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Structural and Physiological Characteristics of Rhamnogalacturonan II from Fruit Wines

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Abstract To characterize the polysaccharides which exist as soluble forms in fruit wines, crude polysaccharides were isolated from red, white, raspberry, wild grape, and pear wine, respectively. Among them, the crude polysaccharide (RW-0) in red wine showed the highest yield and considerable amounts of thiobarbituric acid (TBA)-positive materials. The pectic polysaccharide RW-2 was purified to homogeneity from RW-0 by subsequent size-exclusion chromatography using Sephadex G-75 and its structure was characterized. RW-2 consisted of 14 different monosaccharides which included rarely observed sugars in general polysaccharides, such as 2-O-methyl-fucose, 2-O-methyl-xylose, apiose (Api), 3-C-carboxy-5-deoxy-L-xylose (aceric acid, AceA), 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), and 3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha). Methylation analysis indicated that RW-2 comprised at least 20 different glycosyl linkages such as 3,4-linked fucose, 2,3,4-linked rhamnose, 3'-linked apiose, and 2,3,3'-linked apiose, being characteristic in rhamnogalacturonan II (RG-II). High performance size-exclusion chromatography indicated that RW-2 mainly comprised RG-II of higher molecular weight (12,000), and that the changes of molecular weight to apparent 7,000 under less than pH 2.0 were observed. These analyses indicated that the higher molecular weight polysaccharide in RW-2 was mainly present as a RG-II dimer.

Keywords: red wine, structure, polysaccharide, rhamnogalacturonan II, dimer

Introduction

It is widely agreed that moderate intake of alcohols such as fruit wines, in particular, red wine, can improve the cardiovascular health of populations (1, 2). Red wine has been postulated to be beneficial, in part due to its natural antioxidant compounds and/or its association with enhancement in serum antioxidant activity (3, 4). Red wine contains high concentrations of polyphenolic substances, particularly flavonoids (5, 6), that have been found to possess different biological properties, such as anti-inflammatory responses, anti-hypertensive, anti-thrombotic effects, and prevention of low density lipoprotein (LDL) oxidation (6).

The health benefits associated with polyphenols have prompted many studies on the ingredients of red wine and their biological activities. Fruit wines contain several low and high molecular weight bioactive substances. Although biologically active substances of the low molecular weight compounds in fruit wines have been studied (3-6), they can not account for all of the ingredients in fruit wines and their observed clinical effects. Of the high molecular weight substances in fruit wines, polysaccharides and their activities have been overlooked mainly because of the fact that polysaccharides are precipitated in alcohol solutions. The possibility that specific polysaccharides may be selectively soluble under the relatively low alcohol concentration of fruit wine (12-14%), still remains.

Pectins refer to a group of closely associated polysaccharides from the primary cell walls and intracellular region of higher plants, and are probably the most complex class of polysaccharides in plant cell wall components (7). Pectins have been used as a source of dietary fiber and a ubiquitous nutritional factor and gelling agent in food manufacturing. Pectins are a family of complex polysaccharides that contain 1,4-linked α-D-galacturonic acid (GalA*p*) residues. Pectins comprise a family of homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and substituted galacturonans (rhamnogalacturonan II, RG-II) (7, 8). HG is a linear chain of 1,4-linked α-D-GalA*p* residues in which some of the carboxyl groups are methylesterified. HGs may, depending on the plant source, also be partially O-acetylated at C-3 or C-2 (10). By digestion with endo-α-(1→4)-polygalacturonase (endo-PGase), RG-I, and RG-II have been obtained (7, 8). RG-I is composed of a rhamnogalacturonan core with neutral carbohydrate side chains such as arabinan, galactan, arabinogalactans, and related oligosaccharides (11). Endo-PGase-released RG-II (12) is a low molecular weight (5-10 kDa) polysaccharide that contains 12 different glycosyl residues linked together by more than 20 different glycosyl linkages (13). RG-II contains the rarely observed sugars, D-apiose (Api), L-aceric acid (3-*C*-carboxy-5-deoxy-L-xylose, AceA), 2-*O*-methyl L-fucose (2-Me-Fuc), 2-*O*-methyl D-xylose (2-Me-Xyl), L-galactose (L-Gal), 2-keto-3-deoxy-D-*lyxo*-heptulosaric acid (Dha), and 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo) in addition to GalA, D-glucuronic acid (GlcA), L-rhamnose (Rha), D-galactose (Gal), L-arabinose (Ara), and L-fucose (Fuc) (13).

In the present study, we describe the isolation of RG-II from red wine by precipitation and one column chromatography. We also describe the structural characterization of RG-II and demonstrate the existence of dimeric RG-II in red wine.

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Materials and Methods

Experimental materials Several fruit wines such as red

wine, white wine, raspberry wine, wild grape wine, and pear wine (all produced in 2004), were purchased commercially at a market in Seoul, Korea. Pectinase from *Aspergillus niger* was purchased from Sigma (St. Louis, MO, USA) and poly (1,4-α-D-galacturonide) glycohydrolase (endo-PGase, EC 3.2.1.15) was purified from pectinase (Sigma) by the procedure of Thibault and Mercier (14). Sephadex G-75 was obtained from Pharmacia (Uppsala, Sweden), and Sep-Pak C18 cartridges from Waters Associates (Milford, MA, USA).

General analytical methods Total carbohydrate, uronic acid, and protein were determined using phenol-H_2SO_4 (15), *m*-hydroxydiphenyl (16), and Bradford's method (17) with Bio-Rad dye (Bio-Rad Co. Hercules, CA, USA), respectively, using galactose, galacturonic acid, and bovine serum albumin as the respective standards. The content of Kdo and Dha were colorimetrically determined through the modified thiobarbituric acid (TBA) method (18). The sugar component of polysaccharides was analyzed by alditol acetate method after hydrolysis of polysaccharides with 2 M trifluoroacetic acid (TFA) for 1.5 hr at 121°C (19, 20) and analyzed via gas-liquid chromatography (GLC) using the procedure of Zhao *et al.* (21). The composition of Kdo and Dha were determined through GLC as alditol acetates according to the modified methods of York *et al.* (22) and Stevenson *et al.* (23). Briefly, the samples were partially hydrolyzed partially under mild acid conditions (0.1 M TFA, 100°C, 1 hr) and the resulting hydrolyzates were reduced with $NaBD_4$. The samples were then treated with 2 M TFA for 1 hr at 121°C, and the resulting lactones were reduced with $NaBD_4$ under neutral conditions. The products were further treated with 2 M TFA and $NaBD_4$ to reduce Dha completely. After acetylation, the resulting carboxyl-reduced alditol acetates were analyzed through GLC (GC M600D; Young-Lin Co., Gyeonggi, Korea) using an SP-2380 capillary column (0.2 μm film thickness, 0.25 mm i.d. × 30 m; Supelco, Bellefonte, PA, USA). The molar percentage was calculated from the peak areas and response factors using the flame-ionization detector (FID). High performance size-exclusion chromatography (HPSEC) was performed on an HPLC-9500 instrument (Young-Lin Co.) equipped with combined columns of Asahi-pak GS-510+GS-320+GS-220 (Asahi Chemical Industry Co. Ltd., Japan). Ten μL of each polysaccharide solution (10 mg/mL) was analyzed by using an isocratic mobile phase (0.2 M NaCl) at a flow rate of 0.5 mL/min and room temperature. Molecular weights of polysaccharides were estimated from the calibration curve constructed for standard pullulans (P-400, 200, 100, 50, 20, 10, and 5, Showa Denko Co. Ltd., Tokyo, Japan).

Isolation and purification of polysaccharides from fruit wines Each fruit wine (2 L) was concentrated to 400 mL using a vacuum rotary evaporator (Eyela, Tokyo Rikakikai Co., Tokyo, Japan). Total colloids were precipitated by the addition of two volumes of cold ethanol containing 60 mM HCl to the concentrated wines. The precipitate was then dissolved in water and dialyzed using Spectra/Por 2 (molecular weight cut off, MWCO, 12,000-14,000; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The salt-free solution was finally lyophilized to give the crude polysaccharide fraction from fruit wine. The crude polysaccharide fraction from red wine (RW-0) was applied to a column (2.5×90 cm) of Sephadex G-75 equilibrated with 50 mM acetate buffer (pH 5.2), and were eluted with the same buffer. Two major purified polysaccharides (RW-1 and RW-2) were obtained, and lyophilized after desalting by dialysis using Spectra/Por 7 (MWCO, 2,000; Spectrum Laboratories Inc.). TBA-positive material was found in the retarded fraction (RW-2).

Methylation analysis Methylation analysis was performed according to the Hakomori method (24, 25), and the methylated products were recovered using the modified procedure of Waeghe *et al.* (26). Uronic acids in the methylated polysaccharides were reduced by $LiB(C_2H_5)_3D$ in THF (1 mL, room temperature, 1 hr, Super-Deuteride; Aldlich, Milwaukee, WI, USA) (27). The resulting partially methylated alditol acetates were analyzed via GLC and GLC-mass spectrometry (GLC-MS) using an SP-2380 capillary column (Supelco) as described previously (21), identified by their fragment ions and relative retention times, and their molar percentages were estimated from the peaks and response factors (28).

Mineral determination The B, Ca, K, and Na content was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) performed with a Jarrell-Ash 965 Atomcomp Plasma Emission spectrometer (Thermo Jarrell Ash Corp., Franklin, MA, USA). Samples of wine RG-II (3-5 mg/mL in water) were injected into the nebulizer of the spectrometer and analyzed according to the manufacturer's instructions.

Results and Discussion

Isolation and purification of polysaccharides from fruit wines The composition of soluble polysaccharides from five kinds of commercially-produced fruit wines, was investigated. Among red wine, white wine, raspberry wine, wild grape wine, and pear wine, the crude polysaccharide in red wine (366 mg/L yield) and the considerable amounts (1.4%) of TBA-positive materials it possessed was selected for further experiments (Table 1). When RW-0, the crude polysaccharide from red wine, was applied to a column of Sephadex G-75, it gave two major peaks (RW-1 and RW-2) of carbohydrate containment as shown in Fig. 1. Interestingly, the retarded fraction (RW-2) contained relatively high amounts of TBA-positive material and all carbohydrate and uronic acid in RW-2

Table 1. Yields of crude polysaccharide isolated from various fruit wines

Fruit wines	Yield (mg/L)
Pear wine	122
Red wine	366
White wine	185
Raspberry wine	98
Wild grape wine	125

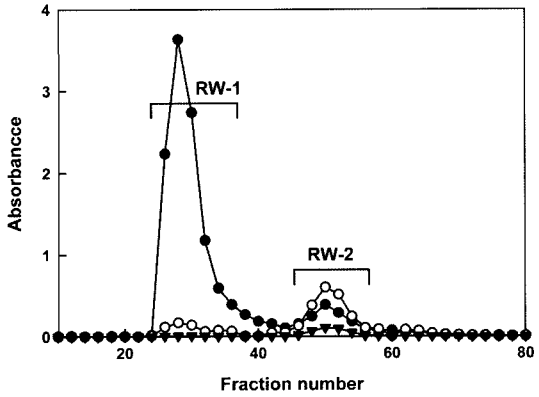

Fig. 1. Elution pattern of the crude polysaccharide (RW-0) isolated from red wine using Sephadex G-75. RW-2 was subjected on a Sephadex G-75 column (2.5×90 cm) and eluted with 50 mM acetate buffer (pH 5.2) at a flow rate of 0.2 mL/min. ●, Carbohydrate (490 nm); ○, Uronic acid (520 nm); ▼, TBA-positive material (500 nm).

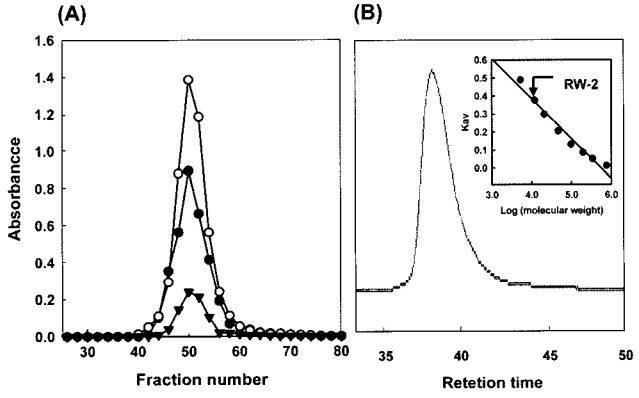

Fig. 2. Gel permeation chromatogram and HPSEC profile of RW-2 purified from the crude polysaccharide of red wine. (A) RW-2 was subjected on a Sephadex G-75 column (2.5×90 cm) and eluted with 50 mM acetate buffer (pH 5.2) at a flow rate of 0.2 mL/min. (B) RW-2 was injected into combined columns (0.76 ×30 cm, each) of Asahi-pak GS-510+GS-320+GS-220 and then eluted with 0.2 M NaCl at a flow rate of 0.5 mL/min. ●, Carbohydrate (490 nm); ○, Uronic acid (520 nm); ▼, TBA-positive material (500 nm).

seemed to co-elute with the TBA-positive peak. Since KDO is a unique component of RG-II among several plant polysaccharides, the TBA-positive reaction in RW-2 indicates the possibility that RW-2 contains a RG-II polysaccharide with KDO residues. When the RW-2 fraction was reapplied on the Sephadex G-75 column, it was eluted as a single peak with symmetry (Fig. 2A). It also gave single HPSEC peak on Asahi-pak GS-510+GS-320+GS-220 columns, and its molecular weight was estimated to be 12,000 Da (Fig. 2B).

Chemical characteristics of RW-2 RW-2 mainly contained neutral sugar and uronic acid in addition to small amounts of TBA-positive material, whereas protein was not detected (Table 2). Sugar analysis indicated that RW-2 consisted of 6 kinds of unusual sugars, namely 2-Me-Fuc, 2-Me-Xyl, Api, AceA, Kdo, and Dha which were characteristic monosaccharide constituents in RG-II (29)

Table 2. Chemical composition of RW-2 fraction purified from the crude polysaccharides of red wine (%)

Chemical composition	RW-2	Mineral composition	RW-2
Yield from total wine polysaccharide (RW-0)	26.2	B	0.07
		Ca	1.71
Neutral sugar	63.0	K	0.93
Uronic acid	34.4	Na	0.47
TBA-positive material	2.7	Mg	0.08
Protein	0.0	Fe	0.09
Component sugars[1)]			(mol%)
2-Me-Fuc	3.5	Man	5.5
Rha	16.6	Gal	12.9
Fuc	3.1	Glc	3.8
2-Me-Xyl	3.6	GalA	30.7
Ara	11.5	GlcA	3.6
Api	1.4	Dha	Detected
AceA	1.1	Kdo	2.7

[1)]Monosaccharides were analyzed using alditol acetates. Mol% was calculated from the detected total carbohydrate.

of plant cell wall polysaccharides in addition to Fuc, Ara, Rha, Man, Gal, Glc, GlcA, and GalA (Table 2). Although the Api and AceA residues were estimated as relatively small amounts, the acid labile nature of apiosyl residues may account for their low yield as an alditol acetate derivative, and low yields of aceric acid had previously been observed (30).

RG-II, along with RG-I and HG, constitute pectin in the plant cell and is released from the walls of plant cells by treatment with endo-PGase (29). RG-II is a highly branched polysaccharide that has been found in the primary cell walls of all higher plants, so far examined, such as sycamore (*Acer pseudoplatanus*) (31), rice (*Oryza sativa*) (32), Douglas fir (*Pseudotsuga menziensii*) (33), in the commercial enzyme preparation Pectinol AC (34), and in the leaves of *Panax ginseng* C.A. Meyer (30). However, digestion with endo-PGase gave no oligogalacturonides from RW-2 and the molecular weight of the product did not change (data not shown). These results indicate that RW-2 was similar to a typical RG-II (29) and that these polysaccharides also existed as the free form of RG-II-type polysaccharide in red wine but not as the conjugated form with RG-I and HG. It is also possible the red wine itself or some microbes during fermentation and aging process contain potent endo-PGase activity, and therefore RW-2 may be generated as a free form by this endogenous enzyme.

In contrast, RW-1, the fast-eluting fraction in Fig. 1 was mainly composed of mannose and showed different chemical properties compared to RG-I. Therefore, it was assumed that RG-I released from grape-pectins may be precipitated and removed by filtration-process during wine production. The occurrence of the free form of RG-II-type polysaccharide in red wine had previously been observed in the leaves of *P. ginseng* (30).

Glycosyl linkage composition of RW-2 Methylation analysis indicated that RW-2 contained at least 20 different glycosidic linkages as shown in Table 3. Methylation analysis of RW-2 showed the occurrence of terminal 2-Me-Xyl, terminal 2-Me-Fuc, 2,3,4-linked Rha, 3'-linked Api, and 2,3,3'-linked Api, being characteristic glycosidic linkages in RG-II (29). No significant difference was observed in the composition of the glycosidic linkages among RG-IIs originating from other sources. Dha and Kdo are both degraded under the acidic conditions used to cleave glycosidic linkages (35) and their methylated products were not identified in this study. Recently, and O'Neil *et al.* (36) have found that RG-II from sycamore and pea contained 2,3,3'-linked Api which contributed to the dimerization of RG-II. Methylation analysis also indicated that RW-2 contained 2,3,3'-linked Api (Table 3). These results suggest that RW-2 mainly comprise RG-II structures.

RW-2 from red wine as a dimer When RW-2 was analyzed by HPSEC, RW-2 gave single peak having an apparent molecular weight of 12,000 (Fig. 2B). However, RW-2 was eluted with some tailing on side of low molecular weight. Kobayashi *et al.* (37), Ishii and Matsunaga (38), O'Neill *et al.* (36), and Kaneko *et al.* (39) have reported that RG-II from radish, sugar beet pulp, sycamore and pea stem, and bamboo shoot dimerize by forming a complex with borate, and that the 2,3,3'-branched Api in RG-II is involved in the formation of the RG-II dimer containing borate diester (36). We also have reported the existence of the borate-RG-II complex in the leaves of *P. ginseng* (40). After RW-2 had been treated with buffers with diverse pH ranges (1.0-8.0) for 30 min, the products were analyzed on HPSEC. Under conditions where pH is less than 2.0, the peak of high molecular weight (12,000) in RW-2 gradually disappeared, and peaks having an apparent molecular weight of 7,000 were observed (Fig. 3). Methylation analysis indicated that RW-2 also contained 2,3,3'-branched Api, which has been suggested to form a complex with borate in RG-II (36) (Table 3). The boron content of RW-2 was estimated to 0.07%(w/w) by ICP (Table 2). Therefore, it was assumed that RW-2 polysaccharide in red wine also might contain borate-RG-II dimer as RG-IIs obtained from other sources.

Table 3. Methylation analysis of RW-2 fraction purified from the crude polysaccharide of red wine

Glycosyl residue	Position of methyl group	Deduced glycosidic linkage	RW-2 (mol%)[1)]
Ara	2,3,5	T-Ara*f*[2)]	9.12
	3,4	2-Ara*p*[3)]	1.06
	4	2,3-Ara*p*	4.27
2-Me-Xyl	3,4	T-2-Me-Xyl*f*	4.32
AceA	3	2-AceA*f*	3.18
Api	2,3	3'-Api*f*	4.18
	~	2,3,3'-Api*f*	2.22
Fuc	2	3,4-Fuc*p*	2.69
2-Me-Fuc	3,4	T-2-Me-Fuc*p*	4.70
Rha	2,3,4	T-Rha*p*	6.65
	3,4	2-Rha*p*	3.49
	2,4	3-Rha*p*	5.16
	~	2,3,4-Rha*p*	3.04
Gal	2,3,4,6	T-Gal*p*	4.29
	3,6	2,4-Gal*p*	5.09
GlcA	3,4	2-GlcA*p*	3.54
GalA	2,3,4	T-GalA*p*	13.94
	2,3	4-GalA*p*	10.24
	2	3,4-GalA*p*	5.05
	3	2,4-GalA*p*	3.77
Total			100.00

[1)]Calculated from the peak areas and molecular response factors of each partially methylated alditol acetate in GLC and GLC-MS. [2)]T-Ara*f* means non-reducing terminal arabinofuranoside. [3)]2-Ara*p* means 2-linked arabinopyranoside.

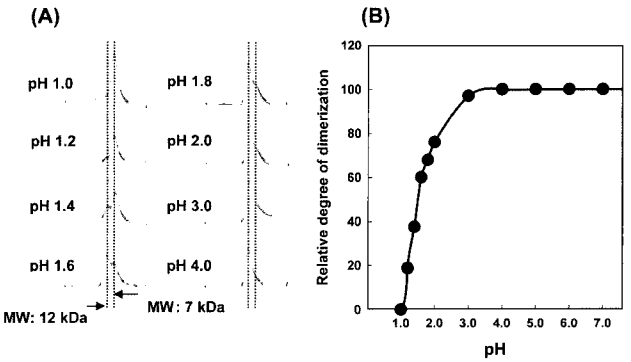

Fig. 3. Effect of pH on the changes in molecular weight of RW-2 purified from red wine. (A) Elution patterns on HPSEC using Asahi-pak GS-320+GS-220 of RW-2 and after treatment with buffers at each pH for 30 min. (B) Relative dimerization degree (RDD) of RW-2 at each pH. RDD = [(height of fast-eluted peak - height of retarded peak) / (height of fast-eluted peak)] × 100.

The results of this study have demonstrated that red wine is an abundant source of RG-II and fruit wines may serve as a viable commercial source for RG-II. Fruit wines have become the material of choice in many studies where large amounts of RG-II are required because gram quantities of RG-II are readily obtained using a small number of purification steps as shown in this study. Recently, the health-promoting activities of RG-IIs such as inhibition of lead absorption (41), macrophage Fc receptor expression-enhancing activity (30), intestinal immune system modulating activity (42), and so on, have been reported. Further research on pharmaceutical activities of RG-II from fruit wines waits.

Acknowledgments

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