Isolation and Characterization of Arabinogalactan-protein from the Fruit of *Lycium chinense* Mill

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Two kinds of arabinogalactan-protein (Cp-1-C and -D) were obtained from the fruit of *Lycium chinense* Mill. In Cp-1-C, the carbohydrate was composed of arabinose and galactose at a ratio of 3:1, and *O*-glycosidically linked to both the serine and threonine residues of protein. Its molecular weight was 42,000. In Cp-1-D, the ratio of arabinose to galactose was 1:1, and the *O*-glycosidical junction between the carbohydrate and protein was composed of serine residue. The molecular weight was 23,000. From the results of structural characterization, both Cp-1-C and -D were assigned to type II arabinogalactan-protein (AGP), where a (1→6) linkage of galactose occurred mainly at the branching point in the case of Cp-1-C and mainly at the linear part in Cp-1-D.

The fruit of *Lycium chinense* Mill (gouqizi in Chinese, and kuko in Japanese) has been used as a herb and has been known as a somewhat famous ingredient since ancient times in China as well as Japan. Recently, a water extract of the fruit was found to have a range of pharmacological functions such as the enhancement of immune response,¹⁻³⁾ the inhibition of apoptosis induced by dextroamphetamine,⁴⁾ and the restoration to genetic wounding,⁵⁾ and the polysaccharide in the extract was estimated to be an active component.⁶⁾ We previously reported that the polysaccharide extracted with water from the fruit was separated mainly to three fractions (Cp-1, -2 and -3) by DEAE-cellulose column (HCO_3^- form), two of which (Cp-1 and -2) were presumed to be composed mainly of the arabinogalactan-protein (AGP).⁷⁾ In this paper, we report the further purification of Cp-1 and characterization of the resultant carbohydrate-protein.

MATERIAL AND METHODS

Material. Commercially available fruit of *Ly-*

cium chinense Mill, which was harvested and air-dried in the Ningxia province of China in 1996, was extracted with water at 4°C, and the extract submitted to DEAE-cellulose column chromatography (HCO_3^- form). The polysaccharide fraction (Cp-1) eluted with 0.1 M NH_4HCO_3 was used for the experiment. The yield of Cp-1 was 0.54% of the dried fruit.

General methods. Total sugar was determined by the phenol-sulfuric acid method,⁸⁾ and galacturonic acid by the carbazole-sulfuric acid method.⁹⁾ Protein content was estimated by a modified Lowry method.¹⁰⁾ A glycosyl residue (neutral sugar) of the polysaccharide was analyzed as alditol acetate after acid hydrolysis.¹¹⁾ Gas-liquid chromatography (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 spectrometer (GC-MS) was conducted with a Shimadzu GC-MS QP-1000 apparatus equipped with a CBP-1-M25-025 capillary column (0.2 mm×25 m) programmed from 160 to 230°C at 3°C/min,

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and the spectra were recorded at an ionizing potential of 70 eV. Infrared (IR) spectra were recorded with a Perkin Elmer 2000 FT-IR infrared spectrometer system. The specific rotations were measured with a Horiba polarimeter SEPA-300. Amino acid residues of the protein were determined after acid hydrolysis (6 M HCl, 110°C, 16 h, N₂ atmosphere) using a Millipore LC-module 1 analysis system equipped with a column of PICO-TAG (3.9×150 mm, Waters Co.). Liquid chromatography at about 7.4 Mpa (HPLC) was performed in a JASCO apparatus (880-PU pump, 860-CO column oven, and 830-RI detector). For the estimation of molecular weight of the polysaccharide, a Shodex OHpak KB-805 (8×300 mm) column was used and operated at 45°C with a solvent flow rate of 0.1 M NH₄HCO₃ at 0.8 mL/min. The peak areas were estimated with a System Instrument SIC Chromatocorder 11. ¹H- and ¹³C-NMR spectra were recorded at 399.956 (¹H) and 100.578 MHz (¹³C) with a Varian FT-NMR unity inova 400. Chemical shifts are given in δ values.

RESULTS AND DISCUSSION

Isolation of arabinogalactan-protein (AGP) from Cp-1.

A sample (30 mg) of Cp-1 was submitted to DEAE-cellulose column (B₄O₇²⁻ form, 2.6×50 cm) chromatography being monitored by the phenol-sulfuric acid method. The column was eluted with water and then stepwise with 0.02, 0.04, 0.06, 0.08 M and saturated Na₂B₄O₇ at a flow rate of 30 mL/h. Four kinds of polysaccharide fraction (Cp-1-A, -B, -C and -D) were obtained (Fig.1). Each fraction was dialyzed against running water and lyophilized. Some of the properties of each fraction are shown in Table 1. A glycosyl residue analysis of the fractions suggested that Cp-1-A should be mainly composed of arabinoxylan (ara: xyl=1:1) and Cp-1-B mainly araban. Cp-1-C (and -D) was composed of arabinose and galactose at a ratio of 3:1 (and 1:1) together with a small amount of fucose, xylose, mannose, glucose and galacturonic acid. The last two fractions were considered to be the arabinogalactan-protein (AGP) anticipated in the previous paper since they also con-

Fig. 1. Elution profile of Cp-1 on a DEAE-Cellulose column.

Cp-1 was dissolved in distilled water and loaded on a DEAE-Cellulose column (2.6×50 cm, B₄O₇²⁻ form). After being washed with distilled water, the column was eluted with 0.02–0.08 M sodium borate, saturated sodium borate and 0.1 M sodium hydroxide successively. Fractions were collected and analyzed for total sugar.

Table 1. Some properties of Cp-1-A, -B, -C and -D.

	Cp-1-A	Cp-1-B	Cp-1-C	Cp-1-D
Yield ^a	1.8	24.8	31.1	42.3
[α] _D (c = 0.2, H ₂ O)	—	+14.5	−5.0	−12.0
Mw×10 ⁴	1.0	1.1	4.2	2.3
Protein (%)	1.0	5.4	5.5	3.7
Total sugar (%)	87.8	89.4	92.4	90.7
GalA (%)	4.3	2.7	7.1	8.6
Ara	28.0	70.7	66.0	41.4
Gal	5.13	8.4	22.7	42.7
Fuc	3.7	7.7	4.4	2.5
Xyl	23.0	4.1	0.8	2.0
Man	8.3	1.9	1.5	2.5
Glc	8.4	1.9	0.9	1.0

^aPercentage to dry-weight basis of Cp-1.

tained a protein at 5.5 and 3.7%, respectively. Many workers have perceived that AGPs have never failed to be accompanied by a small amount of various neutral and acidic sugars.^{12–14)} When considering it, such monosaccharide could be a component of Cp-1-C (and -D), though its location in the polysaccharide could not be clarified. It is well-known that two kinds of arabinogalactan and AGP, type I (arabino-4-galactan) and II (arabino-3,6-galactan), have been isolated from various plants,^{15,16)} and in most of them the ratio of arabi-

nose to galactose is ca. 1:2. In both samples, however, the ratio of arabinose to galactose was more than one (ara/gal=3 in Cp-1-C, and 1 in -D). Such AGP has been found in turnip (ara/gal=3.2)¹⁷⁾ and the root of radish (=1.4),¹³⁾ which have been assigned to type II.

Linkage analysis of Cp-1-C and -D by methylation.

Five milligrams of Cp-1-C (and -D) was methylated by a modified Hakomori procedure.¹⁸⁾ The product was confirmed to show no absorption for the hydroxyl group in its IR spectrum, and it hydrolyzed with 90% formic acid and then with 0.25 M sulfuric acid. The partially methylated sugar thus obtained was converted to alditol acetate for GLC and GC-MS analyses. GLC gave some peaks as shown in Fig. 2, and six of them were identified by their retention times and mass spectrum as shown in Fig. 3. These are summarized in Table 2. The ratio of methylated arabinose to galactose was 2.72 (=66.7/24.5) in Cp-1-C and 1.11 (=49.4/44.6) in Cp-1-D. This is coincident with the result of the glycosyl residue analysis (Table 1). In both samples, almost half of the arabinose was found to occur at the non-reducing terminal unit in furanose form and the remainder was linked through *O*-3 or *O*-5, the ratio of *O*-3 and *O*-5 linkage being ca. 1:1. Moreover the ratio of 3-*O*-substituted galactose to total galactose was almost the same in both samples [22% (=5.5/24.5) in Cp-1-C, and 21% (=9.4/44.6) in Cp-1-D]. It is interesting that the linkage of galactose through *O*-6 occurs at the same rate but at a different kind of galactosyl residue in Cp-1-C and -D. That is to say, it occurred mainly at 3-*O*-substituted galactose in Cp-1-C to yield 3,6-di-*O*-substituted galactose, consequently giving the branching domain of the polysaccharide. In the case of Cp-1-D, it occurred mainly at the galactose located in the non-reducing terminal yielding 6-*O*-substituted galactose, consequently giving the linear domain of the polysaccharide. AGP in Cp-1-C, therefore, should be a polysaccharide branched more than that in Cp-1-D. It could be concluded that the fraction of Cp-1 contained two kinds of AGP and that both of them should be classified to type-II AGP.

Fig. 2. Gas chromatograms of partially methylated alditol acetates from Cp-1-C and -D.

A: 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-arabinitol, B: 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methyl-arabinitol, C: 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-arabinitol, D: 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol, E: 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-galactitol, F: 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-galactose.

Table 2. Methylation analysis of Cp-1-C and Cp-1-D.

Alditol acetate of linkage		Cp-1-C	Cp-1-D
2,3,5-Me ₃ -L-Ara	L-Araf-(1→	34.3	27.8
2,5-Me ₃ -L-Ara	→3)-L-Araf-(1→	17.8	11.6
2,3-Me ₂ -L-Ara	→5)-L-Araf-(1→	14.6	10.0
Total		66.7	49.4
2,4,6-Me ₃ -D-Gal	→3)-D-Galp-(1→	5.5	9.4
2,3,4-Me ₃ -D-Gal	→6)-D-Galp-(1→	5.8	25.5
2,4-Me ₂ -D-Gal	→3,6)-D-Galp-(1→	13.2	9.7
Total		24.5	44.6

¹H- and ¹³C-NMR analysis of Cp-1-C (and -D).

The spectra were recorded at room temperature in D₂O. Water soluble TMS (sodium 3-trimethylsilylpropionate-2,2,3,3-*d*₄) was used as the internal standard. In the ¹H-NMR spectrum, signals at 5.27 (and 5.26) and 5.10 (and 5.09) were easily recognized from other signals, and each was assigned to terminal and 3-(and 5-)*O*-substituted arabinofuran-

Fig. 3. Mass spectrum of each peak of Cp-1-C (and -D) on a gas chromatogram.

osyl residues in α -L-form. In a similar way, signals at 4.48 (and 4.45), 4.45 (and 4.50) and 4.52 (and 4.53) were respectively assigned to 3-*O*-, 6-*O*- and 3,6-di-*O*-substituted β -D-galactopyranosyl

residues (Table 3). In the ^{13}C -NMR spectrum, signals at 112.10 and 110.25 ppm were assigned to anomeric carbons of terminal and 3-(and 5-)*O*-substituted α -L-arabinofuranosyl residues, respective-

ly. The signal at 106.60 ppm was due to anomeric carbons of 3-*O*-, 6-*O*- and 3,6-di-*O*-substituted β -D-galactopyranosyl residues. The assignment of other signals is shown in Table 4. The results were consistent with those of other arabinogalactan (type II) and supported by methylation analysis.

Carbohydrate-protein linkage analysis by reductive alkaline degradation.¹⁶⁾

Cp-1-C (and -D) contained protein at 5.5% (and 3.7%). To learn the amino acid residue to which the carbohydrate was linked, the sample was subjected to reductive alkaline degradation followed by amino acid analysis. Five milligrams of Cp-1-C (and -D) in 0.25 M NaOH containing 0.5 M NaBH₄ (1.0 mL) was kept at 45°C for 6 h. The results of the amino acid analysis for the native and degraded proteins are listed in Table 5. In both samples, five kinds of amino acids (serine, proline, glutamic acid, glycine and alanine) were found to form about 74% of the total amino acid residues.

Table 3. ¹H-NMR chemical shift of anomeric proton of Cp-1-C and Cp-1-D.

Glycosidic linkage		Cp-1-C	Cp-1-D	Ref. ^a
Arabinose	terminal	5.27	5.26	5.23
	3- <i>O</i> -substituted	5.10	5.09	5.07
	5- <i>O</i> -substituted	5.27	5.26	5.23
Galactose	3- <i>O</i> -substituted	4.50	4.48	4.48
	6- <i>O</i> -substituted	4.45	4.45	4.46
	3,6-di- <i>O</i> -substituted	4.53	4.52	4.52

^aRef. 21).

Of these amino acids, serine and alanine, on reductive alkaline degradation, varied to a considerable degree in constitutional mole percentage. In the case of Cp-1-C, about 5 moles (=27.20–21.91) of serine residue were lost and about 5 moles (=17.24–12.66) of alanine residue gained. In the case of Cp-1-D, these values were approximately 4 moles for each amino acid. The decrease in mole percentage of serine was almost equal to the increase in that of alanine in both samples, suggesting that the carbohydrate should link *O*-glycosidically to the serine residue of the protein. The data, moreover, indicate that about 20% [(27.20–21.91)/27.20] of the serine residue is bound to carbohydrate in Cp-1-C. It is approximately 15% in Cp-1-D. It is interesting that almost half of the threonine residue (55%=2.55/5.71) could also be a *O*-glycosidical junction between carbohydrate and protein in Cp-1-C, while having no relation in Cp-1-D. The concurrence of two kinds of carbohydrate-protein linkage in the molecule have been reported for AGP from the leaves of *Acacia robusta*¹⁹⁾ and *Cannabis sativa*.¹⁴⁾ In both cases, serine and hydroxyproline were junctions between the carbohydrate and protein. A threonyl junction was proved for such AGP as one from the seed of radish,²⁰⁾ but the concurrence of serinyl and threonyl junctions in AGP have not yet been reported. AGP in Cp-1-C might be the first example for the concurrence of serinyl and threonyl junctions in a molecule.

Table 4. ¹³C-NMR chemical shift of Cp-1-C (and -D).

Glycosidic linkage	Chemical shifts (ppm)*					
	C-1	C-2	C-3	C-4	C-5	C-6
α -L-Araf						
terminal	112.10(110.9 ^b)	84.8(83.0 ^b)	79.4(78.2 ^b)	86.7(85.5 ^b)	65.7(62.9 ^b)	
3-linked	110.25(109.3 ^c)	84.2(81.0 ^c)	84.8(85.1 ^c)	84.2(84.1 ^c)	64.1(62.4 ^c)	
5-linked	110.25(108.5 ^d)	84.2(81.8 ^d)	79.4(77.2 ^d)	84.2(83.2 ^d)	72.3(67.9 ^d)	
β -D-Galp						
3-linked	106.6(105.3 ^b)	72.7(71.6 ^b)	84.2(81.8 ^b)	71.4(70.1 ^b)	79.4(76.8 ^b)	64.1(62.7 ^b)
6-linked	106.6(105.3 ^b)	72.7(72.4 ^b)	76.4(74.3 ^b)	71.4(70.3 ^b)	76.3(75.0 ^b)	72.7(71.0 ^b)
3,6-linked	106.6(104.8 ^b)	72.7(71.6 ^b)	86.7(81.8 ^b)	71.4(70.1 ^b)	76.3(75.0 ^b)	72.7(71.0 ^b)

*Values in parentheses are published data (^b Ref. 21), ^c Ref. 22), ^d Ref. 23)).

Table 5. Amino acid analysis of native and reductive alkaline degraded Cp-1-C and -D.

Amino acid	Cp-1-C	Cp-1-C*	Cp-1-D	Cp-1-D*
Asp	5.21	6.65	4.76	4.63
Glu	10.27	13.03	10.47	11.25
Ser	27.20	21.91	26.36	22.57
Gly	12.77	12.96	14.34	13.64
Arg	2.69	3.31	2.82	2.70
Thr	5.71	2.55	5.24	4.92
Ala	12.66	17.24	14.18	18.38
Phe	1.28	1.26	1.89	1.90
Pro	10.80	11.39	9.28	8.63
Tyr	0.20	0.37	0.29	0.19
Val	3.76	2.77	3.42	3.63
Met	0.58	0.52	0.40	0.68
Lys	0.78	0.51	0.96	0.89
Ile	3.38	2.79	3.41	3.55
Leu	2.69	2.41	2.18	2.44
Total	100.00	100.00	100.00	100.00

*Reductive alkaline degraded sample.

Molecular weight estimation.

Cp-1-C (and -D) was dissolved in 0.1 M NH_4HCO_3 and the solution was subjected to HPLC. Dextran T-70 (mol. wt. 70,000), T-40 (43,500), T-20 (20,400), and T-10 (10,500), products of Pharmacia Fine Chemicals, were used to calibrate molecular weight. The elution profile of Cp-1-C (and -D) showed a single peak, and from its elution time, the molecular weight was determined to be 42,000 (and 23,000). This data is very close to the molecular weight reported for the type II AGP from the root of radish (25,000)¹³ (and turnip, 40,000).¹⁷⁾

For Type II AGP, some biological functions have been reported.^{12,14,16,21)} Although Cp-1-C (and -D) was not examined for any biological functions, some medicinal actions of *Lycium chinense* fruit could be corroborated to some extent by the detection of two kinds of type II AGP in the fruit. The biological activity of the AGPs in Cp-1-C (and -D) as well as AGP-like compound(s) in Cp-2 presumed in a previous paper are now being studied.

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クコ果実のアラビノガラクトタン-プロテインについて

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クコの実からの冷水抽出物を DEAE-cellulose column chromatography に供したところ, 2 種類の arabinogalactan-protein (AGP) が得られた. 一つは arabinose:galactose=3:1 からなり, 分子量は 42,000 で, 糖は蛋白質の serine および threonine の 2 種のアミノ酸残基とグリコシド結合している糖蛋白質であり, もう一つは arabinose:galactose=1:1 からなり, 分子量は 23,000 で, 糖は蛋白質の serine 残基にグリコシド結合している糖蛋白質であることが明らかになった. さらに構造解析の結果, 両者はともに分岐した type II に属する arabinogalactan であるが, 大きな相違点は (1→6) 結合が主に分岐として存在 (Cp-1-C) するか直鎖として存在 (Cp-1-D) するかの違いであることが明らかとなった.