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Improved Quantitative Analysis of Oligosaccharides from Lichenase-Hydrolyzed Water-Soluble Barley β-Glucans by High-Performance Anion-Exchange Chromatography

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Cereal β-glucan is a linear biopolymer linked by β-(1,3)/(1,4)-glycosidic bonds. More specifically, the β-(1,4)-linked glucose chain is interrupted with β-(1,3)-linkages in cereal β-glucan structure. Elucidation of the exact length and distribution of linear β-(1,4)-linked portion facilitates the understanding of the fine structure of cereal β-glucan. A HPAEC assisted by lichenase treatment has been used for the structural and quantitative analysis of cereal β-glucan. The absence of authentic standard oligosaccharides, putatively 3-*O*-β-cellobiosyl-D-glucose (DP3) and 3-*O*-β-cellotriosyl-D-glucose (DP4), was a potential problem to the characterization of β-glucan structure. In this study, two major lichenase-hydrolyzed products were generated from the barley β-glucan, and putative 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose were separated and highly purified by recycling preparative HPLC technology. Structural analysis of highly purified putative 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose was performed by TLC and LC-MS analysis. Two putative DP3 and DP4 displayed the nonreducing end/(1,4)/(1,3) linkage ratios of 1:0.96:0.90 and 1:2.18:1.16, respectively; the molecular masses (*m*/*z*) of their sodium adducts were 527.0 and 689.0, respectively. Using these structurally confirmed oligosaccharides, the exact amounts of β-glucan lichenase hydrolysates from domestic barley cultivars were quantified. The amount of two major DP3 and DP4 accounted for only 71.4−73.3% of water-extractable β-glucan fraction, and the (1,4)/(1,3) linkage ratios of the extracted β-glucans were almost identical in the range of 2.24−2.25 among the barley cultivars tested.

KEYWORDS: Barley β-glucan; lichenase; HPAEC; oligosaccharides; recycling preparative HPLC

INTRODUCTION

Primary cell walls of the Poaceae family of higher plants comprise cellulosic microfibrils embedded in a matrix that consists predominantly of glucuronoarabinoxylans and (1,3)/(1,4)-β-D-glucans (*1*). Smaller amounts of xyloglucans, pectic polysaccharides, glucomannans, and structural proteins may also be present. The (1,3)/(1,4)-β-D-glucans are especially abundant in inner cell walls of the starchy endosperm in plants. In barley they constitute about 75% of cell walls in the starchy endosperm of grain, but are considerably less abundant in walls of other tissues. In other words, whole barley contains 4−11% of arabinoxylans, 3−7% of β-glucan, and smaller amounts of cellulose and lignin (*2*−*4*).

β-D-Glucan has not been widely applied in foods as an ingredient or a stabilizer yet. Oat β-glucan as whole oats and oat bran has been used in breakfast cereals and snack foods. Recently, purified oat β-glucan dispersion was used in the cosmetic industry as a moisturizing agent and a thickener (*4*). Even though excessively high viscosity of β-glucan is not desirable for the brewing process or feeding efficiency in poultry diets, this rheological property of β-glucans could be useful in the food industry. The biological activities of β-glucan have been studied extensively as well as its physical functionality and applicability as a food ingredient. An effective retarding effect of barley β-glucan on bile acid and glucose was proven in vitro by evaluating their diffusion rates (*5*). Barley β-glucan lowered cholesterol level in hamsters (*6*) and humans (*7*, *8*) and had hypolipodemic effects in rats (*9*). It was recently reported that the inclusion of high-viscosity barley β-glucan in the diet induced a greater proportion of Lactobacilli in the rat cecum (*10*). Even though many studies have been pursued to

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elucidate β-glucan's biological role, the mechanism of its functionality in vivo still is not well understood.

Water-soluble (1,3)/(1,4)-β-D-glucans from barley and oat endosperm have been characterized in detail. The water-soluble fraction consists of 1000 or more glucosyl residues connected in linear chains via (1,3)- and (1,4)-β-D-glucosidic linkages. The ratio of (1,4) to (1,3) linkages is in the range of 2:1 to 3:1, and most of the polysaccharide is composed of groups of two or three contiguous (1,4)-β-glucosyl residues separated by single (1,3)-β-glucosyl residues. Barley β-glucans are composed of cellotriosyl and cellotetraosyl units joined by single (1,3) linkages. However, in the water-soluble (1,3)/(1,4)-β-D-glucans from barley or other cereals, about 10% of the polysaccharide consists of block of up to 10 or more contiguous (1,4)-β-glucosyl residues. Thus, distinct longer regions of adjacent (1,4)-β-glucosyl residues are dispersed along the polysaccharide chain (*1*).

Up to now various analytical techniques, such as paper chromatography, high-performance liquid chromatography (HPLC), and high-performance anion-exchange chromatography (HPAEC), have been applied to determine the lichenase-hydrolysate profile of cereal β-glucans (*11*−*14*). The HPLC methods have been typically used to analyze carbohydrate profiles in food systems and seemed to be promising. However, these methods with RI detection did not resolve oligosaccharides with a degree of polymerization (DP) of >3. To overcome this limitation, HPAEC has been used to analyze a series of glucose-based linear chains with DP > 50. In 1994, Wood et al. successfully applied it to the characterization of the oligosaccharide profiles of lichenase-hydrolyzed barley β-glucan (*12*). Still this technique had a critical problem to quantitatively determine each oligosaccharide peak with a pulsed-amperometric detector (PAD) because the weight response factors rapidly decreased from DP2 to DP6 (*15*−*17*). Recently, matrix-assisted laser desorption−ionization mass spectrometry (MALDI-MS) was developed to quantify individual (1,3)/(1,4)-mixed-linked oligosaccharides (*14*). The basic principle of the MALDI-MS analysis was to produce the summation of Na^+ and K^+ adducts of the mother molecule and Na^+ adduct of anhydrous mother molecule. The authors reported that an excellent linear correlation was obtained on a weight basis. However, this method still used maltooligosacchride-based DP series for calibrating the mass system, and this MS-based quantification was still known to be error prone even though it is a very rapid and more sensitive approach.

The ultimate goal of our study was to develop a precise and accurate quantification method of cereal β-glucans using the HPAEC method. To achieve our purpose, we determined the structure of β-glucan extracted from domestic barley varieties in Korea and quantified the β-glucan contents using a commercial assay kit. Furthermore, the main β-glucan hydrolysates obtained by lichenase treatment, which were 3-*O*-β-cellobiosyl-D-glucose (DP3) and 3-*O*-β-cellotriosyl-D-glucose (DP4), were prepared by recycling preparative chromatography. These authentic standard oligosaccharides were applied to the quantification of the oligosaccharides produced by lichenase hydrolysis using HPAEC, and the content of barley β-glucans was estimated on the basis of the quantified amounts of DP3 and DP4 from lichenase-treated barley samples.

MATERIALS AND METHODS

Enzyme and Barley Samples or Materials. Three barley varieties, Ohl, Gang, and Gwang-an, were harvested in June 2005 and were kindly provided from Honam Agricultural Research Institute (HARI), National Institute of Crop Science (NICS), Iksan, Jeonbuk, Korea. Whole barley samples were milled with a commercial blender (HR2860, Philips), and the resulting barley flours were used in our study. α-Amylase (A3306) and Pronase (P3910) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) for the extraction process of β-glucan. A Megazyme β-glucan (mixed linkage) assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) was applied to determine the content and purity of β-glucan. A Whatman K6 TLC plate (20 × 20 cm) was used for linkage analysis, and 2,3,4,6-tetra-*O*-methyl-D-glucose (Sigma, S960225), β-1,3-glucan from *Euglena gracilis* (Fluka, 89862), and α-cyclodextrin (Sigma, C4642) were used as reference materials. Lichenase (Megazyme, E-LICHN), a (1,3)(1,4)-β-D-glucan-4-glucanohydrolase (EC 3.2.1.73), was purchased from Megazyme International Ltd.

Extraction and Purification of Water-Soluble Barley β-Glucans. Water-soluble barley β-glucan was extracted from barley flour following a modified method of Wood et al. (*18*, *19*). Briefly, barley flour (5 g) was dispersed in water, the pH was adjusted to 10.0 using 20% sodium carbonate, and then the β-glucan was extracted for 30 min in a shaking incubator (45 °C, 125 rpm). After the supernatant was separated by centrifugation at 15000*g* for 15 min, the extraction process above was repeated twice with the remaining insoluble materials, and then the extract was acidified to pH 4.0 using 2 M HCl to precipitate proteins. After removal of protein precipitates, the supernatant was treated with final 50% (v/v) of isopropyl alcohol, and the precipitates were dried in a drying oven at 60 °C for 24 h and used as crude β-glucan. The 0.3% (w/v) crude β-glucan was completely dispersed in water with boiling, and 30% (w/v) of ammonium sulfate was added to the dispersion. The resulting precipitate was redissolved with heating and dialyzed using a membrane tube (Spectrum Medical Industry; MWCO 6000−8000). The retentate was adjusted to pH 6.9 and treated with α-amylase (1013 units/100 mL of β-glucan solution) at 40 °C for 1 h. The α-amylase was heat-inactivated, and the reaction mixture was reacted with Pronase (70 units/100 mL of β-glucan solution) at pH 7.5 and 40 °C for another 1.0 h. Again, the enzyme was heat-inactivated and the precipitate was discarded by centrifugation at 15000*g* for 15 min. The resulting supernatant was dialyzed against water and lyophilized and then used as purified β-glucan for further analysis (*18*, *19*).

The β-glucan content of barley flour and the purity of β-glucan extract were determined by using a modified AOAC method, 995.16, which was adopted by the Megazyme β-glucan (mixed linkage) assay kit. Either barley flour (100 mg) or extract (10 mg) was dispersed in 0.2 mL of 50% (v/v) ethanol, and 4.0 mL of 20 mM sodium phosphate buffer (pH 6.5) was added. The mixture was fully dispersed in a boiling water bath for 3 min and incubated with 10 units of lichenase at 50 °C for 1 h. Sodium acetate buffer (200 mM, pH 4.0) was added to make 10 mL of total volume. After centrifugation at 10000*g* for 10 min, the supernatant (0.1 mL) was incubated with 0.2 unit of β-glucosidase at 50 °C for 10 min, and the amount of released glucose was assayed by a 2700 SELECT Biochemical Analyzer (YSI Life Science, Yellow Springs, OH).

Preparation of Authentic DP3 and DP4 by Recycling Preparative HPLC. Fifty grams of barley flour was dissolved in 250 mL of distilled water, then 3 M sodium carbonate (pH 10.0) was added to make pH 10.0 of the mixture, and then the mixture was incubated for 24 h. After the addition of the same volume of isopropyl alcohol, the sample dispersion was centrifuged at 15000*g* for 15 min and the supernatant discarded. The residue was redissolved in 250 mL of 20 mM sodium phosphate buffer (pH 5.0) and incubated with 40 units of lichenase at 50 °C for 72 h. Again, the solution was treated with the same volume of isopropyl alcohol, and the residue was removed after centrifugation at 10000*g* for 15 min. The supernatant was concentrated using a rotary evaporator at 40 °C, and DP3 and DP4 were separated and purified by injecting the concentrated solution into a recycling preparative HPLC (model LC-9104; JAI Ltd., Tokyo, Japan). The HPLC system equipped with an RI detector was connected to two hydrophilic preparative columns of JAIGEL W-251 and W-252 (20 ∅ × 500 mm) in this order. The peak signals corresponding to DP3 and DP 4 were monitored during the analysis, and each highly pure oligosaccharide was prepared with HPLC grade water as an eluent by a recycling mode at the flow rate of 3.0 mL/min.

Linkage Analysis of Barley β-Glucan and Authentic Oligosaccharides: Thin-Layer Chromatography (TLC). Both β-glucan extract and authentic oligosaccharides were methylated using the Hakomori method (*20*). Sodium hydride (25 mg) was dissolved in 0.5 mL of dimethyl sulfoxide (DMSO) and incubated at 90 °C for 30 min. This DMSO reagent was freshly made just before TLC analysis. The sample (10 mg) was dissolved in the DMSO reagent and was incubated at 25 °C for 3 h. Iodomethane (0.8 mL) was added to the DMSO reagent and stirred overnight at room temperature. Then, 2 mL of water was added to the DMSO solution. This mixture was extracted three times with 2 mL of chloroform ($CHCl_3$), and water was removed by sodium sulfate anhydrous. The $CHCl_3$ layer was evaporated using nitrogen gas; 1 mL of 4 M trifluoroacetic acid (CF_3CO_2H, TFA) was added to 1 mL of water, and the solution was transferred to a reaction vial that was heated for 2 h at 121 °C by a heating block. The sample was dried with a gentle nitrogen gas flow. Aliquots (1−3 μL) were placed on Whatman K6 TLC plates using a micropipet; the TLC plate was developed at room temperature with acetonitrile/$CHCl_3$/methanol (3:9:1, v/v/v). The plate was dried next to ascent. The compounds were developed on the plate by spraying the plate with a solution containing 0.3 g of *N*-(1-naphthyl)ethylenediamine and 5 mL of concentrated sulfuric acid in 100 mL of MeOH. The plate was dried and then placed in an oven for 10 min at 120 °C; blue-black spots appeared on a white background. The preparation method of methylated standard sugars was described previously in detail (*20*−*22*). Quantitative analysis of O-methylated sugars was achieved by scanning the plate with a Bio-Rad Gel-Doc system [Bio-Rad Laboratories, Segrate (Milan), Italy].

Structural Analysis and Quantification of Lichenase-Hydrolyzed Barley β-Glucan: HPAEC. The β-glucan sample (10 mg) was dissolved in 10 mL of sodium phosphate buffer (20 mM; pH 5.0) and incubated with 8 units of lichenase at 40 °C for 90 min. The reaction mixture was filtered through a disposable membrane filter (0.45 μm, MFS) and was directly injected into an HPAEC system (DX300 series, Dionex Corp., Sunnyvale, CA) (*23*). An analytical column (Carbopac PA1, 4 × 20 mm) was connected to the system, and two eluents, A and B, 150 mM NaOH and 150 mM NaOH + 600 mM NaOAc, respectively, were used at the flow rate of 1.0 mL/min. The linear gradient of eluent B was applied up to 50% from 0.01 to 50 min of elution time after 15 min of regeneration with 100% eluent A for the analysis. The potentials (E) and duration times (t) of the PAD were set at $E_1 = 0.05$ V ($t_1 = 300$ ms), $E_2 = 0.6$ V ($t_2 = 120$ mV), and $E_3 = -0.15$ V ($t_3 = 300$ ms). The absolute amount of oligosaccharides produced by lichenase hydrolysis was determined by comparison with the standard curves prepared with authentic DP3 and DP4 that were highly purified in our study.

Molecular Weight Analysis of Authentic DP3 and DP4: Liquid Chromatography−Mass Spectrometry (LC-MS). Mass spectra of the purified authentic DP3 and DP4 were obtained on an Agilent 1100 series MSD (Agilent Technologies, Palo Alto, CA) using positive electrospray mode. Sample solution was delivered to the electrospray source at a flow rate of 800 μL/min, and the injection volume was 10 μL. The eluent used was 50% (v/v) of acetonitrile in deionized water. The fragmentor voltage was set at 150 V while the capillary voltage was maintained at 4 kV. The source temperature was maintained at 350 °C. The drying gas flow in the source chamber was 11.5 L/min, and the detection scanning range was from m/z 100 to 1500.

Statistical Analysis. Statistical analysis was performed using SAS for Windows version 8.1. Statistical significance in the difference among the values was evaluated by Duncan's test. The significance level was $P < 0.05$.

RESULTS AND DISCUSSION

Extraction and Purification of Water-Soluble Barley β-Glucans. The β-glucan contents in the selected barley varieties were determined following a modified AOAC method 995.16 with the Megazyme β-glucan (mixed linkage) assay kit. Basically this method can be used to quantify total β-glucan content in barley, that is, both water-extractable and non-water-extractable fractions. Total β-glucan contents of Gang, Ohl, and Gwang-an barley cultivars were 6.39, 6.18, and 6.12% (w/w, dry basis), whereas their water-extractable β-glucan contents were 1.80, 2.00, and 1.49%, respectively (**Table 1**). These results showed that the total β-glucan contents of Korean domestic varieties were comparable with the values (3.6−6.3%) reported in other studies (*2*, *14*). Meanwhile, the water-extractable β-glucan contents were somewhat less than those of previous studies (*2*, *14*). The purity of the extracted β-glucans was determined to be 89.3 (Gang), 97.9 (Ohl), and 91.5% (Gwang-an). Greater β-glucan purity obtained in our study could explain the lower yield of water-extractable fractions. In this study, it was shown that Ohl barley had the greatest amount of water-extractable fraction of β-glucan with the highest purity of β-glucan among the barley samples tested.

Table 1. β-Glucan Content in Whole Grain of Selected Barley Varieties

barley cultivar	β-glucan content[a] (%)	extractable amount (%)	purity of β-glucan[b] (%)
Gang	6.39 (0.07)[c] a[d]	1.80 (0.02) b	89.3 (2.5) b
Ohl	6.18 (0.04) b	2.00 (0.01) a	97.9 (1.5) a
Gwang-an	6.12 (0.02) b	1.49 (0.03) c	91.5 (1.5) b

[a,b] The content and purity of β-glucan were determined by using Megazyme β-glucan (mixed linkage) assay kit, AOAC method 995.16. [c] The number in parentheses is the standard deviation. [d] Values within the same column with different letters are significantly different ($P \leq 0.05$).

Structural Determination of β-Glucan by TLC and HPAEC. The partial structural information of the purified barley β-glucans was obtained by TLC analysis. Barley β-glucan samples were methylated according to the Hakomori method (*20*) and hydrolyzed into individual sugar units by 2 M TFA treatment. Compared to other structurally confirmed carbohydrates, two distinct spots on the TLC plate were characterized to be 2,3,6-tri-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-glucose. This result indicated that the β-glucan structure of barley was exclusively composed of two types of β-(1,3) and β-(1,4) linkages (**Figure 1**). On the basis of the TLC spot intensity measured by an image analyzer, it was shown that the β-glucan of Ohl barley consisted of the greatest β-(1,4) to β-(1,3) linkage ratio of 2.36:1 and that Gang β-glucan had the lowest (**Table 2**). This TLC method has an advantage over other analysis methods because the large number of samples can be analyzed simultaneously.

Lichenase is a specific enzyme on cereal β-glucan structure, and it can cleave the β-(1,4) linkage just after β-(1,3) from the nonreducing end. Thus, this enzyme has been widely used to determine the β-glucan structure and even quantify β-glucan contents in cereals. When the lichenase hydrolysates of barley β-glucans were analyzed using HPAEC, almost identical chromatographic profiles were obtained from all three samples. When 20 μL of the hydrolysate sample (0.1%, w/v) was injected into the HPAEC system, two major product peaks on the HPAE chromatogram were speculated to be DP3 and DP4 oligosaccharides (**Figure 2**). If the unidentified peaks smaller than DP3 were ignored on the chromatograms, the percent weight fraction of the DP3 and DP4 peaks reached 88−92% (data not shown). These values were comparable to the results of previous studies (*14*, *23*−*25*). However, this quantification method accounted for only the relative amounts of both oligosaccharides based on the sum of the detectable peak areas. Moreover, the peak area cannot explain accurate weight fraction because the intensity of the PAD signal gradually decayed as the chain length increased (*15*−*17*). Thus, it is prerequisite to obtain authentic

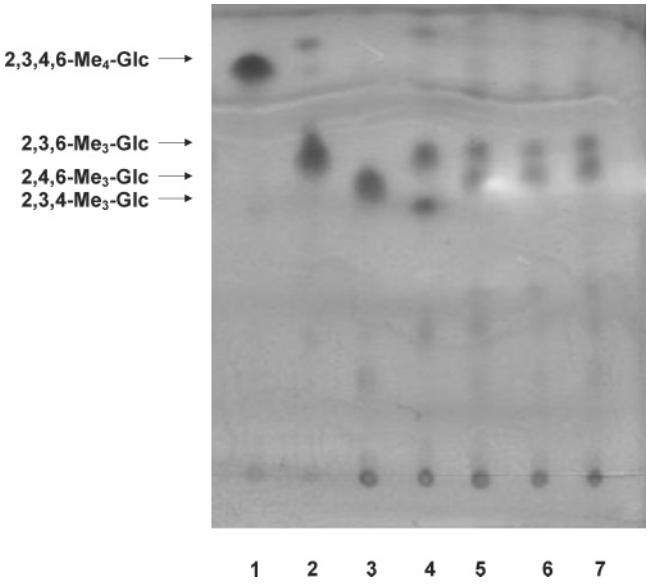

Figure 1. TLC analysis of methylated and acid-hydrolyzed barley β-glucans for the linkage determination. Lanes: 1, 2,3,4,6-Me_4-glucose; 2, 2,3,6-Me_3-glucose from α-CD; 3, 2,4,6-Me_3-glucose from β-1,3-glucan; 4, 2,3,6-Me_3-glucose and 2,3,4-Me_3-glucose from pullulan; 5, methylated and acid-hydrolyzed glucan from Gang barely; 6, methylated and acid-hydrolyzed glucan from Ohl barley; 7, methylated and acid-hydrolyzed glucan from Gwang-an barley.

Table 2. β-Glucan Linkage Ratio Determined by Spot Intensity Analysis[a]

barley cultivar	linkage	spot intensity (%)	ratio of (1,4)[b]/(1,3)[c]
Gang	(1,4)	60.80	1.55
	(1,3)	39.20	
Ohl	(1,4)	70.23	2.36
	(1,3)	29.77	
Gwang-an	(1,4)	65.75	1.92
	(1,3)	34.25	

[a] Spot intensity was determined by an image analyzer. [b] β-(1,4) linkage of purified β-glucan. [c] β-(1,3) linkage of purified β-glucan.

standard materials of DP3 and DP4 before the absolute amount of each oligosaccharide would be quantified in lichenase-hydrolyzed barley β-glucans.

When total β-glucan contents in barley flour were measured by using the Megazyme assay kit, a noticeable amount of precipitation consistently occurred during the lichenase treatment step. It was previously reported that substantial precipitation occurred during the lichenase hydrolysis of barley β-glucan (*26*), possibly due to the exposure of a sparingly soluble long linear (1,4)-linked region in the β-glucan structure. The β-(1,4)-linked chain in β-glucan is structurally identical to cello-oligosaccharide, the solubility of which is very low. If these linear chains with a β-(1,3)-linked stub were released by lichenase from β-glucan, molecular association among the linear chains became easier. Thus, the longer chains precipitated more rapidly. From this observation, it was suggested that a lower proportion of β-(1,3)-linkage interruption in β-glucan structure might result in lower solubility. We did not observe the oligosaccharides of more than DP9 from lichenase-hydrolyzed products because of either PAD detection limitation or possibly their low solubility. Even though a small amount of longer DPs was detected from the concentrated hydrolysates, it was possible that a larger proportion of them would be precipitated.

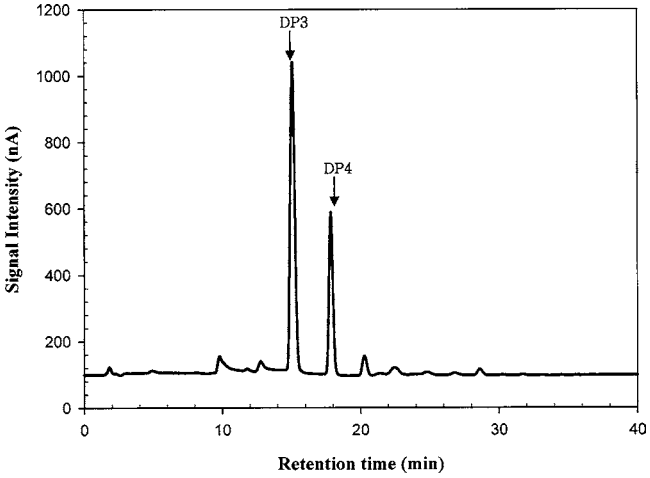

Figure 2. HPAEC elution profile of barley β-glucan hydrolysate produced by lichenase treatment.

Preparation and Structural Determination of Highly Pure Authentic DP3 and DP4. To determine absolute amounts of two major oligosaccharides released from β-glucan by lichenase hydrolysis, authentic standard oligosaccharides (DP3 and DP4) derived from the lichenase-hydrolyzed β-glucans were prepared and highly purified using a recycling HPLC system (**Figure 3**). From 50 g of Gang barley cultivar, 3.5 and 8.9 mg of DP3 and DP4 were finally obtained, respectively, and their purities were approximately 92% of total peak area detected from the HPAEC. We did not note their yields at this point. Thus, further study of optimizing the separation process would be required to increase the yield of highly pure oligosaccharides. Because the number of glucose residues in the tentatively designated DP3 and DP4 was only speculated from the HPLC retention time comparison to either malto- or cello-oligosaccharides, the molecular weights of both DP3 and DP4 were confirmed by the LC-MS analysis (**Figure 4**). Their mass spectra showed that the molecular masses (*m*/*z*) of their sodium adducts were exactly determined to be 527.0 (DP3 + Na^+) and 689.0 (DP4 + Na^+), respectively (**Figure 4A**,**B**). It was reported that positive ESI-MS spectra produced oligosaccharide ions predominantly as sodium adducts (*27*). Linkage analysis by TLC was also performed to confirm the structure of highly purified oligosaccharides. TLC analysis of methylated and TFA-hydrolyzed oligosaccharides clearly displayed three distinct spots corresponding to 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,4,6-tri-*O*-methyl-D-glucose from the top to the bottom on the plate (**Figure 5**). When the intensity of each spot was determined by color density analysis, the ratio of nonreducing end to (1,4) to (1,3) was 1:0.96:0.90 for the DP3 fraction and 1:2.18:1.16 for DP4 (**Table 3**). Two different structures, 3-*O*-cellobiosyl-glucose and 3-*O*-glucosyl-cellobiose, of highly pure DP3 could be possible from the TLC result, whereas one of them may not exist because lichenase has been known to hydrolyze the β-(1,4) linkage just after the β-(1,3) linkage. Therefore, it was concluded that the purified sample was 3-*O*-cellobiosyl-glucose. Again, two different structures were possible for highly pure DP4. Considering the factors of linkage ratio and lichenase specificity, however, 3-*O*-cellotriosyl-glucose would be the best candidate rather than 3-*O*-cellobiosyl-cellulose.

Absolute Quantification of Lichenase Hydrolysates of Barley β-Glucans. Standard curves of structurally confirmed 3-*O*-cellobiosyl-glucose and 3-*O*-cellotriosyl-glucose were prepared and compared with those of malto-DP3 and -DP4 and

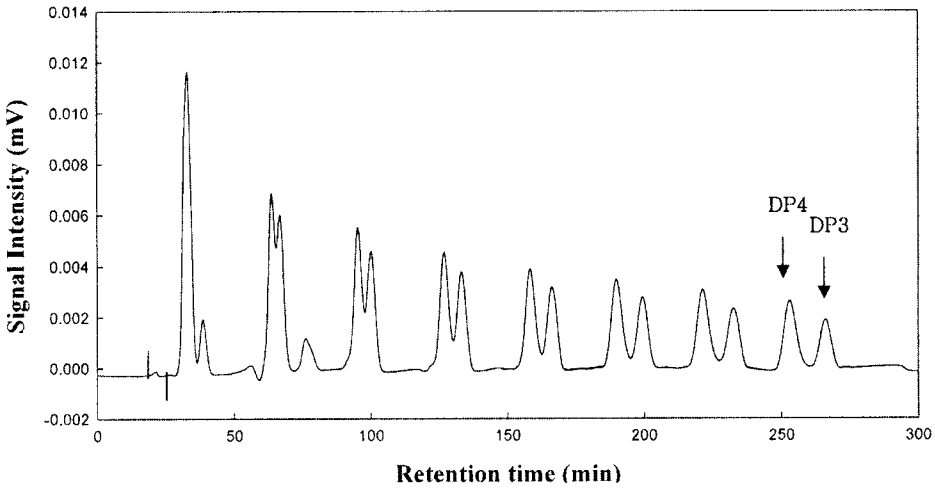

Figure 3. Separation and purification of putative DP3 and DP4 from lichenase-hydrolyzed barley β-glucan by using a recycling preparative HPLC.

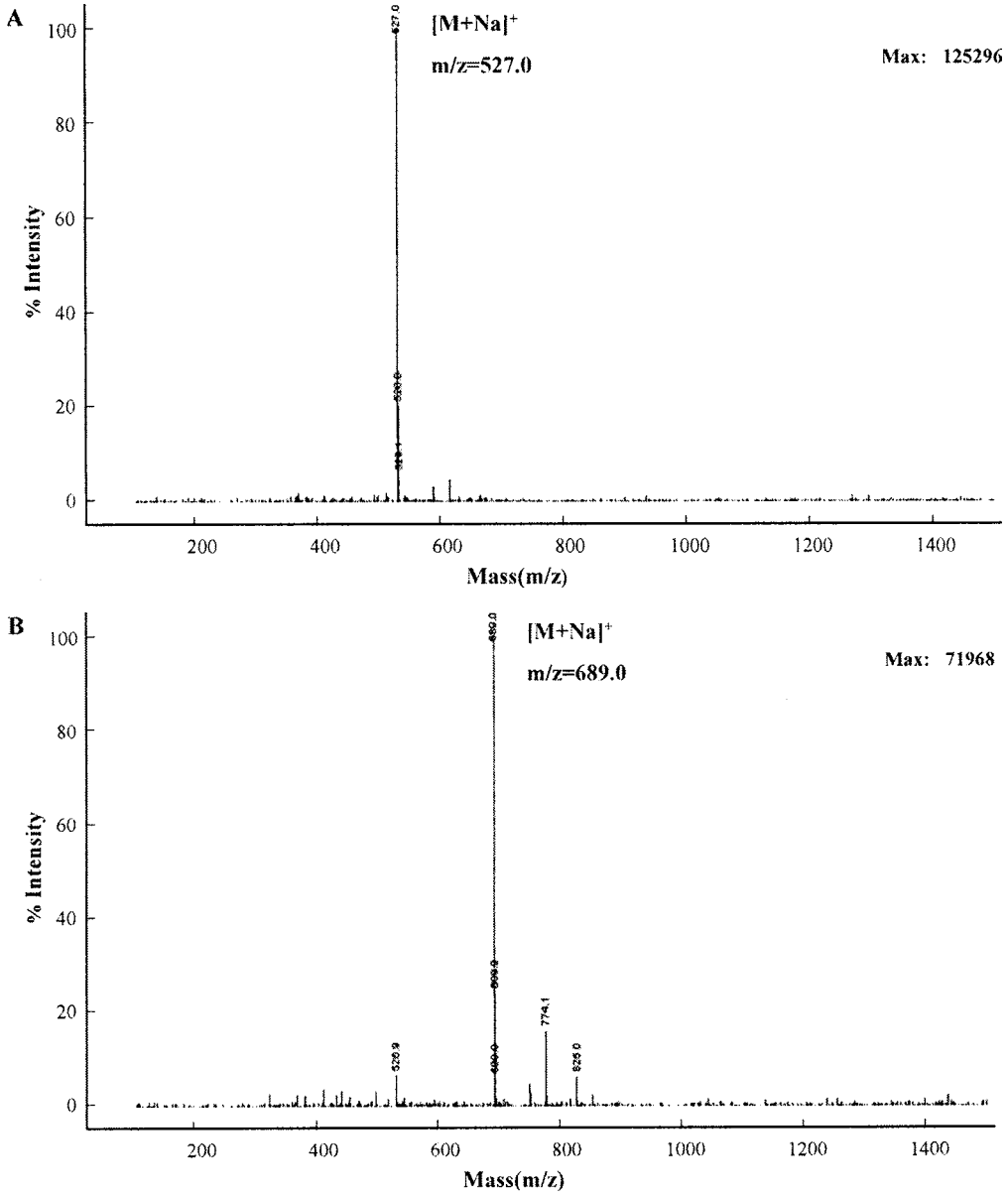

Figure 4. Molecular weight determination of highly purified putative DP3 and DP4 by LC-MS analysis.

cello-DP3 and -DP4 (**Figure 6**). The peak area of trioses on the HPAEC profile showed that the PAD signal intensity of 3-*O*-cellobiosyl-glucose was lower than those of maltotriose and cellotriose (**Figure 6A**). This difference in PAD signal intensity

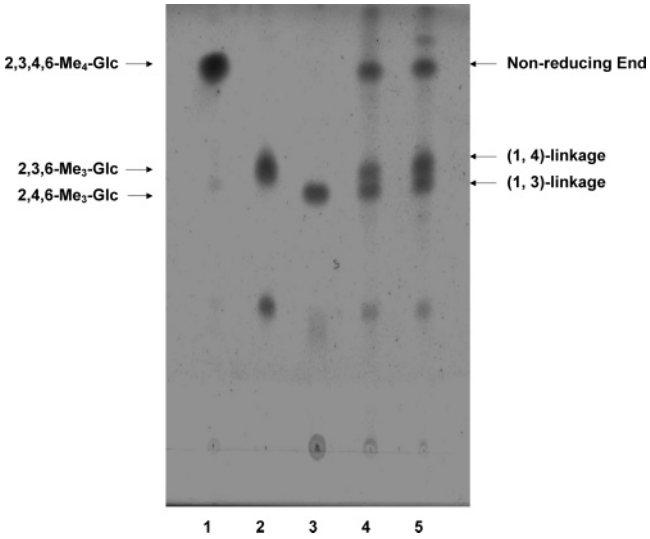

Figure 5. TLC analysis of methylated and acid-hydrolyzed highly purified DP3 and DP4 for the linkage determination. Lanes: 1, 2,3,4,6-Me$_4$-glucose; 2, 2,3,6-Me$_3$-glucose from α-CD; 3, 2,4,6-Me$_3$-glucose from β-1,3-glucan; 4, methylated and acid-hydrolyzed DP3 and DP4 from 3-*O*-β-cellobiosyl-D-glucose; 5, methylated and acid-hydrolyzed DP3 and DP4 from 3-*O*-β-cellotriosyl-D-glucose.

Table 3. Linkage Ratio Consisting of 3-*O*-β-Cellobiosyl-D-glucose and 3-*O*-β-Cellotriosyl-D-glucose by Spot Intensity Analysis

	2,3,4,6-Me$_4$-Glc (%)	2,3,6-Me$_3$-Glc (%)	2,4,6-Me$_3$-Glc (%)	end[a]:(1,4)[b]:(1,3)[c]
DP3[d]	35.03	33.58	31.38	1:0.96 :0.90
DP4[e]	23.01	50.20	26.79	1:2.18 :1.16

[a] Glucose residue at nonreducing end. [b] β-(1,4) linkage of purified β-glucan. [c] β-(1,4) linkage of purified β-glucan. [d] 3-*O*-β-cellobiosyl-D-glucose. [e] 3-*O*-β-cellotriosyl-D-glucose.

was more obvious when 3-*O*-cellotriosyl-glucose was compared with maltotetraose and cellotetraose (**Figure 6B**). Thus, the actual amounts of oligosaccharides would be underestimated when maltooligosacharide-based standard curves are used for quantitative analysis of 3-*O*-cellobiosyl-glucose and 3-*O*-cellotriosyl-glucose produced by lichenase hydrolysis. On the basis of the standard curves of authentic DP3 and DP4, therefore, the weight percentages of β-(1,3)/(1,4)-mixed-linked DP3 and DP4 were calculated from lichenase-hydrolyzed oligosaccharides. As shown in **Table 4**, the weight fractions of DP3 and DP4 were obtained within very narrow ranges from 45.7 to 46.5% and from 25.7 to 26.8%, respectively, among the β-glucan samples. These two major hydrolyzed products accounted for 71.4−73.3% of total β-glucan content, which was determined by a lichenase-based assay kit. Actually, the relative weight percent fraction of DP3 plus DP4 was around 90% on the HPAE chromatogram as discussed above. When we considered the specific hydrolysis pattern of lichenase on β-glucan structure, the linkage ratio of (1,4) to (1,3) was identical among the samples tested. Compared to other reported studies, this linkage ratio of 2.24−2.25 was exactly matched to 2.24 by HPLC and 2.23 by MALDI-MS even though absolute amounts of DP3 and DP4 were quantified to be quite different (*14*). This linkage ratio could be an indicator or fingerprint of specific plant-based β-glucan. Barley samples having more diverse genetic background should be analyzed to confirm the constant linkage ratio. When the absolute amounts were

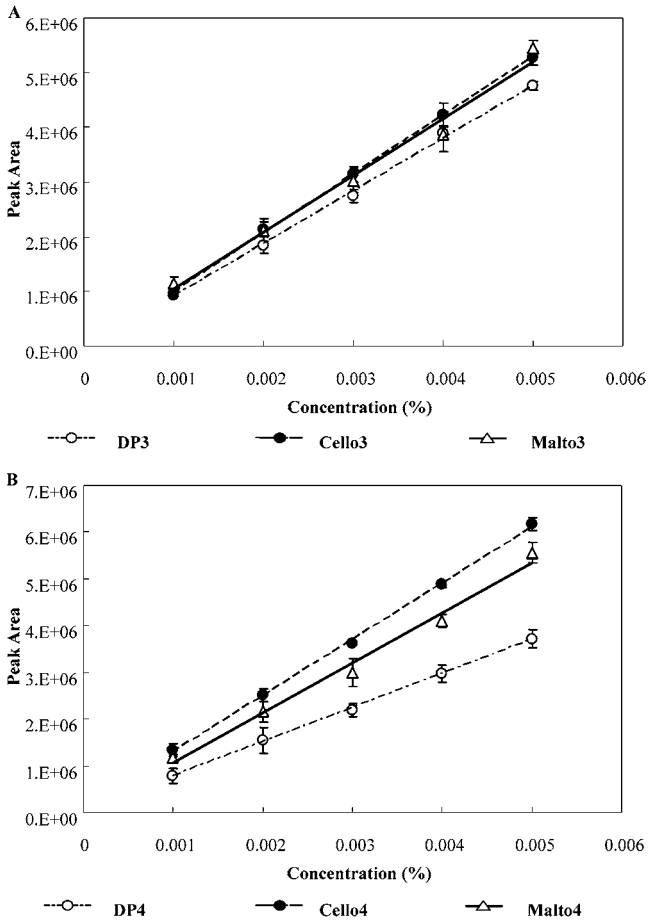

Figure 6. Comparison of standard curves among glucose-based DP3s (**A**) and DP4s (**B**).

Table 4. Absolute Contents of Oligosaccharides in Lichenase-Hydrolyzed Water-Soluble β-Glucan from Three Barley Varieties

	DP3 (%)	DP4 (%)	total amount of DP3 and DP4 (%)	weight ratio of DP3/DP4	molar ratio of DP3/DP4	(1,4)[a]/(1,3)[b]
Gang	46.47 a[c]	26.82 a	73.29 a	1.73 a	2.29 a	2.25 a
SD	0.79	1.17	1.96	0.05	0.06	0.00
Ohl	45.73 a	26.73 a	72.47 a	1.71 a	2.26 a	2.25 a
SD	0.42	0.99	1.41	0.05	0.06	0.01
Gwang-an	45.74 a	25.67 a	71.41 a	1.78 a	2.35 a	2.24 a
SD	0.58	0.63	1.21	0.02	0.03	0.00
HPLC[d]	55.74	31.06	86.80	1.79	2.37	2.24
SD	2.94				0.16	
MALDI-MS[e]	57.55	31.97	89.52	1.80	2.38	2.23
SD					0.14	

[a] β-(1,4) linkage of purified β-glucan. [b] β-(1,4) linkage of purified β-glucan. [c] Values within the same column with different letters are significantly different ($P \leq 0.05$). [d,e] Data were adopted from ref *14*.

compared, more than 10% of DP3 and 5% of DP4 were lower than the results of the other work. From the results, the response sensitivity of authentic β-(1,3)/(1,4)-mixed-linked DP3 was smaller than those of malto- and cello-DP3s (**Figure 6**). These discrepancies in the response sensitivity were even prominent when DP4s were compared within the concentration range from 0.1 to 0.5%. Therefore, a more careful analytical method should

be considered to quantify the building blocks of β-glucan structure.

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