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Research Article

Structural characteristics of a red ginseng acidic polysaccharide rhamnogalacturonan I with immunostimulating activity from red ginseng

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ABSTRACT

Background: Many researchers reported that the various immune activities of red ginseng are due to acid polysaccharides. But, the exact structural characteristics of the acidic polysaccharide in red ginseng have not been fully elucidated. Therefore, we isolated the acidic polysaccharide from red ginseng and characterized the structural property of the active moiety of this polysaccharide, which contributes to the immunostimulatory activity of red ginseng.

Methods: A polysaccharide (RGP-AP-I) was purified from red ginseng via size-exclusion chromatography using Sephadex G-100. Immunostimulatary activity of RGP-AP-I was investigated via anti-complementory and macrophage stimulatory activity. The structure of RGP-AP-I was characterized by HPLC, sugar composition, β-glucosyl Yariv reagent and methylation analysis.

Results: Peritoneal macrophages stimulated using RGP-AP-I significantly augmented the production of various cytokines such as interleukin (IL)-6, IL-12, and tumor necrosis factor (TNF)-α. The primary structure of RGP-AP-I was elucidated by assessing its sugar composition and methylation analysis. RGP-AP-I is a 96 kDa acidic polysaccharide, and comprises nine different monosaccharides, which mainly include sugars such as rhamnose (Rha, 9.5%), galacturonic acid (GalA, 18.4%), galactose (Gal, 30.4%), and arabinose (Ara, 35.0%). RGP-AP-I exhibited an considerable reaction with the β−glucosyl Yariv reagent, revealing the presence of arabino-β-3,6-galactan. Methylation analysis indicated that RGP-AP-I comprises 21 different glycosyl linkages, such as 3-, 4-, 6- and 3,6-linked Galp; 5-linked Araf; 2,4-linked Rhap; and 4-linked GalAp, which are characteristics of rhamnogalacturonan I (RG-I).

Conclusion: we assumed that the immunostimulatory activity of RGP-AP-I may be due to the RG-I structure, which comprises a main chain with a repeating linkage unit, [→2)-Rhap-(1→4)-GalAp-(1→] and three groups of side chains such as (1→5)-linked arabinan, (1→4)-linked galactan, and arabino-β-3,6-galactan, which branch at the C(O)4 positions of Rha residues in the main chain of RGP-AP-I.

1. Introduction

Representative herbal medicine Korean ginseng (the root of *Panax* ginseng Meyer) is ethnopharmacologically used in the East Asian countries such as Korea and China [1]. They have been considered to be a supplementary herbal medicine that exerts modulatory activities in numerous diseases such as cardiovascular diseases [2], cancer [3], diabetes [4], and hypertension [5]. Active constituents of ginseng have been reported to be ginsenosides, polyphenols, flavonoids, and polysaccharides [6]. Among these, ginsenosides have been recognized as the most active ingredients, having various pharmacological activities [6]. In contrast, the pharmacological efficacies of ginseng polysaccharides have not been as actively investigated as those of ginsenosides.

Meanwhile, fresh ginseng is easily degraded by several physical factors. Considering its characteristics, red ginseng (RG) is frequently processed from fresh ginseng *via* simple or continuous steaming/drying process. In general, the processed RG has

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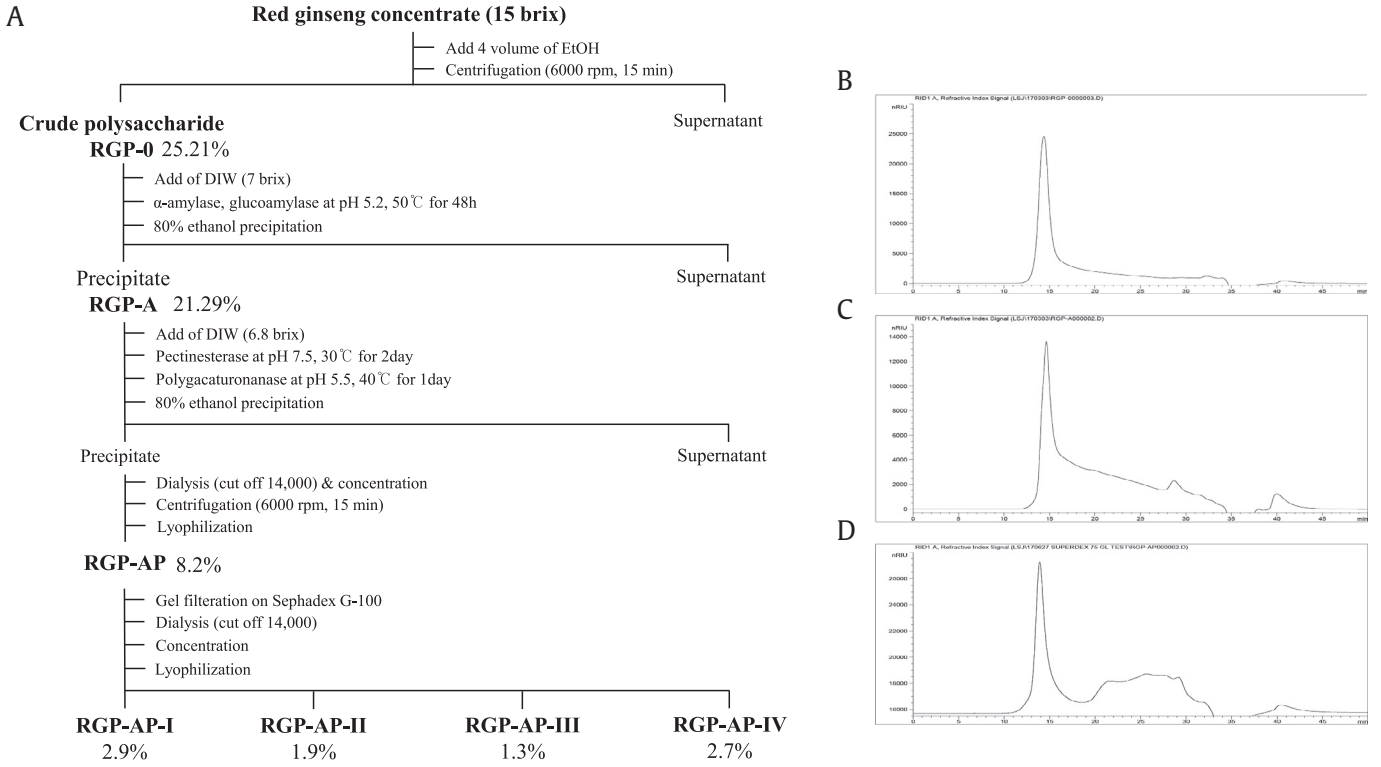

Fig. 1. Purification scheme of active polysaccharides from red ginseng concentrate. (A) Purification scheme of polysaccharides with different molecular weights from enzyme digests of red ginseng concentrate. Elution pattern of (B) RGP-0, (C) RGP-A, and (D) RGP-AP on high-performance size-exclusion chromatography (HPSEC) linked with Superdex 75 GL column.

significantly higher biological activity and fewer side effects than the fresh and white ginsengs [7,8]. Recently, many researchers reported that the various immune activities of RG are due to acid polysaccharides [1,9–11]. But the exact structural characteristics of the acidic polysaccharide in RG have not been fully elucidated because of their complexity and heterogeneity.

At present, an analytical technique for the structural characterization of natural polysaccharides is necessary; however, owing to their diversity and structural complexity, this characterization seems difficult. Appropriate characterization involves the determination of sugar compositions, configurations, anomerizations, ring forms, glycosyl linkages, and sequences of the polysaccharides [12]. Methylation is widely used to determine the linkage of complex carbohydrates; it involves the hydrolysis of a polysaccharide to a partially methylated alditol acetate (PMAA) and is carried out via GC–MS [13].

In this study, we isolated the acidic polysaccharide from RG and characterized the structural property of the active moiety of this polysaccharide, which contributes to the immunostimulatory activity of RG.

2. Materials and methods

2.1. Material and animals

RG concentrates (15 brix) were supplied by the Korean Ginseng Corporation (KGC Co., Daejeon, Republic of Korea). Carbohydrate-digesting enzymes, such as α-amylase from *Aspergillus oryzae*, amyloglucosidase from *Aspergillus niger,* and pectinesterase from orange peel were all obtained from Sigma (St. Louis, MO, USA), whereas polygalacturonase from *Aspergillus aculeatus* was purchased from Megazyme (Bray, Ireland). Sephadex G-100 was obtained from GE Healthcare (Uppsala, Sweden). Sheep red blood cells were obtained from Innovative Research, Inc. (Novi, MI, USA). Dulbecco's Modified Eagle's Medium, fetal bovine serum, and other cell culture supplements were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other analytical reagents were purchased from Sigma (USA).

Female Balb/c mice (6 weeks old) were purchased from SaeronBio Inc. (Uiwang, Gyeonggi, the Republic of Korea). They were housed in a specific pathogen–free room maintained at a constant temperature (20 ± 1°C) and humidity (55%) in a clean rack with 12 h of light and dark cycles. Water and pellet diet were supplied *ad libitum*. All animal experiments were carried out according to the instructions by the Ethics Committee for Use of Experimental Animals at Kyonggi University (admission number: 2017-007).

2.2. Isolation and purification of polysaccharide from RG concentrates

Isolation and purification of polysaccharides from RG concentrate were carried out via ethanol precipitation, enzyme hydrolysis, and size exclusion chromatography (Fig. 1). Briefly, 95% cold ethanol (EtOH) was added to the RG concentrate, and precipitate was collected and redissolved with water, followed by dialysis for 3 days using a dialysis membrane (cutoff: 12,000–14,000; Sigma). The solution was lyophilized to obtain a crude polysaccharide (RGP-0) from RG concentrate. To remove the starch-like materials, the lyophilized RGP-0 was suspended in the solution (pH 5.2), α-amylase (3000 units) and amyloglucosidase (300 units) were added, and hydrolysis was performed at 50°C for 48 h. After heating for 15 min, the enzyme reaction was quenched, and four volumes of 95% EtOH were then added to precipitate the polysaccharide fraction from the enzyme digest of RGP-0. Dried polysaccharide, which was named as RGP-A, was prepared after dialysis and lyophilization as mentioned previously. To remove homogalacturonan (HG), RGP-A was treated with pectinesterase for 48 h (pH 7.5; 30°C) and was then hydrolyzed with polygalacturonase for 24 h (pH 5.5; 40°C). The enzymatic hydrolysates were eventually prepared as dried

RGP-AP by the same method as mentioned previously. RGP-AP was applied to a column (3 × 120 cm) packed with Sephadex G-100, and elution was carried out with 50 mM ammonium formate buffer (pH 5.5). Four major polysaccharides subfractions (RGP-AP-I, RGP-AP-II, RGP-AP-III, and RGP-AP-IV) were obtained followed by desalination using dialysis membrane and lyophilization (Fig. 2 (A)).

2.3. General analysis

The contents of total carbohydrate, uronic acid, 2-keto-3-deoxy-D-manno-octulosonic acid–like materials, and protein in the polysaccharide samples were measured by phenol–sulfuric acid method [14], *m*-hydroxybiphenyl method [15], modified thiobarbituric acid method [16], and Bradford method [17] with protein assay dye (Bio-Rad laboratories, Hercules, CA, USA), respectively. Sugar compositions were determined by the 1-phenyl-3-methyl-5-pyrazolone derivatives method [18,19] by HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with SPD-20A UV/VIS detector and Acclaim 120 C18 column (Thermo Fisher Scientific). The optimal analytical condition of the column was maintained at 30°C temperature. The 1-phenyl-3-methyl-5-pyrazolone derivative elution was carried out with a mixture of 82% phosphate buffer (0.1 M, pH 6.7) and 18% acetonitrile at a flow rate of 1 mL/min, and UV absorbance of the eluate was monitored at 245 nm.

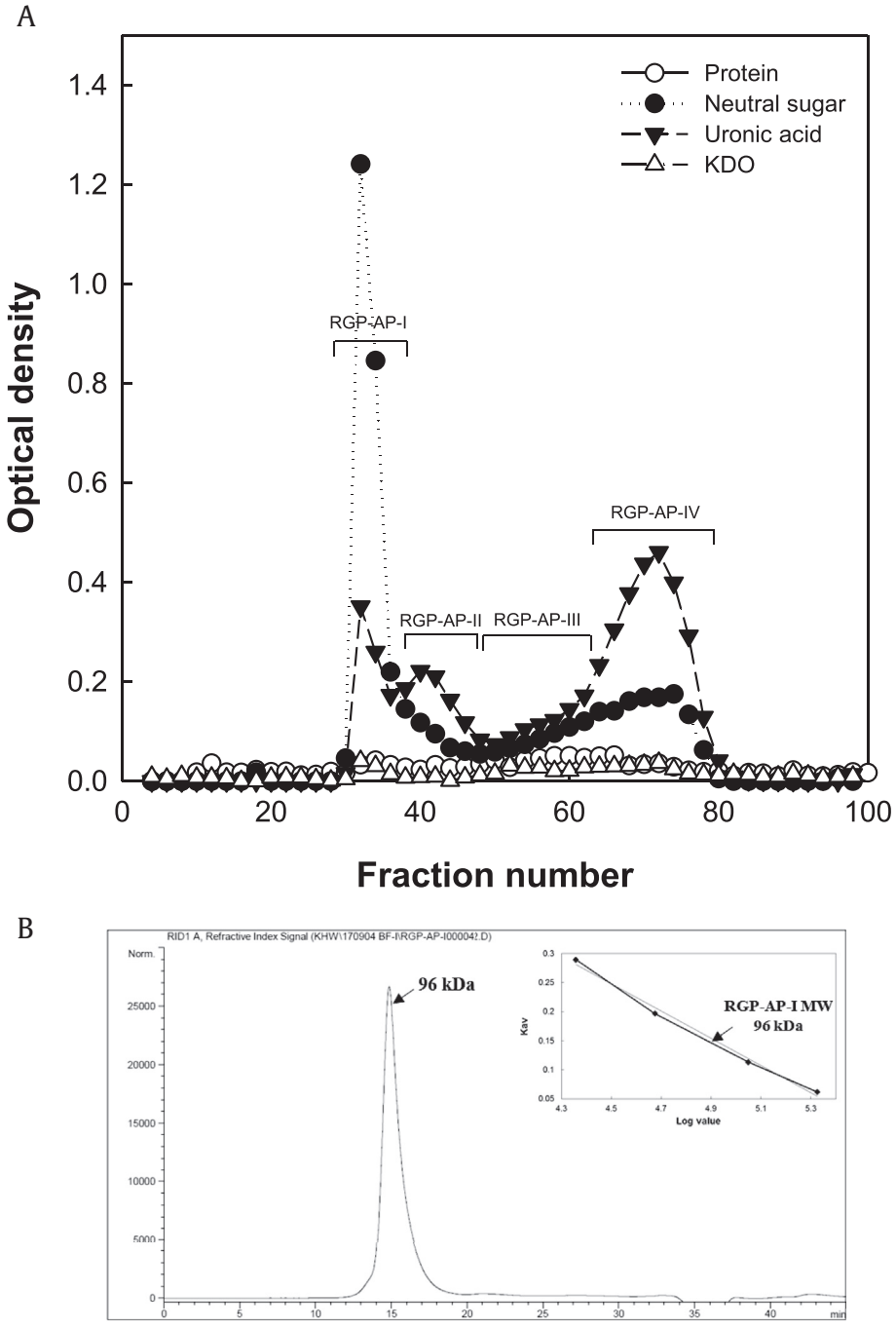

Fig. 2. (A) Elution profile of RGP-AP by gel permeation chromatography using Sephadex G-100 packing and (B) molecular weight determination of RGP-AP-I. RGP-AP was subjected to Sephadex G-100 column (3 × 90 cm) and was eluted with 50 mM ammonium formate buffer (pH 5.5) at a flow of 1 mL/min. To determine the molecular weight, high-performance size-exclusion chromatography was performed using Superdex 75 GL column. KDO, 2-keto-3-deoxy-D-manno-octulosonic acid.

Table 1
Chemical properties and sugar components of polysaccharides purified from red ginseng concentrate

Chemical composition	RGP-0	RGP-A	RGP-AP	RGP-AP-I	RGP-AP-II	RGP-AP-III	RGP-AP-IV (%)
Neutral sugar	74.3 ± 0.3	51.8 ± 1.2	52.4 ± 1.2	67.7 ± 0.6	51.7 ± 0.0	48.6 ± 0.9	34.2 ± 0.5
Uronic acid	24.6 ± 0.2	46.8 ± 0.8	46.5 ± 0.8	29.8 ± 1.1	45.0 ± 0.4	46.8 ± 0.3	62.5 ± 3.9
Protein	1.0 ± 0.5	1.2 ± 0.5	1.1 ± 0.5	2.5 ± 0.2	3.2 ± 0.0	4.6 ± 0.3	3.4 ± 0.2
KDO-like material[1]	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
Component sugar[2]							(Mole%)[3]
Rhamnose	1.9 ± 0.1	4.4 ± 0.2	4.9 ± 0.3	9.5 ± 0.2	9.3 ± 0.3	6.8 ± 0.3	1.4 ± 0.0
Fucose	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.8 ± 0.0	0.5 ± 0.1
Arabinose	6.7 ± 0.2	16.4 ± 0.4	22.0 ± 1.0	35.0 ± 0.4	25.4 ± 0.8	19.6 ± 0.5	4.2 ± 0.1
Xylose	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Mannose	0.3 ± 0.0	0.1 ± 0.0	0.7 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.4	0.5 ± 0.1
Galactose	8.4 ± 0.1	21.3 ± 0.4	23.2 ± 0.7	30.4 ± 0.2	19.9 ± 0.5	24.6 ± 0.7	10.5 ± 0.3
Glucose	56.3 ± 0.1	9.0 ± 0.4	2.3 ± 0.2	1.7 ± 0.0	1.2 ± 0.2	2.6 ± 0.1	4.1 ± 0.1
Galacturonic acid	24.1 ± 0.5	46.3 ± 0.6	44.4 ± 2.4	18.4 ± 1.1	38.6 ± 2.0	38.9 ± 2.1	74.4 ± 0.5
Glucuronic acid	0.5 ± 0.0	0.6 ± 0.0	0.9 ± 0.1	1.5 ± 0.1	1.2 ± 0.0	1.4 ± 0.0	0.7 ± 0.1

[1] KDO, 2-keto-3-deoxy-D-manno-octulosonic acid.
[2] Monosaccharides were analyzed using alditol acetates.
[3] Mole % was calculated from the detected total carbohydrate.

High-performance size-exclusion chromatography of the polysaccharide sample was carried out using an Agilent 1260 Infinity LC system (Agilent Technologies Co., Ltd., Palo Alto, CA, USA) according to the method described by Lee et al. [20].

2.4. Anticomplementary activity

The anticomplementary activity was measured by the method reported by Mayer [21]. In brief, sample was mixed with normal

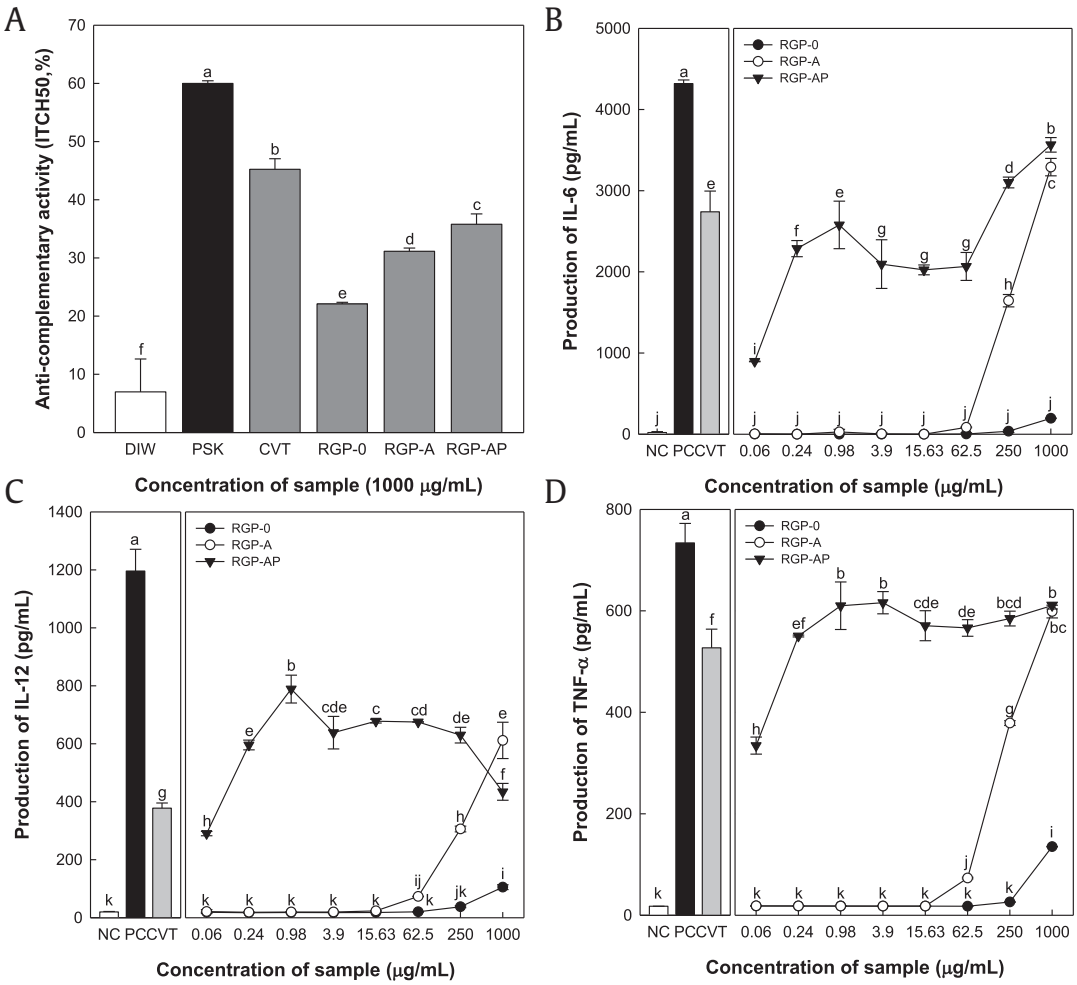

Fig. 3. Effect of RGP-0, RGP-A, and RGP-AP on the (A) anticomplementary activity and cytokine production of macrophage-stimulating cytokines such as (B) IL-6, (C) IL-12, and (D) TNF-α of murine peritoneal macrophages. The anticomplementary activity was presented as the inhibition of 50% total complementary hemolysis (ITCH, 50%) by Mayer's method. Polysaccharide K (PSK, 1 mg/mL) and CVT-E002 (CVT, 1 mg/mL) were used as immunostimulatory positive controls. Production of macrophage-stimulatory cytokines was analyzed in the cell culture medium of peritoneal macrophage. Only medium and LPS (5 μg/mL) were used as the negative control (NC) and positive control (PC), respectively. Means with different superscript letters indicate significant differences at $p < 0.05$ by Duncan's multiple range test. IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide.

human serum (complement sauce) with gelatin veronal buffer (pH 7.4) and activated at 37°C. After 30 min, IgM from hemolysin-sensitized sheep erythrocytes (EA cells, 1×10^8 cells/mL) were added to the mixture and incubated at 37°C for 1 h. Two and a half milliliters of phosphate-buffered saline was added to terminate the reaction and centrifuged at 800 ×g for 10 min. Optical density of the supernatant was measured at 412 nm. Polysaccharide K (PSK) from *Coriolus versicolor* and CVT-E002 (sold commercially as Cold-FX, Valeant Pharmaceuticals International, Canada) from *Panax quinquefolium* were used positive controls [22,23]. The anti-complementary activity has been expressed as follows:

Total hemolytic complement (TCH_{50}) (%) = TCH_{50} (control) − TCH_{50} (treated with sample)/TCH_{50} (control)

2.5. Assay of cytokines on murine peritoneal macrophages

To determine the immunostimulatory activity of polysaccharide samples, peritoneal macrophages were isolated from Balb/c mice injected with 5% thioglycollate medium, as previously described [24]. The collected macrophages were suspended in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 1% penicillin/streptomycin (Thermo Fisher Scientific) plated at a density of 2.5×10^6 cells/mL into 96-well culture plates (100 μL). After 2 h, polysaccharide samples (100 μL) were added to the cells and incubated at 37°C for 24 h. The concentration of cytokines [IL-6, IL-12, and tumor necrosis factor (TNF)-α] accumulated in the culture medium was determined using enzyme-linked immonusorbent assay kits (BD Biosciences, San Diego, CA, USA; eBioscience, San Diego, CA, USA) according to the manufacturer's protocols.

2.6. Determination of type II arabinogalactan by β-D-glucosyl Yariv reagent

The amount of arabino-β-D-3,6-galactan (type II arabinogalactan; AG-II) in the polysaccharide fractions were analyzed using the β-D-glucosyl Yariv reagent (Biosupplies, Parkville, Australia), according to the method reported by van Holst and Clarke [25]. Five microliters of the sample was loaded into the gel and incubated at 25°C. After 12 h, the area of the produced red circle was calculated based on the determination of coefficient of gum arabic as a standard reference.

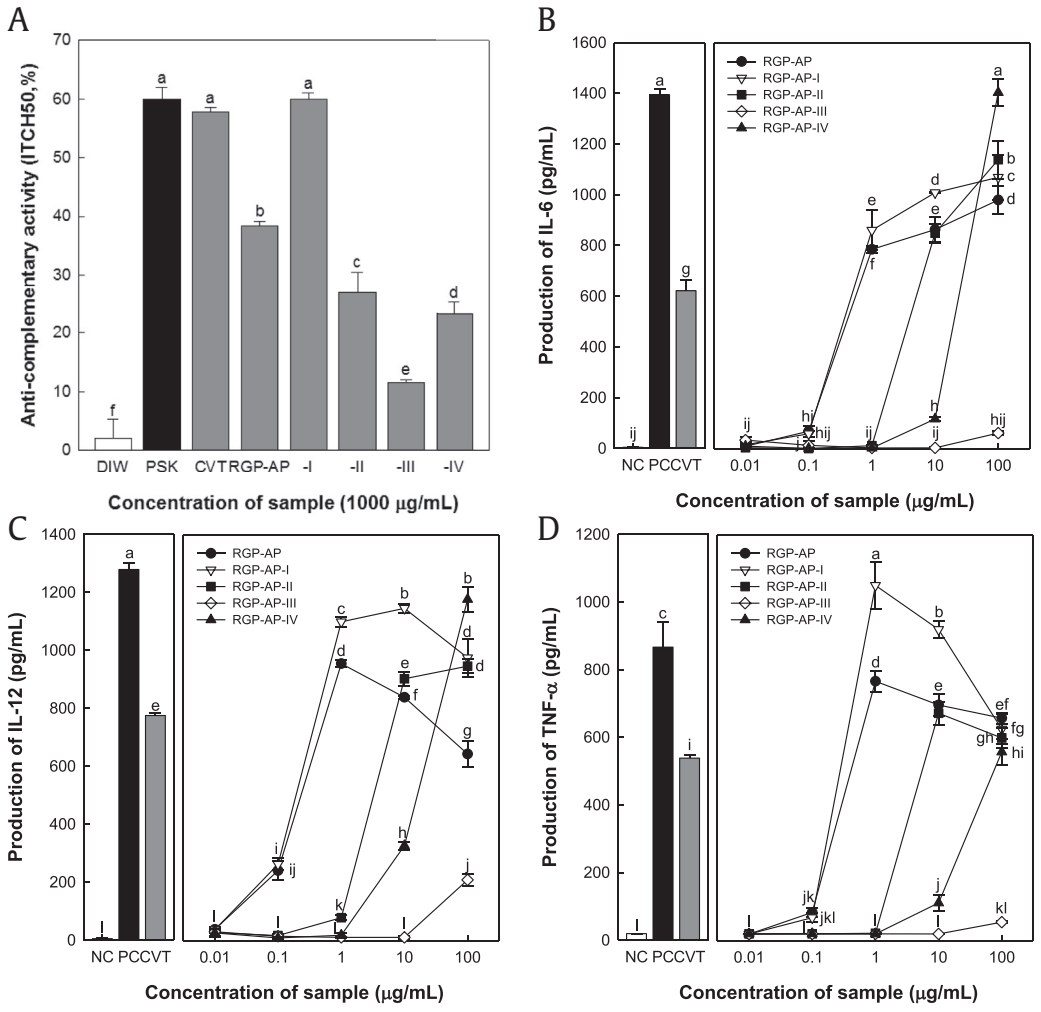

Fig. 4. Effect of RGP-AP and its subfractions on (A) anticomplementary activity and production of macrophage-stimulating cytokines such as (B) IL-6, (C) IL-12, and (D) TNF-α of murine peritoneal macrophages. The anticomplementary activity was presented as the inhibition of 50% total complementary hemolysis (ITCH, 50%) by Mayer's method. Polysaccharide K (PSK, 1 mg/mL) and CVT-E002 (CVT, 1 mg/mL) were used as immunostimulatory positive controls. Production of macrophage-stimulatory cytokines was analyzed in the cell culture medium of peritoneal macrophage. Only medium and LPS (5 μg/mL) were used as the negative control (NC) and positive control (PC), respectively. Means with different superscript letters indicate significant differences at $p < 0.05$ by Duncan's multiple range test. IL, interleukin; TNF, tumor necrosis factor.

2.7. Glycosidic linkage analysis

To determine the glycosidic linkage of the polysaccharide, methylation was carried out according to the methods described by Hakomori [26] and Pettolino et al [13], with slight modifications. In brief, polysaccharide fraction was dissolved in dimethyl sulfoxide, and methylsulfinyl carbanion with iodomethane (Sigma) was added to prepare the methylated polysaccharide. The unmethylated parts were removed using a Sep-pak C18 cartridge (Waters, Dublin, Ireland), and the methylated sample was hydrolyzed by 1 M trifluoroacetic acid (Sigma) for 90 min at 121°C. Reduction was carried out with sodium borodeuteride (Sigma) for 240 min, and then acetylation was carried out with acetic anhydride for 180 min at 121°C. The resulting partially methylated alditol acetates (PMAAs) were analyzed by GC–MS (Agilent, Santa Clara, CA, USA), and the glycosidic linkage of the polysaccharide fraction was calculated based on the method of Lee et al [27].

2.8. Statistical analysis

All statistical analyses evaluated were analyzed by a one-way analysis of variance and Duncan's multiple range test. All data are presented as the mean ± standard deviation (SD).

3. Results and discussion

3.1. Characteristics of RG polysaccharide

3.1.1. Preparation of polysaccharide fraction from an RG concentrate

Initially, RGP-0 was isolated from an RG concentrate with a yield of 25.21 w/w%. As mentioned in Table 1, RGP-0 comprised neutral sugar (74.3%) and uronic acid (24.6%), including mainly glucose (Glc; 56.3%) and GalA (24.1%) as the monosaccharide components. The high Glc content suggested that RGP-0 may comprise a large amount of a starch-like polysaccharide (α-1,4-glucan). To remove this starch-like polysaccharide from RGP-0, treatment with specific

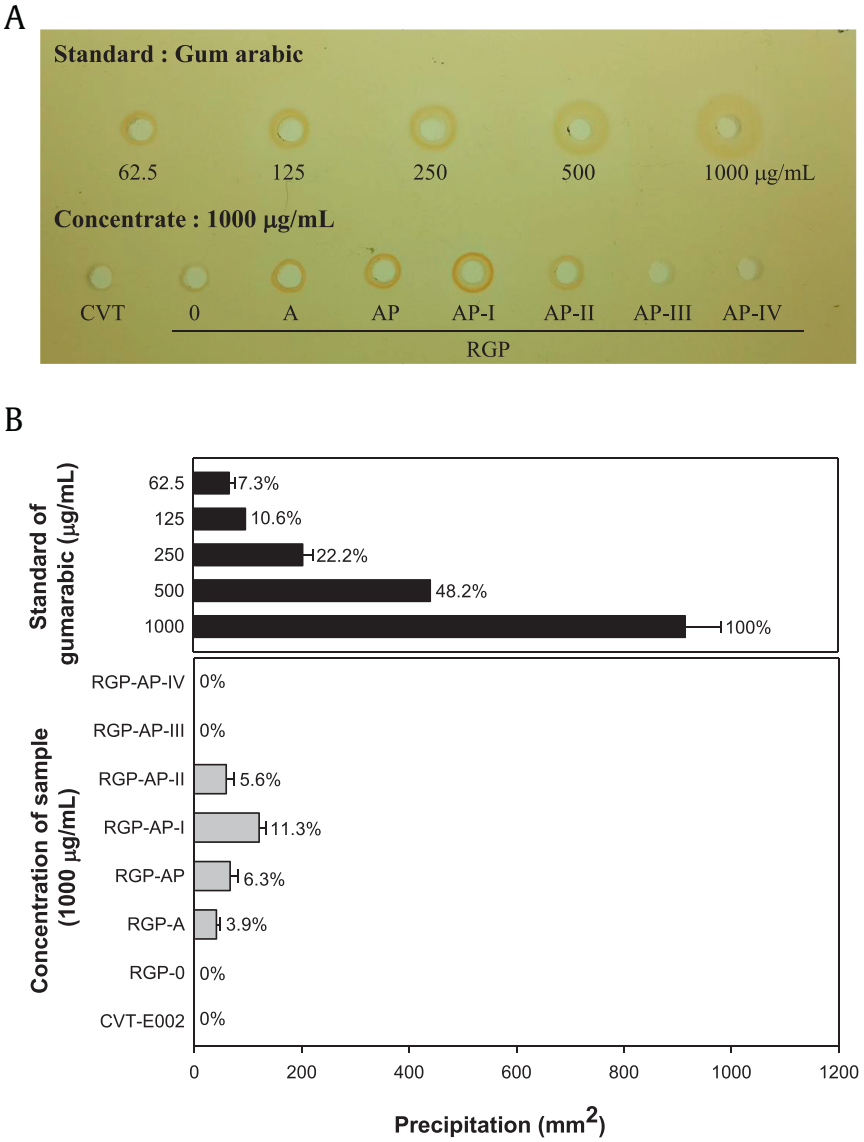

Fig. 5. (A) Single radial gel diffusion and (B) reactivity between β-glucosyl Yariv reagent and polysaccharide fractions isolated from red ginseng concentrate. Gum arabic was used as a standard material.

enzymes such as α–amylase and amyloglucosidase was carried out to yield a starch-deprived polysaccharide fraction (RGP-A, yield 21.29% against RGP-0). The Glc content (9.0%) was dramatically decreased in RGP-A, whereas the GalA (46.3%), galactose (Gal; 21.3%), and arabinose (Ara; 16.4%) contents were enriched *via* the catalytic action of starch-depriving enzymes (Table 1). Large amounts of GalA, Gal, and Ara strongly suggested that RGP-A consists primarily of pectic polysaccharide, which comprises three major groups such as HG, rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II).

To appropriately characterize the ginseng polysaccharide structure, RGP-A was further treated with pectinesterase, which catalyzes the deesterification of the methyl-esterified HG region of the pectic polysaccharide into pectate and methanol [28]. As a result of the pectinesterase digestion, RGP-AP (8.2% yield against RGP-A) was prepared (Table 1). The chemical component and sugar analyses suggested that Gal (23.2%) and Ara (22.0%) contents were slightly increased in RGP-AP, whereas Glc (2.3%) content was greatly diminished compared with that of RGP-A. Interestingly, GalA content was rarely affected by pectinesterase digestion.

3.1.2. *Effect of enzymatic digestion on immunostimulatory activity of RG polysaccharide*

The anticomplementary activity and production activities of immunostimulatory cytokines in polysaccharide samples isolated through the enzymatic digestion of the RG concentrate were estimated and compared. As presented in Fig. 3A, the anticomplementary activities of RGP-0, RGP-A, and RGP-AP were 22.1%, 31.1%, and 35.8%, respectively; whereas, those of PSK and CVT, which were used as positive controls, were 60.0% and 45.2%, respectively. In addition, the effects of the three polysaccharide samples on the production of macrophage-stimulating cytokines, such as IL-6, IL-12, and TNF-α were quantified and expressed in Fig. 3B–D. Considering the same concentration range, RGP-A increased the production of the three cytokines more than RGP-0, suggesting that the removal of starch could presumably enhance the macrophage activity of RGP. Meanwhile, although RGP-0 and RGP-A induced the production of the three cytokines in high concentration ranges (above 62.5 μg/mL), RGP-AP exhibited greater production of the three cytokines in low concentration ranges (0.06∼0.98 μg/mL). Interestingly, the production of the three cytokines peaked when RGP-AP stimulus was provided to RAW 264.7 cells at a concentration of 0.98 μg/mL; however, it decreased at higher doses. Collectively, these results indicated that enzymatic digestion is a useful method for enhancing the anticomplementary activity of RGP. Considering the association analysis with sugar composition, as presented in Table 1, the small amounts of Glc in RGP-AP suggest that glucans, including starch, are not important moieties for the immunostimulatory activity of RGP-AP. Consequently, the amylase digestion of the RG concentrate is a useful method for significantly enhancing its immunostimulatory activity.

3.1.3. *Chemical property and immunostimulatory activity of subfractions retrieved from RGP-AP*

As the immunostimulatory polysaccharide (RGP-AP) exhibited a relatively higher activity than RGP-0 and RGP-A, it was further fractionated into four subfractions via gel permeation chromatography using Sephadex G-100 (Fig. 2A), that is, RGP-AP-I (yield; 2.9% against RG concentrate) was eluted in a void volume, RGP-AP-II (1.9%) and RGP-AP-III (1.3%) were eluted in intermediate volumes, and RGP-AP-IV (2.7%) was obtained in an inner volume. To compare the immunostimulatory activity of the subfractions, the anticomplementary and macrophage stimulatory activities were reestimated. As presented in Fig. 4A, RGP-AP-I increased the anticomplementary activity to similar level with the positive control (PSK and CVT), whereas the other fractions exhibited decreased activity compared with that of RGP-AP. The results of the production of macrophage stimulatory cytokines such as IL-6, IL-12, and TNF-α were illustrated in Fig. 4B–D, respectively. Among four fractions, only the highest-molecular-weight fraction, RGP-AP-I showed significantly increased production of IL-6, IL-12, and TNF-α compared with those of RGP-AP, at doses above 1 μg/mL. Interestingly, the production of IL-12 and TNF-a showed decreased tendency at doses above 10 μg/mL in both RGP-AP and RGP-AP-I, suggesting that treatment of them to RAW 264.7 cells was more suitable at dose of 1 μg/mL. Other fractions beside RGP-AP-I did not show enhanced production activity of three cytokines compared with those of RGP-AP. Therefore, we preferred RGP-AP-I as an immunostimulatory polysaccharide and carried out further analysis to identify its appropriate structure.

Table 1 lists the chemical composition of subfractions isolated from RGP-AP. The maximum high-molecular-weight fraction, RGP-AP-I, comprised a large proportion of neutral sugars (67.7%), consisting mainly of Ara (35.0%), Gal (30.4%), and GalA (18.4%). In comparison with RGP-AP, the proportion of neutral sugars such as Rha, Ara, and Gal was considerably enriched in RGP-AP-I; however, the content of uronic acid was decreased by more than 15% in RGP-AP-I compared with that in RGP-AP, and the major uronic acid was confirmed as being GalA (18.4%) rather than glucuronic acid (0.9%). The high proportion of Ara and Gal suggests that RGP-AP-I

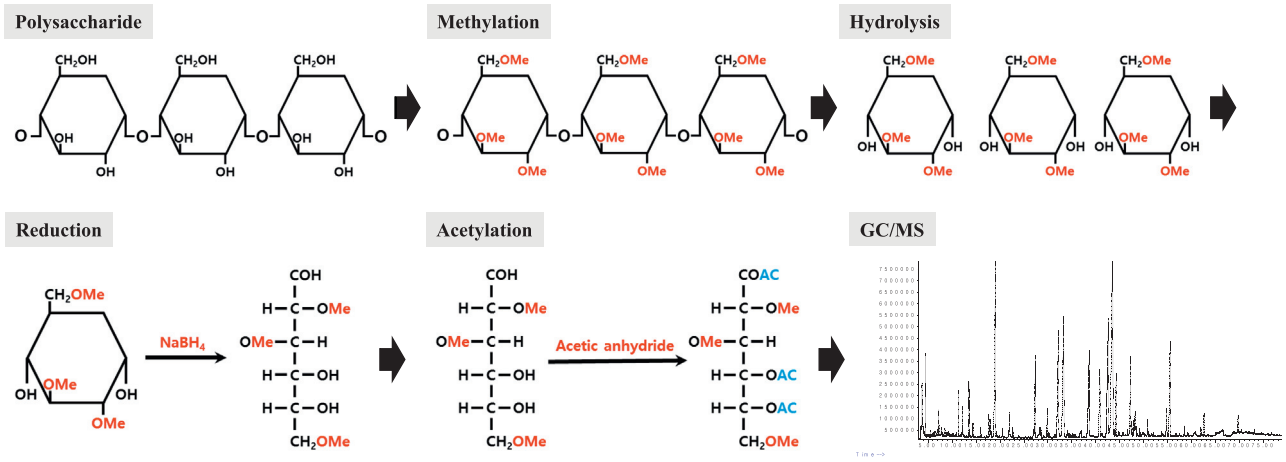

Fig. 6. Principle of methylation analysis for determination of sugar linkages of polysaccharides.

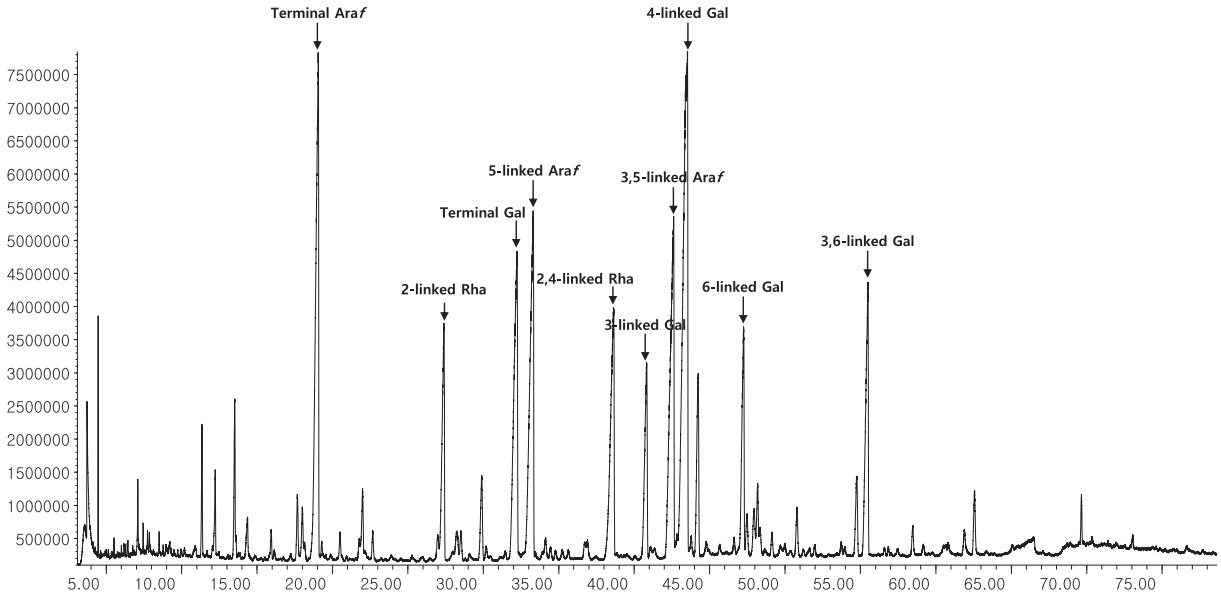

Fig. 7. Total ion chromatograms of GC–MS for partially methylated alditol acetate of RGP-AP-I purified from red ginseng concentrate.

presumably possesses few side chains, such as arabinan, galactan, or arabinogalactan (AG), which are attached to the backbone of RG-I [29]. Meanwhile, the determination of the molecular weight distribution of the RGP-AP-I fraction via HPLC is illustrated in Fig. 2 (B). RGP-AP-I appeared as a symmetrical, single peak, and its molecular weight was estimated to be 96 kDa according to the calibration curve prepared using a dextran reference at various concentrations.

3.2. Structural characterization of RGP-AP-I

3.2.1. Yariv reagent assay of polysaccharide isolated from RG concentrate

After estimating that all polysaccharide fractions contained different proportions of Ara and Gal, we analyzed single radial gel diffusion assay with β-glucosyl Yariv reagent for the identification and quantification of AG-II. The Yariv reagent has the ability to precipitate polysaccharides containing AG-II. Depending on the length of the Gal chain, a precipitate will form if the Gal units contain branching at positions 3 and 6 [25,30]. As presented in Fig. 5, a positive correlation was revealed between the Yariv reagents in the standard reference gum arabic, and the area of precipitation measured by the square of the diameter of the halo formed in the radial diffusion assay. The amount of AG-II in a sample was determined by a calibration curve based on the square value of the halo formed using gum arabic [29]. Although RGP-0 rarely possessed AG-II moiety in their structure, AG-II contents were enriched in its enzyme-digested fractions, RGP-A and RGP-AP (3.9% and 6.3%, respectively). Furthermore, the immunostimulatory subfraction, RGP-AP-I, exhibited higher content of AG-II (11.3%) among all polysaccharide samples tested. This result suggested that AG-II moiety is important for expressing the immunostimulatory activity in RGP. The amount of AG-II in RGP-II was calculated to be a 5.6%, whereas AG-II moiety is not included both in RGP-III and RGP-IV.

Table 2
Methylation analysis of glycosidic linkages of RGP-AP-I purified from red ginseng concentrate

Glycosyl residue	Deduced glycosidic linkage	RGP-AP-I (mole%)[1]
Arabinose	Terminal (*f*)	9.9
	Terminal (*p*)	1.2
	2 (*f*)-	0.3
	5 (*f*)-	7.8
	3,5 (*f*)-	7.3
	2,3,5 (*f*)-	0.4
Xylose	Terminal (*p*)	0.3
Galactose	Terminal (*p*)	6.3
	3-	2.7
	4-	12.9
	6-	2.8
	3,4-	0.6
	2,4-	0.4
	4,6-	0.8
	3,6-	3.6
	3,4,6-	0.3
Glucose	4-	2.0
Rhamnose	2-	3.0
	3,4-	0.8
	2,4-	4.4
Galacturonic acid		18.4

[1] Calculated from the peak area and the molecular response factors of each partially methylated alditol acetate in GC.

3.2.2. Methylation analysis of RGP-AP-I

The structural characterization of polysaccharides requires the identification of sugar compositions, anomeric configurations, ring forms, glycosyl linkages, and sequences. Glycosyl linkages are confirmed by NMR, FT-IR, and methylation analysis. In particular, the methylation-based method is well established and is widely used for determining the structure of the polysaccharide [12]. In this method, all the free hydroxyl groups of the sample (polysaccharide) are methylated, and the methylated polysaccharide is hydrolyzed using trifluoroacetic acid. The partially methylated monosaccharide is reduced by $NaBH_4$ and then acetylated to PMAAs [13]. This method follows the same basic procedures as summarized in Fig. 6.

The immunostimulatory subfraction RGP-AP-I was analyzed by GC–MS to identify the active structure of RG. RGP-AP-I comprises 20 types of neutral glycosyl linkages (Fig. 7 and Table 2), consisting mainly of galactopyranosyl (Gal*p*), arabinofuranosyl (Ara*f*), and rhamnopyranosyl (Rha*p*) linkages (30.4, 26.9, and 8.2 mole %, respectively), in addition to small amounts of glucopyranosyl (Glc*p*)

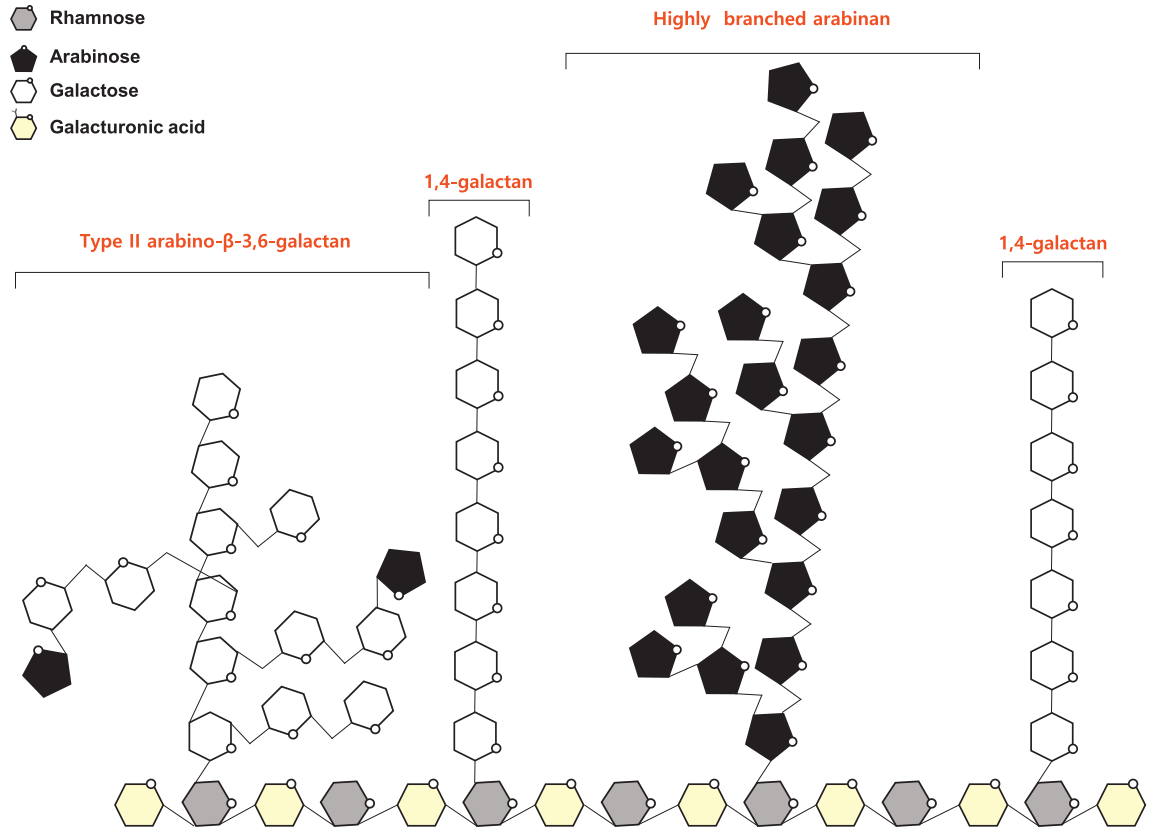

Fig. 8. A possible structure of the immunostimulatory polysaccharide (RGP-AP-I) isolated from red ginseng.

and xylopyranosyl (Xyl*p*) linkages (2.0 and 0.3 mole %, respectively). Unfortunately, the linkage confirmation of GalA was not carried out in this study because they are not detectable as alditol acetate unless they are prereduced to their neutral sugar counterparts [13]. Nevertheless, the result of sugar composition (Table 1) provided the information about glycosidic linkages of GalA*p*. Commonly, GalA*p* is composed of (1→4)-linked backbone in pectic material. RG-I in pectic material has a repeating disaccharide [→4)-D-GalA*p*-α-(1→2)-Rha*p*-α-(1→]$_n$. Although the glycosidic linkage of GalA was not detected in methylation analysis, 2- and 2,4-linked Rha linkages assumed α-(1→2)-Rha*p* residues attached with various side chains at C4 position of RG-I backbone [31–33]. Moreover, a high proportion of 4-linked Gal*p* linkages (12.9%) was postulated as linear galactan of [→4)-D-Gal-(1→4)-D-Gal-(1→], whereas Ara*f* residues (5-linked, 3,5-linked and terminal Ara*f*; 7.8%, 7.3% and 9.9%, respectively) were estimated as highly branched arabinan side chains. In addition, 3-linked (2.7%), 6-linked (2.8%), and 3, 6-linked (3.6%) Gal*p* linkages were identified as arabino-β-3,6-galactan as observed in Yariv reagent assay. This three-type side chain should be attached to the backbone of RG-I at C(O)4 position because of the presence of 2,4-linked Rha*p* linkage.

Based on the aforementioned results, we assumed that the RGP-AP-I purified from RGP comprises a typical RG-I structure, which includes several side chains such as galactan, highly branched arabinan, and arabino-β-3,6-galactan. The proposed structure of RGP-AP-I is illustrated in Fig. 8.

4. Conclusion

Many researchers have reported pharmacological activities such as radioprotective [34], antifatigue [35], anticancer [36], and immunological activities [37] of *P. ginseng* polysaccharides, and they investigated active structure of polysaccharides [38]. Tomoda et al [37] reported that the active polysaccharide fraction (S-IIA) isolated from the root of *P. ginseng* is mainly comprised of L-Ara, D-Gal, D-Glc, and D-GalA and has a 100-kDa molecular weight and a relatively high content of α-3,5-barached L-Ara and β-1,4-linked D-Gal residues. Moreover, Zhang et al [39] reported that water-soluble polysaccharides isolated from *P. ginseng* include pectic arabinogalactans and pectins enriched in RG-I, HG, and starch-like glucans. In addition, several studies have demonstrated that the immunostimulatory functions of RG could be due to the presence of an acidic polysaccharide. Nevertheless, the exact structural features of these acidic polysaccharides have not been fully elucidated. Furthermore, the structure of the RG polysaccharide has rarely been studied in comparison with that of wild ginseng. Therefore, in this study, we isolated an acidic polysaccharide with enhanced immunostimulatory activity and elucidated the structure of the key moiety of the polysaccharide, contributing to its immunostimulatory activity. RGP-AP-I was purely isolated via starch and homogalacturonan elimination and gel filtration chromatography, which indicated enhanced macrophage-stimulatory and anticomplementary activities than the other fractions. RGP-AP-I was estimated as having a molecular weight of 96 kDa and mainly comprised Ara, Gal, and GalA. As a result of methylation analysis, RGP-AP-I was found to comprise a pectic RG-I backbone highly substituted with arabinan, linear galactan, and arabino-β-3,6-galactan branches. Based on these results, the active immunostimulatory structure of RGP was suggested, and this may be the first research of its kind on RG polysaccharide.

Conflicts of interest

The authors claim no conflicts of interest.

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