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Structural features of immunostimulatory polysaccharide purified from pectinase hydrolysate of barley leaf

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ABSTRACT

Four polysaccharide fractions were isolated from young barley leaves treated with or without pectinase followed by ethanol fractionation. Among the polysaccharide fractions, BLE-P isolated from pectinase digested with a high molecular weight had the most enhanced macrophage stimulatory activity, indicating that pectinase digestion of barley leaf is a useful method for enhancement of its activity. BLE-P was further purified by column chromatography to identify the chemical and structural properties. BLE-P-I eluted in void volume fraction showed potent macrophage stimulatory activity. Monosaccharide composition and linkage analysis indicated that at least three kinds of polysaccharide, that is, glucuronoarabinoxylan (GAX; 40–45%), rhamnogalacturonan-I (RG-I) with branching mainly involving a type II arabinogalactan (AG-II) side chain (30–35%), and linear glucan such as starch and cellulose (less than 10%) coexisted in BLE-P-I. Given the association with macrophage stimulatory activity, it is likely that the GAX and to the RG-I polysaccharide branched with an AG-II side chain may be important for expression of the activity in barley leaf.

1. Introduction

In vascular plant (angiosperms and gymnosperms), the cell wall is a dynamic and highly specialized network formed by a heterogeneous mixture of cellulose, hemicelluloses, pectins, and to a lesser extent, proteins and phenolic compounds [1]. Monocotyledon families belonging to angiosperm can be divided into two groups depending on the presence or absence of ferulic acid ester-linked to their unlignified cell wall. As the major type of non-cellulosic polysaccharide in the cell wall of monocotyledon, one group having ferulic acid ester is the hemicelluloses and the other group not having ferulic acid ester is the pectic polysaccharides [2,3]. Hemicelluloses are polysaccharide in plant cell walls that have β-(1 → 4)-linked backbones with an equatorial configuration which include heteroglucans, heteroxylans, heteromannans, and β-(1 → 3, 1 → 4)-glucans [4]. Especially, heteroxylans are the major non-cellulosic polysaccharides possibly in the lignified secondary walls in the commelinid monocotyledons [3]. Pectic substances are complex high molecular mass glycosidic macromolecules in the unlignified cell wall and are the major components of the middle lamellae in the commelinid monocotyledons. Three major pectic polysaccharides groups, namely, homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II), are recognized, all containing D-galacturonic acid to a greater or a lesser extent [5].

Barley (*Hordeum vulgare* L.) belonging to commelinid monocotyledon is widely distributed and cultivated in eastern Asia and used in foodstuffs such as bread and cakes [6]. It is well known that the cell wall polysaccharide of barley grain is generally rich in β-(1 → 3, 1 → 4)-glucan, whereas other grains such as rye and wheat cell walls contain higher levels of arabinoxylan (AX) [7]. Young barley leaf is a well-known component of a green-colored drink named "Aojiru" in Japan, which is harvested 2 weeks after seeding [8]. Recently, several animal and human studies revealed that the young barley leaf has beneficial activities such as antiulcer, antioxidant, hypolipidemic, antidepressant, and antidiabetic effects [6,9–11]. However, the physiological effects of barley leaf have not been investigated to the same extent as those of barley grain. Furthermore, no studies have reported the structural characteristics of polysaccharides such as the linkage position, kinds of side chain, or the anomeric configuration in barley leaf.

Pectinases comprise an enzyme group that catalyzes pectic substance degradation through depolymerization (hydrolases and lyases) and deesterification (esterases) reactions. The well-known

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pectinolytic enzymes are homogalacturonan-degrading enzymes, especially polygalacturonanase [12]. In the literatures, various pharmacological activities have been observed in pectic polysaccharides isolated from plants, and the activities appear to be dependent on the RG-I and RG-II regions rather than the HG region [13–15]. Therefore, the hydrolysis of the HG region using pectinase will easily release the RG-I and RG-II regions, which are expected to be promising biologically active moieties [16].

Therefore, in this study, young barley leaf treated with pectinase to enhance the immunostimulatory activity, and the pectinase-digested polysaccharide was characterized by isolation, purification and structural investigation.

2. Materials and methods

2.1. Preparation of polysaccharide fractions from pectinase-treated barley leaf

Young barley leaves harvested 2 weeks after seeding were cultivated in Gyeonggi-do, Korea, and commercial pectinase (Rapidase C80MAX) was purchased from Vision Corporation (Seongnam, Gyonggi, Korea). The dried barley leaf (1 kg) was suspended in 20 L of water followed by pH adjustment to 4.5; 20 mL of commercial pectinase was then added. The mixture was incubated for 3 days at 50 °C, followed by heating at 100 °C for 15 min to inactivate the enzyme. The pectinase–hydrolysate of barley leaf was centrifuged at 7,000 rpm for 20 min to remove insoluble precipitate, and then clear supernatant was precipitated by the addition of four volumes of 95% cold ethanol to obtain a crude polysaccharide. The rapid isolation method for fractionation of the crude polysaccharide according to size of molecular weight was referred to Lee et al. [17]. Briefly, the crude polysaccharide was suspended with 65% ethanol and vigorously mixed, and then centrifuged at 7,000 rpm for 20 min. The ethanol-insoluble precipitate was dialyzed using a Spectra/Por 2 membrane (molecular weight cutoff, MWCO 12,000–14,000; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) to obtain relative high molecular-weight polysaccharide fraction, whereas ethanol-soluble supernatant was not dialyzed to yield relative low molecular-weight polysaccharide fraction. Both supernatant and precipitate were lyophilized to yield polysaccharide fractions (BLE-S and BLE-P) separated by the size of molecular weight from pectinase-treated barley leaf (Fig. 1). BLW-S and BLW-P were also prepared from hot water extract without the pectinase digestion by the same procedure to give pectinase-untreated polysaccharide fractions from barley leaf as respective controls (Fig. 1).

2.2. Purification of immunostimulatory polysaccharide fraction isolated from pectinase-treated barley leaf

The lyophilized BLE-P (300 mg) was dissolved in water and fractionated by gel filtration chromatography involving Sephadex G-100 (GE Healthcare, Uppsala, Sweden) packed into a column (2.5 cm i.d. × 90 cm). Ammonium formate buffer (50 mM at pH 5.5) was used as an eluent at a flow rate of 1 mL/min. The resulting eluates were fractionated into four different subfractions through the analysis of the total carbohydrate, uronic acid, protein, and 2-keto-3-deoxy-manno-octulosonic acid (KDO)-like materials described below (see Section 2.3). Each subfraction was dialyzed using a Spectra/Por 2 membrane (MWCO 12,000–14,000; Spectrum Laboratories Inc.) to remove buffers, and then lyophilized to obtain four purified polysaccharide subfractions (BLE-P-I, BLE-P-II, BLE-III, and BLE-P-IV).

2.3. Analytical methods

The contents of total carbohydrate, uronic acid, protein, and KDO-like materials in the column eluates were measured by the phenol-sulfuric acid method [18], *m*-hydroxybiphenyl method [19], Bradford method [20] with protein assay dye (Bio-Rad laboratories, Hercules, CA, USA), and the modified thiobarbituric acid (TBA) positive method [21], respectively, using galactose (Gal), galacturonic acid (GalA), bovine serum albumin (BSA), and KDO as the respective standards. Compositions of aldoses and uronic acids of the polysaccharide were analyzed by the alditol acetate derivatives method [22] with a slight modification using GC on an ACME-6100 GC system (Young-Lin Co., Anyang, Korea) equipped with a SP-2380 capillary column (0.2 μm film, 0.25 mm i.d × 30 m; Supelco, Bellefonte, PA, USA). Briefly, alditol and aldonic acids were successively separated by means of Sep-Pak QMA cartridge (Waters, Massachusetts, USA), following acid-hydrolysis and $NaBH_4$ reduction of polysaccharide. The alditols eluted with deionized water were converted to alditol acetates by acetylation, whereas the absorbed aldonic acids were eluted and converted aldolactones with 1 M HCl. The resulting lactones were then reduced to alditols under neutral conditions and acetylated to form the alditol acetates. The analysis was performed with the following temperature schedule: 60 °C → 220 °C (30 °C/min), 220 °C for 8 min, 220 °C → 250 °C (8 °C/min), 250 °C for 15 min. Response factors of individual monosaccharides were determined by dividing each peak area to the area of the internal standard (*myo*-inositol) peak, and the respective molar ratio of monosaccharides was determined from the peak areas and response factors which calculated by internal standard peak.

2.4. Methylation analysis

Methylation analysis was performed according to the methods of Hakomori [23] and Kim et al. [24] with slight modifications. Briefly, after 10 μL of glycerol (Sigma) was added to the 800 μL of polysaccharide solution (mg/mL) for the enhancement of solubility and permethylation, the solution was dried under a nitrogen-flushing heating block (Eyela MG-2200, Tokyo, Japan). After the sample was methylated by addition of methylsulfinyl carbanion with DMSO and methyl iodide, the methylated product was recovered using a Sep-pak C_{18} cartridge (Waters, Dublin, Ireland) eluted with EtOH. The product was hydrolyzed with 1 M trifluoroacetic acid for 4 h at 121 °C and then reduced with $NaBH_4$ followed by acetylation. The resulting partially methylated alditol acetates (PMAAs) were dissolved with 10 μL of acetone (Sigma) and analyzed using a GC–MS (6890 GC/5975 MSD; Agilent, Santa Clara, CA, USA) equipped with a SP-2380 capillary column. The analysis was performed with the following temperature schedule: 60 °C for 1 min, 60 °C → 150 °C (30 °C/min), 150 °C → 180 °C (1 °C/min), 180 °C → 231 °C (1.5 °C/min), 231 °C →250 °C (30 °C/min), 250 °C for 10 min. PMAAs were identified by their fragment ions and relative retention times, and their mole percentage was estimated from the peak areas and response factors on a flame ionization detector (FID).

2.5. Cell culture and macrophage stimulatory activity of polysaccharide

Roswell Park memorial Institute (RPMI)-1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and fungizone (amphotericin B) were obtained from Gibco BRL Co. (Grand Island, NY, USA). RAW 264.7 murine macrophage cell line was obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 100 U penicillin, 100 μg/mL streptomycin, and 10%