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Quantitative comparison of acidic polysaccharides in the endosperm of two major varieties of rice

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Abstract Rice endosperm, the portion that remains after milling, is the part of the rice seed that is primarily consumed as a source of nutrients. There have been many studies on polysaccharides, such as hemicellulose, cellulose, and pectins, derived from the cell walls of various plant groups. It has been reported that the acidic polysaccharide fractions, which contain water-soluble pectins that have been shown to have pharmacological effects *in vivo* and *in vitro*, have common chemical structures that include galacturonic acid polymers, rhamnose, arabinose, and galactose. However, few studies have been conducted on the acidic polysaccharides contained in the endosperm of rice. In this study, we quantitatively compared the differences in the acidic polysaccharide contents from samples from two of the main varieties of rice consumed as staple foods, *japonica* and *indica*, using a colorimetric method. Rice samples were collected from 39 different regions in Korea, China, Thailand and Vietnam. Acidic polysaccharide fractions were obtained by precipitation of the alcohol-insoluble residue (AIR) and enzyme treatment of each sample. The total amount of carbohydrates and uronic acid in each acidic polysaccharide fraction were measured using the phenol-sulfuric acid method and the carbazole-sulfuric acid method, respectively. The differences in the total polysaccharide contents in the acidic polysaccharide fractions were not statistically significant ($p = 0.07$), but the uronic acid contents were significantly different between the two groups ($p = 0.04$).

Key words: rice endosperm, acidic polysaccharide, pectin, *japonica* rice, *indica* rice, colorimetric quantification

1. Introduction

Rice (*Oryza sativa* L.) has been used for thousands of years as a main food source in many countries. It is estimated that rice was cultivated for the first time in India, Thailand, Myanmar, North Vietnam (Indochina peninsula) or China approximately 8,000 to 15,000 years ago.[1,2] Currently, more than 3.5 billion people worldwide consume milled rice as staple food on a daily basis.[1] More than 715 million tons of paddy rice, equivalent to 4.8 million metric tons of milled rice, is produced annually across more than 100 countries.[3] Although *Oryza sativa* as a species is genetically classified into six categories, there are

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two main varieties of rice consumed staple foods, *japonica* and *indica* varieties. *Japonica* rice is mainly consumed in Korea, China, and Japan, and its grains are round and sticky. In contrast, *indica* rice is mainly cultivated and consumed in some part of southern China, India, and many countries in Southeast Asia such as Vietnam, Thailand, and Laos, and its grains are elongated and less sticky than *japonica* rice.[4]

In flowering plants, the endosperm is the compartment that accumulates the nutrients necessary for the growth of the embryo during the germination process.[5] During the milling process, rice bran, brush, and almost all the outer parts of the rice seeds are removed so that the majority of the consumed rice is endosperm. In the case of wheat, barley, corn or other crops used as staple foods, a portion of the germ is also consumed. Rice endosperm contains a large amount of starch, a polymer consisting of D-glucose molecules linked by α-glycosidic linkages, stored in granule form in its cytoplasm.[6] There have been in-depth studies focused on starch and its derivatives in terms of nutritional quality.[7] characteristics,[8-10] and functionailty.[11] However, various types of polysaccharides, including cellulose, pectins, and hemicelluloses, are derived from cell walls, even if the absolute amounts of these compounds are relatively low compared to starch.[12] These cell wall polysaccharides have inherent phyto-physiological functions, such as cellulose or hemicellulose, which play a role in giving rigidity to the cells, and pectins, which impart fluidity to the cell wall to form a gelatinous matrix.[13,14] The composition of the polysaccharides in cell wall depends on the specific part of the cell wall, the species, the variant, what province the rice was grown in and the local climate, giving each species its distinctive characteristics. It has been reported that plant cell wall polysaccharides may have pharmaceutical activities, such as anti-complementary activity,[15] antioxidant activity,[16] and anti-tumor activity.[17]

The water-soluble polysaccharides can be extracted from the cell walls of leaves, seeds, and fruits of plants.[13] Polysaccharides in this fraction are derived from the cell wall pectin and soluble starch and contain a large number of acidic groups. For this reason, they are called acidic polysaccharides. The pharmacological effects of the acidic polysaccharide fraction have been studied independently in many different plants. The best example is the red ginseng acidic polysaccharides (RGAP). They were reported to have effects related to the immune system, such as stimulating the immune response to macrophage function,[18,19] stimulating B and T cells,[20] and synergistic immunostimulating activity.[21] In addition, other pharmacological effects have been reported for other acidic polysaccharide fractions such as bacterial adhesion inhibitory effects,[22] anti-fatigue activities,[23] and anti-hyperlipidemic effects.[24] In structural terms, Xu *et al.* (2008) proposed that the major polysaccharides contained in the RGAP fraction are pectin-like polysaccharides with galacturonic acid backbones, rhamnose, and arabinose,[25] making them similar to the pectic polysaccharides in rice endosperm reported by Sibuya *et al.* (1984).[26] This suggests that acidic polysaccharides in rice endosperm have pharmacological effects, and it is reasonable to consider acidic polysaccharides in a functional evaluation of rice. Several studies reported the pharmacological functions of polysaccharides extracted from rice bran[16,17] rather than endosperm, the part of rice that we consume the most. Despite this possibility, the analytical studies reported to date on the acidic polysaccharide fractions extracted from rice endosperm are insufficient. In particular, there have been no analytical studies on the content of acidic polysaccharides by region or cultivar.

Most polysaccharides included in the acidic polysaccharide fraction are structurally classified as pectic polysaccharides or pectin, which are one of the most abundant structures in the plant cell wall,[27] and they contain approximately 90 % of the uronic acid in the entire cell wall.[28] Pure acidic polysaccharides can be obtained by completely removing the starch or dextrin from a crude fraction through a multistage process.[25] The structure is based on galacturonan, a polymer of galacturonic acid, which can be sub-classified as homogalacturonan (HG), arabinogalacturonan (AGA), rhamnogalacturonan I (RG-I), and

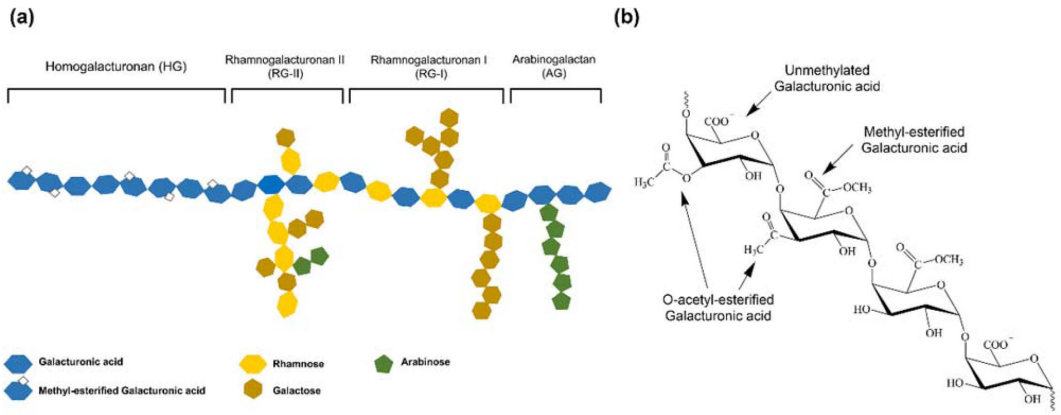

Fig. 1. A representative structure of a polysaccharide chain in the acidic polysaccharide fraction based on previous studies. A) The main structures of the pectic polysaccharide including homogalacturonan (HG), arabinogalacturonan (AGA), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II). Monosa- ccharides components such as galacturonic acid, rhamnose, arabinose, and galactose have been marked with different shapes. B) Schematic representation of the chemical structure of homogalacturonan (HG).

rhamnogalacturonan II (RG-II) depending on the type of additional sugar chains.[29] *Fig.* 1(a) shows representative glycan structures that make up the pectic polysaccharides. Greater than 60 % of the pectic polysaccharides in the cell wall are present in the HG form,[27] a linear polymer of α-1,4-linked-D-galacturonic acids (*Fig.* 1(b)). In the HG chains, the C-6 residues of each galacturonic acid are often methyl esterified and the hydroxyl groups of O-2 and O-3 can be acetylated depending on species.[27] If more than 50 % of the residues are methyl esterified, the polymer is classified as a high methyl-esterified HG; if less than 50 % are esterified, the polymers are classified as low methyl-esterified HG.[13] It is speculated that HG is covalently cross-linked with other pectic polysaccharides, such as RG-I or RG-II, or other cell wall polysaccharides in the hemicellulose family, such as xyloglucans.[30] Due to this internal binding, the pectic polysaccharides of each of HG, RG-I, and RG-II cannot be separated by size-exclusion chromatography unless they are treated with appropriate hydrolytic enzymes.[31] Conventionally, acidic polysaccharide fractions refer to a mixture of these pectic polymers.

One of the inherent properties of acidic polysaccharides is that they contain uronic acids in their residues, which can be used for the quantification of acidic polysaccharides. Chromatographic techniques such as gas-chromatography (GC) methods or high-performance liquid chromatography (HPLC) with pulsed amperometric detection (PAD) for separating and quantifying uronic acid have been used.[32] Fourier transform infrared (FT-IR) spectroscopy based on characteristic IR absorptions of uronic acid residues was also used.[33] However, the most well-known method for quantification is colorimetry using sulfuric acid with a few chromogenic reagents. In the classic carbazole-sulfuric acid method, carbazole molecules can react with galacturonic acid liberated from polymer chains causing the sulfuric acid to emit light in the visible range.[34] The colorimetric method has sufficient linearity to be used for quantification and is fast, simple and intuitive. It does not require complex and costly equipment, and multiple samples can be analyzed at once using a multi-cell plate. Therefore, it is more suitable for quantitative analysis of a large number of samples than chromatographic methods would be.

In this study, we measured the content of the acidic polysaccharides in the endosperms of *japonica* and *indica* rice cultivars collected from four different countries using a colorimetric method. The differences in the acidic polysaccharide content between the two cultivars were compared statistically.

2. Experimental

2.1. White rice samples

All white rice (*Oryza sativa* L.) samples classified as *japonica* were randomly collected in different regions of Korea and China. Samples of white *indica* rice were collected in Thailand and Vietnam. Individual samples were numbered J1 to J10 for the Korean samples and J11 to J20 for the Chinese samples. Likewise, I1 to I11 are samples of *indica* from Thailand and I12 to I19 are samples from Vietnam (*Table* 1). A total of 39 samples from different regions were prepared. After collection, all the samples were subdivided and stored in a deep freezer at -70 °C.

2.2. Chemical and reagents

Amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger*, α-amylase (EC 3.2.1.1) purified from porcine pancreas, and pectinase (EC 3.2.1.15) from *Aspergillus niger* were supplied by Sigma-Aldrich (St. Louis, MO, USA). D-galacturonic acid methyl ester standard was purchased from Carbosynth (Compton, Berkshire, UK). All other monosaccharide standards including D-glucose, D-galactose, D-arabinose, D-rhamnose, and D-galacturonic acid were purchased from Sigma-Aldrich. Diethyl ether, iodine/KI reagent, and pyridine was purchased from Sigma-Aldrich. The HPLC-grade solvents used in the experiments, including ethanol, methanol, and distilled water, were purchased from J. T. Baker® (Phillipsburg, NJ, USA). The all other reagents and chemicals were of analytical grade.

2.3. Preparation of acidic polysaccharide fraction

The acidic polysaccharide fraction was prepared from the cell walls according to the method of Sibuya *et al.*[12] with some modifications (*Fig.* 2). All white rice samples were carefully ground to pass through two sieves (250 and 125 μm) and lyophilized in a freeze dryer (Operon, Gimpo, Gyeonggi, Korea). Lyophilized rice flour (500 mg) was defatted with 3 mL of 1:1 ethanol-ether. After centrifugation, the supernatant was discarded and the remaining solvent was completely removed with a nitrogen purge. The defatted rice flour was suspended in 3 mL of 80 %

Table 1. Information on the varieties and countries of origin of collected white rice samples

Variety	IDs	Country of Origin	Variety	IDs	Country of Origin
japonica	J1	Korea	indica	I1	Thailand
japonica	J2	Korea	indica	I2	Thailand
japonica	J3	Korea	indica	I3	Thailand
japonica	J4	Korea	indica	I4	Thailand
japonica	J5	Korea	indica	I5	Thailand
japonica	J6	Korea	indica	I6	Thailand
japonica	J7	Korea	indica	I7	Thailand
japonica	J8	Korea	indica	I8	Thailand
japonica	J9	Korea	indica	I9	Thailand
japonica	J10	Korea	indica	I10	Thailand
japonica	J11	China	indica	I11	Thailand
japonica	J12	China	indica	I12	Vietnam
japonica	J13	China	indica	I13	Vietnam
japonica	J14	China	indica	I14	Vietnam
japonica	J15	China	indica	I15	Vietnam
japonica	J16	China	indica	I16	Vietnam
japonica	J17	China	indica	I17	Vietnam
japonica	J18	China	indica	I18	Vietnam
japonica	J19	China	indica	I19	Vietnam
japonica	J20	China			

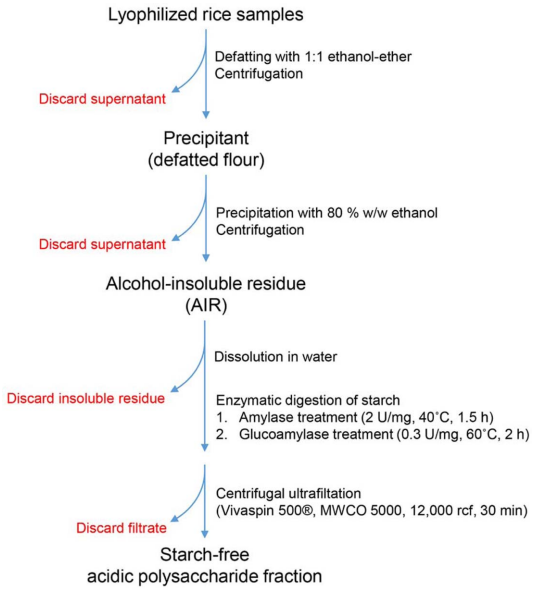

Fig. 2. The preparation scheme for starch-free acidic polysaccharide fractions.

(v/v) ethanol and precipitated. After centrifugation (12,000 g, 15 min), the alcohol-insoluble residue (AIR) was isolated[35] and re-distributed in 3 mL of water. To digest the soluble starch, α-amylase (2 U/mg of carbohydrate) was added, and the mixture was allowed to react for a further 1 h 30 min at 40 ºC. Then, amyloglucosidase (0.33 U/mg of carbohydrate) was added, and the mixture was allowed to react for an additional 2 h at 60 ºC or higher.[35,36] After digestion, the mixture was checked for the presence of starch with iodine/KI stain. The rice starch suspension was filtrated with Vivaspin 500® (Sartorius, Goettingen, Germany) on condition of 12,000 RCF, 30 min and the filtrate was removed. The remaining polysaccharide fraction was air-dried and then dissolved in 500 μL of distilled water for quantification.

2.4. Colorimetric quantification

All colorimetric measurements were performed using an automated microplate reader, SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA) with a management software, Softmax Pro 7.0 (Molecular Devices, Sunnyvale, CA, USA). To determine the total carbohydrate content, we followed the modified phenol-sulfuric acid method in a 96-well microplate format[37] at 490 nm with some modifications. The total uronic acid content in each fraction was determined using the carbazole-sulfuric method[34] at 525 nm. The specificity of the carbazole-sulfuric method was confirmed using six types of monosaccharide standards known to contain pectic polysaccharides, namely, glucose, galacturonic acid, methylated galacturonic acid, rhamnose, arabinose, and galactose.

2.5. Data processing and statistical analysis

After data acquisition using a microplate reader, UV absorbance signals were converted to quantitative values based on D-glucose and D-galacturonic acid standards. Standard calibration curve generation and raw data collection processes were performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and Softmax Pro 7.0. Statistical analysis was carried out using GraphPad Prism version 6.0 (Graphpad Software Inc., San Diego, CA, USA). Unpaired *t*-tests with Welch's correction (two-tailed, unequal variance, Welch-corrected) were conducted individually, with $p < 0.05$ as the level of statistical significance.

3. Results and Discussion

3.1. Comparison of the polysaccharide contents of different varieties

Quantitative analysis of polysaccharides in the acidic polysaccharide fractions was conducted for all white rice samples of *japonica* and *indica* in terms of both uronic acid content and total carbohydrate content. *Fig.* 3 shows the polysaccharide content of each sample in 1 g of lyophilized white rice powder. In the *japonica* samples, the content of uronic acid was between 3 mg and 13 mg per gram of white rice. The mean value was 7.1 mg/g. The total carbohydrate contents were measured by the phenol-sulfuric acid method and varied from 6 mg to 30 mg per gram of white rice. The mean value was 15.0 mg/g. In the *indica* samples, the average contents of uronic acid and total carbohydrates were 5.3 mg and 10.2 mg per gram of white rice, respectively. In both groups,

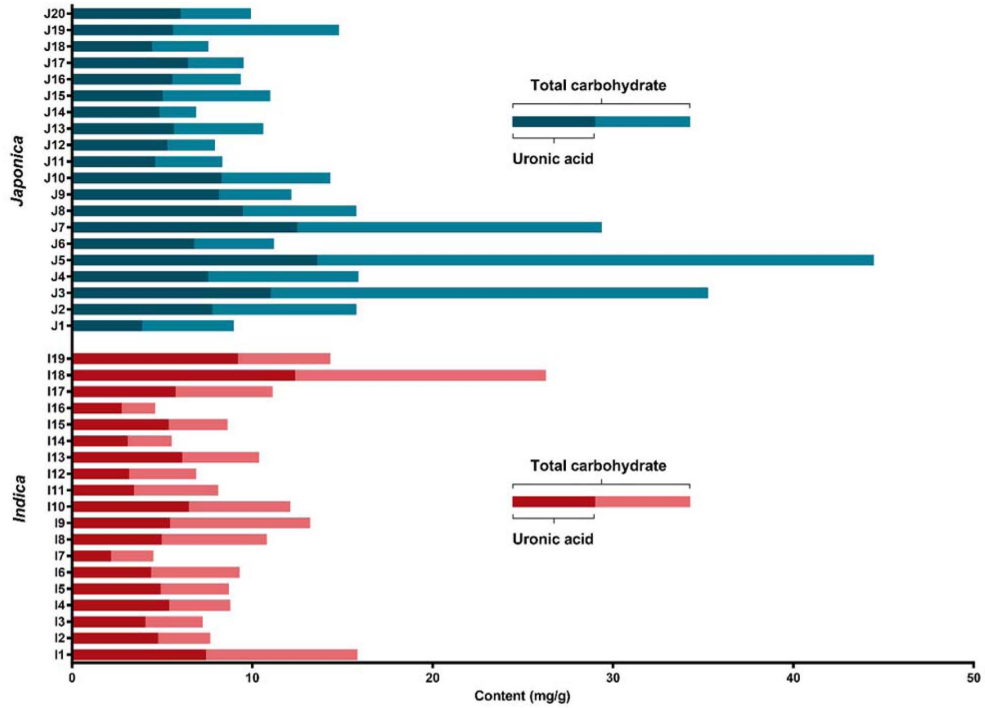

Fig. 3. The amount of uronic acid and total carbohydrate in each sample. The total carbohydrate measured by the phenol-sulfuric acid method was converted into the amount of glucose. The amount of uronic acid measured by carbazole-sulfuric acid method was converted into the amount of galacturonic acid.

the average percentage of uronic acid content was slightly higher than 50 % of the total carbohydrates. This result is in agreement with previous reports that the contents of uronic acid in the pectin fraction of cell wall polysaccharides are approximately 55 % of the total.[12] From a structural point of view, approximately 50 % of the weight of non-uronic acid in the water-soluble acidic polysaccharide fraction may consist of rhamnose, arabinose or other sugars of hemicellulose that are covalently linked to the pectic polysaccharides[13] as well as remaining starch-like polysaccharides.[31] Additional compositional analysis is necessary to verify their structure and monosaccharide composition.

3.2. Regional differences in the polysaccharide contents

Although it is difficult to say that only 8 to 10 samples are representative of an entire country, within-group content variations of the Korean samples were relatively smaller than those of other countries. This tendency was especially prominent in the uronic acid contents. The standard deviation (SD) of uronic acid content among the Korean samples was 0.6, which is significantly lower than that of the Chinese, Thai, and Vietnamese samples (2.9, 1.4, and 3.1, respectively). Since the cultivation area of rice in Korea is smaller than that of other countries and its climate is less diverse,[3] the polysaccharide content may be related to the climate or soil characteristics of the cultivation area. This may also be related to the variations in the sub-genotype caused by the environment. According to genomics research on rice from 82 countries using genome-wide association (GWA) mapping, more than 20,000 segregated single nucleotide polymorphisms (SNPs) were identified within the same variety.[38] The differences in flowering times of rice grown in different environments were found to be related to variations of specific SNPs, such as *HD1*; several other SNPs related to the characteristics of seeds have also been

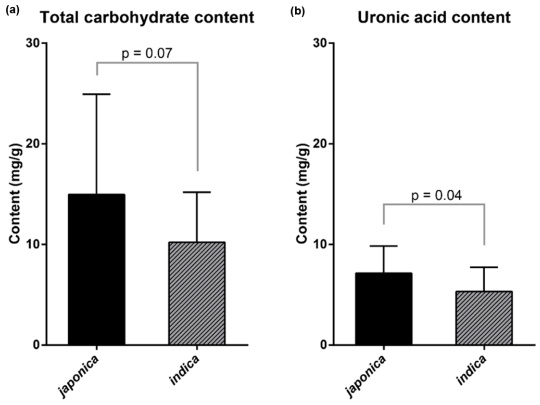

Fig. 4. Comparison of the polysaccharide contents of the two varieties. (a) A comparison of the total carbohydrate contents, p-value of 0.07. (b) A comparison of the uronic acid contents, p-value of 0.04. The significant differences between the two groups were confirmed by unpaired t-tests with Welch's correction.

discovered. The genetic variations caused by the environment may affect the growth, germination and flowering time of the seeds. Since pectin is related to the external resistance of seeds,[13,14] it implies that variations in the acidic polysaccharide contents of rice samples cultivated in more diverse environments should be greater than variations between rice samples grown in similar environments. The relationship between the content of acidic polysaccharides and the genetic variations of rice needs to be further investigated.

3.3. Statistical analysis

Unpaired *t*-tests with Welch's correction were conducted to determine whether the polysaccharide contents were significantly different between the two groups, *japonica* and *indica*. *Fig.* 4 shows the differences in the total polysaccharide contents and uronic acid contents between the two groups. First, for total carbohydrate content, the p-value was 0.07, so it was difficult to say that there was a significant difference in the total carbohydrate contents of the two groups (*Fig.* 4(a)). For the uronic acid contents, however, the p-value was 0.04, which was lower than the 0.05 reference at the 95 percent confidence level (*Fig.* 4(b)). This means that a significantly higher content of uronic acid was observed in *japonica* than *indica*. Since the basic structures forming the pectic polysaccharides are galacturonic acid polymer chains such as HG, this difference in the content of uronic acid is presumably due to differences in the amount of galacturonic acid polymer. Additional qualitative studies using hydrolysis and further stepwise separations are required to determine the differences in the compositions of the individual monosaccharides in both groups.

4. Conclusions

In this study, we conducted quantitative analyses of the polysaccharides in the acidic polysaccharide fractions in two groups of rice varieties, *japonica* and *indica*, based on colorimetric methods for the first time. These results indicate that the total contents of polysaccharides of the two groups were not significantly different, but the contents of uronic acid in the acidic polysaccharide fractions were significantly different between the two groups. Significantly higher uronic acid contents were observed in *japonica* rice than in *indica* rice.

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