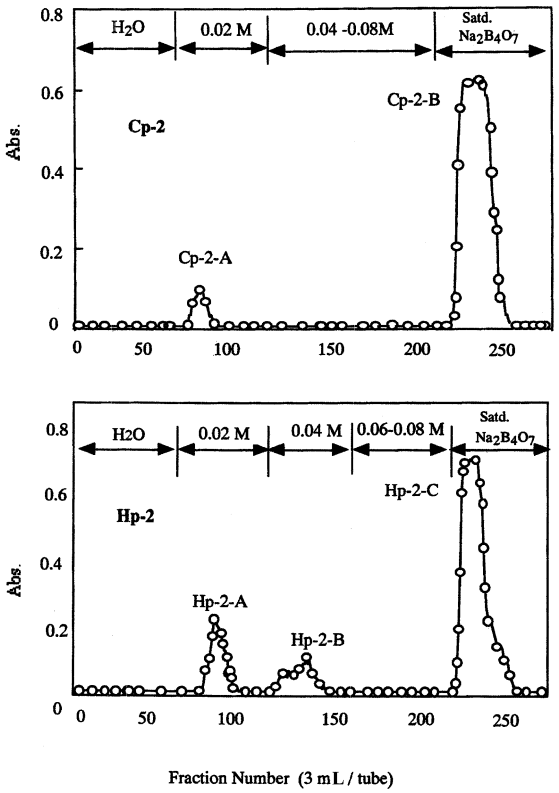

Fig. 2. Elution profiles of Cp-2 and Hp-2 on a DEAE–cellulose column ($B_4O_7^{2-}$ form) Cp-2 and Hp-2 (50 mg each) were subjected to chromatography on a DEAE–cellulose column (2.6 × 50 cm, $B_4O_7^{2-}$ form). After washing with distilled water, the column was eluted successively with 0.02–0.08 M and saturated solution of $Na_2B_4O_7$. Fractions (3 mL/tube) were collected and analyzed for total sugar. (Cp-2 and Hp-2: see Fig. 1).

Gas-liquid chromatography (GLC).—GLC was carried out on a Shimadzu GC-18A apparatus equipped with a flame ionization detector. A capillary column of CBP-10-M25-025 (0.2 mm × 25 m) was used and operated at 220 °C with a gas flow rate of 60 mL/min of nitrogen. Peak areas were measured with a Shimadzu Chromatocorder-21.

Gas chromatography–mass spectrometry (GC–MS).—GC–MS was conducted with a Shimadzu GC–MS QP-5000 apparatus with a class 5000 mass data system. A methyl silicon DB-1 capillary column (30 m × 0.25 mm × 1

μm, J&W Scientific) was used. The column temperature was programmed first at 150 °C for 2 min, and then raised to 250 °C at 5 °C/min. The spectra were recorded at an ionizing potential of 70 eV.

High-performance liquid chromatography (HPLC).—Liquid chromatography at ~7.4 Mpa (HPLC) was performed in a JASCO apparatus (880-PU pump, 860-CO column oven, and 830-RI detector). To estimate the molecular weight of the polysaccharide, a Shodex OHpak KB-805 (8 × 300 mm) column was used and operated at 45 °C with a flow rate of 0.1 M NH_4HCO_3 at 0.8 mL/min. The peak areas were estimated with a System Instrument SIC Chromatocorder 11.

Amino acid analysis.—Amino acid residues of the protein were determined after acid hydrolysis (6 M HCl, 110 °C, 16 h, N_2 atmosphere) using a Millipore LC-module 1 analysis system equipped with a column of Pico-Tag (3.9 × 150 mm, Waters Co.).

NMR spectroscopy.—^1H and ^{13}C NMR spectra were recorded at 399.956 (^1H) and 100.578 MHz (^{13}C) with a Varian FT-NMR Unity Inova 400 instrument. Chemical shifts are given in δ values.

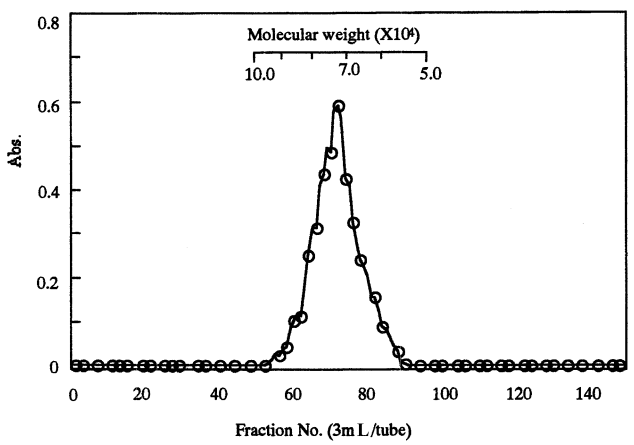

Fig. 3. The molecular weight distribution of Cp-2-B on a Sephacryl (S-300) column Cp-2-B (20 mg) was dissolved in a 0.2 M NH_4HCO_3 and the solution placed on a Sephacryl (S-300) column (2.6 × 90 cm). The column was eluted with the same solvent, and the eluate was collected in 3-mL fractions and analyzed for sugar. Dextran T-40 (mol wt 43,500), T-70 (mol wt 70,000) and T-110 (mol wt 105,000), products of Pharmacia Fine Chemicals, were used to calibrate molecular weight.

3. Results and discussion

Isolation of arabinogalactan–proteins from Cp-2 and Hp-2.—Samples (Cp-2 or Hp-2, 50 mg each) were submitted to DEAE-cellulose column (2.6 × 50 cm, $B_4O_7^{2-}$ form) chromatography, monitored by the phenol–H_2SO_4 method. The column was eluted with water, and then stepwise with 0.02, 0.04, 0.06, 0.08 M and saturated $Na_2B_4O_7$ solution at a flow rate of 30 mL/h. As shown in Fig. 2, Cp-2 gave two polysaccharide fractions (Cp-2-A eluted with 0.02 M $Na_2B_4O_7$, yield 13%, and Cp-2-B eluted with $Na_2B_4O_7$ saturated solution, yield 87%), and Hp-2 three fractions (Hp-2-A eluted with 0.02 M, yield 15.6%, Hp-2-B with 0.04 M $Na_2B_4O_7$, yield 8.0%, and Hp-2-C eluted with $Na_2B_4O_7$ saturated solution, yield 76.4%). Each fraction, after dialysis and lyophilization, was dissolved in 0.1 M NH_4HCO_3 and subjected to HPLC for molecular weight estimation in the same way as mentioned previously.[2] The column was standardized with authentic dextrans from Pharmacia Fine Chemicals. The elution profiles showed single peaks, and the molecular weights were calculated to be 89,000 (Cp-2-A), 71,000 (Cp-2-B), 8000 (Hp-2-A), 11,000 (Hp-2-B), and 120,000 (Hp-2-C). Some other properties of each fraction are shown in Table 1. Arabinose and galactose were detected as the predominant constituents in each fraction. Small proportions of fucose, xylose, mannose, rhamnose, and glucose were also found in the fractions. Since the result was very similar to that of Cp-1-C and -D reported in the previous paper,[2] the high-yield fractions, Cp-2-B and Hp-2-C, were studied in detail; the low-yield fractions were not examined further.

Molecular weight distribution of Cp-2-B and Hp-2-C.—Cp-2-B (and Hp-2-C) (~20 mg) were subjected column (2.6 × 90 cm) chromatography on to Sephacryl (S-300) standardized with dextrans to determine the molecular-weight distribution and the possibility of contamination by other polysaccharide. The solvent used was 0.2 M NH_4HCO_3. Both samples gave a symmetrical peak, when monitored by the phenol–H_2SO_4 method (Fig. 3). The molecular-weight distribution was 56,000–98,000 (for Cp-2-B) and 93,000–

Table 1
Some properties of each fraction isolated from Cp-2 and Hp-2

	Cp-2-A	Cp-2-B	Hp-2-A	Hp-2-B	Hp-2-C
Yield [a] (g/kg)	0.3	1.9	0.3	0.2	1.7
$[\alpha]_D$ ($c = 0.2$, H_2O)		−35.0	−10.0		−23.0
M_w ($\times 10^4$)	8.9	7.1	0.8	1.1	12.0
Protein [b]	11.7	12.5	12.1	10.1	9.3
Galacturonic acid [b]	13.3	11.9	2.9	7.3	24.2
Neutral sugars [b]	71.0	73.6	77.8	75.1	63.3
Arabinose [c]	50.6	45.5	70.6	84.2	49.5
Galactose [c]	22.8	47.4	13.5	10.7	40.8
Fucose [c]	3.0	2.4	4.3		5.9
Xylose [c]	3.7	0.4	3.3	2.1	3.8
Mannose [c]	8.4	1.8	1.8		
Rhamnose [c]	5.9	1.9	1.9		
Glucose [c]	5.6	0.5	4.5	3.1	0.5

[a] Yield to the dried fruit.
[b] Percent dry-weight basis.
[c] Mol %.

158,000 (for Hp-2-C). The fractions giving these peaks had almost equivalent ratios of arabinose to galactose and protein-content to carbohydrate-content. This suggests that Cp-2-B and Hp-2-C are not contaminated with other polysaccharides or protein.

Linkage analysis of Cp-2-B and Hp-2-C by methylation.—Before methylation of the sample, the galacturonic acid residues ($\sim$12% in Cp-2-B and 24% in Hp-2-C, Table 1) were reduced to galactose residues by the method of Taylor and Conrad.[7] In order to distinguish galactose residue thus generated from the native ones, $NaBD_4$ was used as the reductant instead of $NaBH_4$. The native (and carboxyl-reduced) sample (5 mg of each) was repeatedly methylated by a modified Hakomori method.[8] The per-*O*-methylated polymer was completely hydrolyzed with 90% formic acid and then 0.25 M sulfuric acid, and the product reduced with $NaBH_4$, acetylated, and submitted to GLC and GC–MS analyses. As shown in Table 2, seven peaks for Cp-2-B and eight for carboxyl-reduced Cp-2-B were identified by retention times and mass spectra. In the case of Hp-2-C, there were eight and nine peaks, respectively. The ratio of methylated arabinose to galactose was almost 1:1 (45.7:47.8 in Cp-2-B and 48.4:45.3 in Hp-2-C), supporting the results of glycosyl residue analysis (45.5:47.4 and 49.5:40.8, respectively, Table 1). Almost 50% (23.6:45.7, in Cp-2-B) and 30% (15.6:48.4, in Hp-2-C) of the arabinose was found to occur at the non-reducing terminal unit in the furanose form and the remainder was linked through O-3 or O-5, the ratio of O-3 and O-5 linkage being $\sim$1:2 in both samples. The value of 1:2 is different from that found for Cp-1-C and -D, where it was $\sim$1:1. Di-*O*-substituted arabinofuranose occurred only in Hp-2-C. The ratio of 3-*O*-substituted galactose to total galactose in Cp-2-B was $\sim$21% (10.2:47.8), and this is very similar to that in Cp-1-C (22% 5.5:24.5) and -D (21% 9.4:44.6),[2] but different from that for Hp-2-C (14% 6.4:45.3). The percentages of 6-*O*- and 3,6-di-*O*-substituted galactose in Cp-2-B are 27% (13.1:47.8) and 38% (18.3:47.8). These were very similar to those for Hp-2-C [27% (12.2:45.3) and 41% (18.5:45.3), respectively], but somewhat different from those for Cp-1-C [24% (5.8:24.5) and 54% (13.2:24.5)], and -D [57% (25.5:44.6) and 22% (9.7:44.6)].[2] Moreover, the non-reducing terminal group in Cp-1-C and -D was only arabinose, whereas both arabinose and galactose occurred in Cp-2-B (Ara/Gal = 3.8:1.0) and Hp-2-C (Ara/Gal = 1.9:1.0).

In carboxyl-reduced Cp-2-B, another peak was detected in addition to the peaks already mentioned. It was identified by GC–MS analysis as the alditol acetate of 2,3,6-tri-*O*-methyl-D-galactose-6,6-d_2. A specific fragment-ion peak of 235 (instead of a peak at

Table 2
Methylation analysis of arabinogalactan–proteins isolated from the fruit of *L. chinense* Mill.

Methylated alditol acetates	Mol %					
	Cp-1-C [a]	Cp-1-D [a]	Cp-2-B	Cp-2-B [d]	Hp-2-C	Hp-2-C [d]
2,3,5-Me_3-L-Ara [b]	34.3	27.8	23.6	18.9	15.6	12.8
2,5-Me_2-L-Ara	17.8	11.6	7.1	5.1	7.9	6.3
2,3-Me_2-L-Ara	14.6	10.0	15.0	11.4	17.7	15.8
3-Me-L-Ara					7.2	5.6
Total	66.7	49.4	45.7	35.4	48.4	40.5
2,3,4,6-Me_4-D-Gal[c]			6.2	4.2	8.2	5.7
6,6-d_2-2,3,4,6-Me_4-D-Gal				1.1		4.4
2,4,6-Me_3-D-Gal	5.5	9.4	10.2	9.6	6.4	3.2
6,6-d_2-2,4,6-Me_3-D-Gal				2.8		10.5
6,6-d_2-2,3,6-Me_3-D-Gal				8.9		8.9
2,3,4-Me_3-D-Gal	5.8	25.5	13.1	11.2	12.2	10.2
2,4-Me_2-D-Gal	13.2	9.7	18.3	16.2	18.5	12.8
Total	24.5	44.6	47.8	54.0	45.3	55.7

[a] Ref. 2.
[b] 2,3,5-Me_3-L-Ara: 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-L-arabinitol, etc.
[c] 2,3,4,6-Me_4-D-Gal: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol, etc.
[d] Cp-2-B and Hp-2-C: carboxyl-reduced material.

233) was found in the spectrum, and it was altogether similar to that of the non-deuterated derivative. The alditol acetates of 2,3,4,6-tetra- and 2,4,6-tri-*O*-methyl-D-galactose were detected and identified by GC–MS. Some of them were deuterated at C-6. The ratio of deuterated derivatives was calculated from the intensity-ratio of the specific fragment-ions. It was 0.26:1.00 (m/z 207:205) for the non-reducing units and 0.29:1.00 (m/z 163:161) for 3-*O*-substituted units. The ratio of non-reducing terminal D-galacturonic acid to non-reducing terminal D-galactose was also confirmed from another specific fragment-ion; 0.25:1.00 (m/z 163:161). Carboxyl-reduced Hp-2-C was also analyzed in the same way as for Cp-2-B and the results are shown in Table 2. The ratio of non-reducing terminal, 3-*O*- and 4-*O*-substituted D-galacturonic acids is thus 1.0:2.5:8.1 (1.1:2.8:8.9) in Cp-2-B and 1.0:2.4:2.0 (4.4:10.5:8.9) in Hp-2-C (Table 2). The ratio of arabinose, galactose and galacturonic acid (as methylated derivatives) is 1.0:1.1:0.4 [35.4:54.0 $-(1.1+2.8+8.9)$:$(1.1+2.8+8.9)$], and this is very close to that of the native polysaccharide [1.0:1.0:0.3 (45.5:47.4:11.9, Table 1)]. In the case of Hp-2-C, the ratio was 1.0:0.8:0.6 [40.5:55.7 $-(4.4+10.5+8.9)$:$(4.4+10.5+8.9)$] for the methylated sample and 1.0:0.9:0.5 (49.5:40.8:24.2) for the native one. The coincidence of these ratios indicates the completion of carboxyl-reduction and methylation of the polysaccharide. It is well known[9,10] that non-reducing terminal and 4-*O*-substituted galacturonic acid residues occur in the molecule of AGP. The presence of 3-*O*-substituted galacturonic acid has also been noted in the arabinogalactan from Azuki bean.[11] This is the first time to have detected the occurrence of three kinds of galacturonic acid residue (non-reducing terminal, 3-*O*-, and 4-*O*-substituted) in a polysaccharide.

The ratio of 6-*O*- and 3,6-di-*O*-substituted galactoses is 1.0:1.4 (13.1:18.3, Table 2) in Cp-2-B and 1.0:1.5 (12.2:18.5) in Hp-2-C. This ratio for Cp-1-C and -D is 1.0:2.3 (5.8:13.2) and 1.0:0.4 (25.5:9.7), respectively. These results may suggest that the $(1\rightarrow6)$ linkage of galactose is formed preferentially at the 3-*O*-substituted galactosyl residue, yielding AGP (Cp-1-C) and being predominant in the branching domain, and preferably at a non-reducing terminal residue, yielding the product (Cp-1-D) predominant in the $(1\rightarrow6)$-linked linear domain, and almost evenly at both residues, yielding products (Cp-2-B and Hp-2-C) almost equal in quantity for both domains. AGPs from the fruit may thus be divided into

three groups as regards the ratio of galactosyl residues involved in the branching domain and the linear one: (1) AGP being predominant in the branching domain (Cp-1-C); (2) one being predominant in the (1→6)-linked linear domain (Cp-1-D); and (3) one being almost equal in quantity for both domains (Cp-2-B and Hp-2-C). Most of the AGP reported hitherto[9,10,12–14] may be classified into group 1, with the exception of AGP-I and -III from radish root,[14] which are placed in group 3. AGPs in group 2 have never been reported. It is interesting that the fruit of *L. chinense* Mill. contains the AGP (Cp-1-C) placed in group 2.

Carbohydrate–protein linkage analysis by reductive alkaline degradation.—Cp-2-B and Hp-2-C contained 12.5 and 9.3% of protein, respectively, and both were presumed to be glycoprotein. To determine which amino acid residue bore the carbohydrate, each sample was subjected to reductive alkaline degradation followed by amino acid analysis as noted in the previous paper.[2] The results of amino acid analysis for the native and degraded proteins are shown in Table 3. Hydroxyproline, serine, proline, glutamic acid, glycine, and alanine were major amino acids in both samples, being very similar to Cp-1-C and -D.[†] The decrease in mole percentage of serine [3.61 (12.35 − 8.74)] in Cp-2-B is almost equal to the increase in that of alanine [3.55 (15.48 − 11.93)] caused by the reductive alkaline degradation. Similar decreases [5.22 (14.52 − 9.30)] and increases [6.28 (14.42 − 8.14)] were perceived for Hp-2-C. This suggests that the carbohydrate is linked O-glycosidically to the serine residue of the protein. The data for Cp-2-B indicates that ~29.2% [(12.35–8.74:12.35) of the serine residue is bound to carbohydrate. This is much higher than that of Cp-1-C (~24.3%)[‡] and -D (~14%).[‡] The value for Hp-2-C is 36% [(14.52–9.30):14.52]. The data indicate, moreover, that a carbohydrate–protein link to a threonine residue accounts for ~33% [(6.24–4.17):6.24] of all threonine in Hp-2-C. The mole percentage of amino acid(s) linked to carbohydrate are thus 3.61% in Cp-2-B, and 7.29% [5.22 for serine + 2.07 (6.24:4.17) for threonine] in Hp-2-C. These values for Cp-1-C and -D are 6.86% (4.69 for serine + 2.17 for threonine)[‡] and 2.56%[‡]. However, these values may vary a little, since the possibility of an alkali-resistant linkage of AGP to hydroxyproline cannot be ruled out.[12] These percentages suggest that AGPs in the fruit may be divided to two groups, namely a low percentage (2–4%) group (Cp-1-D and Cp-2-B) and a high percentage (6–8%) group (Cp-1-C and Hp-2-C).

^1H and ^{13}C NMR analysis.—The spectra were recorded at room temperature in D_2O. Sodium 4,4-dimethyl-4-silapentanoate-2,2,3,3-d_4 was used as the internal standard. The data were almost the same in both samples. In the ^1H NMR spectrum of Hp-2-C, signals at 5.27, 5.21, 5.16, and 5.11 were assigned to anomeric protons of α-L-arabinofuranosyl residues, and signals at 4.45, 4.48, 4.50, and 4.53 to β-D-

Table 3
Amino acid analysis of native and reductive alkaline degraded Cp-2-B and Hp-2-C

Amino acid	Mol %			
	Cp-2-B	Cp-2-B [a]	Hp-2-C	Hp-2-C [a]
Asp	6.12	6.46	6.40	6.66
Glu	10.51	10.70	7.84	7.93
Hyp	16.30	15.62	15.28	14.52
Ser	12.35	8.74	14.52	9.30
Gly	12.61	12.83	10.30	11.14
His	1.20	0.83		
Arg	1.58	1.82	5.52	5.48
Thr	6.67	6.51	6.24	4.17
Ala	11.93	15.48	8.14	14.42
Pro	7.52	8.02	9.32	9.16
Tyr	0.69	0.79	1.19	0.90
Val	3.26	3.61	4.87	4.73
Met	1.34	1.04	3.38	3.28
Cys	0.19		0.10	0.10
Ile	2.47	2.24	2.30	2.12
Leu	2.92	3.26	2.51	3.05
Phe	1.50	1.56	1.01	1.04
Lys	1.20	1.22	1.10	1.25
Total	100.00	100.00	100.00	100.00

[a] Reductive alkaline degraded sample.

[†] The presence of hydroxyproline was not noted during amino acid analysis in a previous paper (Cp-1-C and Cp-1-D). Reexamination revealed the presence of hydroxyproline and its ratio to serine (mol/mol) was found to be 0.83:1.00 (for Cp-1-C) and 1.20:1.00 (for Cp-1-D).

[‡] This is based on the revised data.

galactopyranosyl residues. Signals at 5.01, 4.98, and 4.96 were attributed to β-D-galacturonic acid residues. In the ^{13}C NMR spectrum of Hp-2-C, signals at 112.1, 110.5, 110.2 and 107.2 were assigned to anomeric carbons of α-L-arabinofuranosyl residues.[2,10,13] Signals of the anomeric carbon atom of β-D-galactopyranosyl[2,13] (and β-D-galacturonic acid[16–18]) residues were recognized at 103.6 to 106.7 (and 100.5 to 101.0). The results indicate the presence of four kinds of arabinose and galactose, and three kinds of galacturonic acid. This is consistent with the result of methylation analysis and with other AGPs (type II).[10,13,15,19,20] Although a variation of AGPs (grouping as already mentioned) was found by methylation studies, it was difficult to be recognize in the spectra. Such heterogeneity of AGPs may indicate that structurally different molecules arise from the cell in different growing-stages, and that they play different roles in the cell.

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