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Novel analysis procedure for red ginseng polysaccharides by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry

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ABSTRACT

Background: Red ginseng polysaccharides (RGPs) have been acknowledged for their outstanding immunomodulation and anti-tumor activities. However, their studies are still limited by the complexity of their structural features, the absence of purification and enrichment methods, and the rarity of the analytical instruments that apply to the analysis of such macromolecules. Thus, this study is an attempt to establish a new mass spectrometry (MS)-based analysis procedure for RGPs.
Methods: Saponin pre-excluded powder of RG (RG-SPEP, 10 mg) was treated with 200 μL of distilled water and centrifuged for 5 h at 1000 rpm and 85 °C. Ethanol-based precipitation and centrifugation were applied to obtain RGPs from the heated extracts. Further, endo-carbohydrase treatments were performed to produce specific saccharide fragments. Solid-phase extraction (SPE) processes were implemented to purify and enrich the enzyme-treated RGPs, while matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) MS was employed for the partial structural analysis of the obtained RGPs.
Results: Utilizing cellulase, porous graphitized carbon (PGC), hydrophilic interaction chromatography (HILIC), and MALDI-TOF/TOF MS, the neutral and acidic RGPs were qualitatively analyzed. Hex_n and Hex_n-18 (cellulose analogs) were determined to be novel neutral RGPs. Additionally, the [Unknown + Hex_n] species were also determined as new acidic RGPs. Furthermore, $HexA_n$ (H) was determined as another form of the acidic RGPs.
Conclusion: Compared to the previous methods of analysis, these unprecedented applications of HILIC-SPE and MALDI-TOF/TOF MS to analyze RGPs proved to be fairly effective for fractionating and detecting neutral and acidic components. This new procedure exhibits great potential as a specific tool for searching and determining various polysaccharides in many herbal medicines.

1. Introduction

Ginseng has been widely utilized as a traditional herbal medicine for thousands of years [1]. Among the 17 kinds of ginseng plants that are distributed globally, some, including *P. ginseng C. A. Meyer, P. quinquefolius L., P. notoginseng (Burkil), P. japonicum Nees, P. trifolius L., and P. pseudoginseng Wall.*, are widely known as pharmacologically proven species. *P. ginseng C. A. Meyer*, mostly cultivated in Korea, is well-known to exert outstanding medicinal effects compared to the other species, and it has been employed as an attractive raw material for the heavy production of red ginseng (RG) in Korea [2,3].

RG has been acknowledged to exhibit excellent pharmacological activities against many diseases. Such effects have been verified mainly by studying RG-derived low-molecular substances, such as saponin, phenolics, and polyacetylene [4,5]. RG polysaccharides (RGPs) have attracted increased attention because of their physiological activities, such as their immunomodulation and anti-tumor effects. RGPs demonstrate the following several outstanding anticancer effects in human: 1) they suppress the propagation of

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cancer cells by strengthening biological immune functions, 2) they induce the death of cancer cells by activating macrophages (natural killer cells) and immunocytes through *in-vivo* detoxification, and 3) they restrain active oxygen species, thus causing normal cell damage by inducing regulatory cytokines [6–8].

However, MS-based studies of RGPs have not successfully revealed the structural characteristics of target RGP molecules. Owing to the large molecular weights of RGPs, existing analysis equipment cannot analyze RGPs intact. Thus, several enzymes have been necessarily introduced to identify the types and structures of RGPs, and some RGPs have been identified [9,10]. Nevertheless, many RGPs are still unknown.

Additionally, a major setback was the lack of specific methods for purifying and enriching sufficient RGPs. Since most RGPs are highly polar water-soluble substances, they can be generally refined and concentrated by organic solvent-based liquid-liquid extraction (LLE) [11]. However, only some RGPs have been obtained with low yields through these purification and enrichment methods.

RGPs are also barely detected with spectroscopic instruments because of their structural complexity and heterogeneity [12,13]. Moreover, some RGPs, which were detected, were only partially analyzed [14]. Despite the advent of liquid chromatography-mass spectrometry (LC-MS), the research on RGPs has not really progressed [15,16]. However, the recent development of matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) MS has increased the possibilities for analyzing RGPs [17,18].

In this study, we presented a novel approach toward purifying, enriching, and profiling RGPs utilizing easily accessible materials and analytical instruments. By combining enzymatic treatment, solid-phase extraction (SPE), and MALDI-TOF/TOF MS, various neutral and acidic RGPs were determined by defining accurate factors, such as the linkages, compositions, mass values, and contents. This study enabled the establishment of a specialized analysis procedure for characterizing high-purity neutral and acidic RGPs. This operational flowchart is presented as a novel tool, which would be applied to the investigation and identification of polysaccharides, that are derived from many other herbal medicines.

2. Materials and methods

2.1. Materials

The "saponin pre-excluded powder of red ginseng" (RG-SPEP) was supplied by Korea Ginseng Corporation (KGC). Various materials for the experiments, such as the enzymes, SPE-cartridges, reagents, solvents, and instruments, were purchased and utilized for all the experiments. Table S1 presents the detailed information of the utilized materials.

2.2. Extraction and collection of RGPs

First, the hot water extraction method (HWEM) was employed in this experiment, which was modified from an existing method [19]. During HWEM, 10 mg of RG-SPEP was treated with 200 μL of distilled water (1:20 (*w/v*)) and centrifuged for 5 h at 1000 rpm and 85 °C utilizing Eppendorf Thermomixer 5350. After this treatment, the heated extracts were centrifuged for 20 min at 1.4×10^4 rpm by Eppendorf Refrigerated Centrifuge 5430R at room temperature (21 °C). Thereafter, only the supernatants were collected from the upper part considering the water-soluble characteristics of most polysaccharides [20].

The supernatants were mixed with ice-cold (−40 °C) absolute ethanol at a 1:4 (*v/v*) ratio as previously described [21]. The 80% ethanol-based solutions were subsequently stored overnight in a refrigerator (−40 °C). The solutions were recentrifuged to precipitate only RGPs. The upper aqueous layers were removed, and the residues were collected and dried by EZ-2 personal evaporator according to the method of a previous study [22].

2.3. Enzymatic digestion of RGPs

RGPs were dissolved in 1 mL of distilled water. Three different endo-carbohydrases (cellulase, pectinase, and α-amylase) were separately utilized with 50 μg of RGPs for each enzyme by modifying conventional methods [23,24]. Table S2 presents and describes the corresponding enzymes. Each enzyme digestion process was performed for 1 h. Afterward, the various enzyme and RGP mixtures were boiled for 2 min at 90 °C to terminate the enzyme digestion.

2.4. Purification and enrichment of RGPs

We selected an SPE method to perform the clean-up process to remove the buffer, detergents, and salts. Additionally, the SPE method was employed to purify and enrich the hydrolyzed RGPs before the MS analysis. Porous graphitized carbon (PGC) and hydrophilic interaction chromatography (HILIC), the two types of cartridges, were utilized to concentrate RGPs since they are typical devices for effectively obtaining various high-purity glycoconjugates (glycans, glycopeptides, glycoproteins, etc.) [25–27]. Each device exhibited different properties; thus, a fractionation protocol was also discriminatively implemented. The individual characteristics and experimental procedures of the devices are presented in Table S3.

2.5. Analysis of RGPs by MALDI-TOF/TOF MS

Here, MALDI-TOF/TOF MS and 2,5-dihydroxybenzoic acid (DHB) were selected to profile and recognize RGPs, respectively, according to previous reports [28,29]. Each SPE of RGPs was reconstituted with 50 μL of distilled water. Before the analyses of RGPs, two types of assays were also employed separately as calibrants for analyzing the neutral and acidic species. Corresponding spotting techniques and analysis modes were performed for each experimental group according to a previous report [30]. The relevant details are summarized in Table S4. To analyze the structure of each compound, tandem MS (MS/MS), which is the main merit of MALDI-TOF/TOF MS, was performed. This technique involves the high-energy collision-induced dissociation (HCD) to fragment RGPs into monomer units.

3. Results

3.1. Extracted RGPs

RGPs belong to the family of free polysaccharides (FPs), which are plant-specific, effective metabolites, that exhibit many physiological activities, such as anti-cancer and immunity-boosting activities [7,8,13]. FPs are generally water-soluble because of their low crystallinity and diverse hydrophilic structural features [20]. As shown in the flowchart (Fig. 1), HWEM and the ethanol precipitation were applied to 10 mg of the RG-SPEPs. Consequently, 2 mg of crude RGPs was successfully produced from the original material in a yield of ~20%.

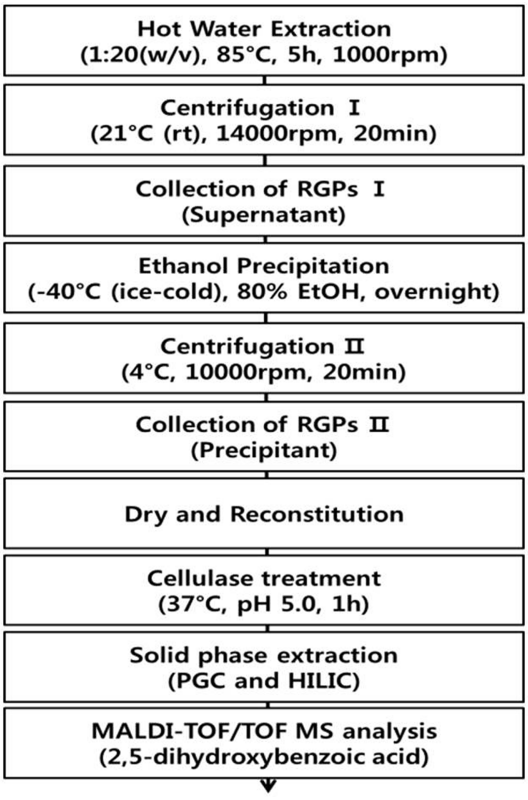

Fig. 1. The new analysis procedure using MALDI-TOF/TOF MS for RGPs.

3.2. Investigation of the enzymatic digestion of RGPs

RGPs possess large molecular weights and complex structures [11,31]. Enzymatic processes were initially indispensable for degrading intact RGPs to produce smaller fragmented saccharides. Such fragments could be accommodated by analysis equipment, including MS instruments [23,32]. In previous studies, some of the fragments, including the Panaxan (Panaxan A, Panaxan B, etc.) and Ginsanan (PA, PB, etc.) series, were identified as neutral and acidic RGPs by several enzymes, such as pectinase and arabinosidase [13,33].

However, there has not been further progress in these studies. It is challenging to determine the most effective enzyme for analyzing RGPs even though there are several types of enzymes for breaking FPs [19,23]. Additionally, the sizes and types of the produced fragments differ greatly depending on the enzyme that was utilized to treat them, thereby adding complexity to the interpretation and deduction of the original structures of RGPs. Above all, the biggest setback is the fact that the enzymatic conditions (temperature, pH, etc.) have not been optimized to determine the most suitable fragmentation for producing the maximum amount of every fragment.

To solve the enzyme-related setbacks, three commonly utilized endo-glycosidases were applied to hydrolyze RGPs. Among the many kinds of enzymes that are utilized in RGP analyses, these enzymes (the endo-glycosidases) easily broke a specific linkage of the glycoconjugates [13,19,23,33]. Further, we utilized these enzymes (cellulase, pectinase and α-amylase) and anticipated the production of a sufficient amount of saccharide fragments, thereby obtain key information for deducing the original structures of RGPs.

Consequently, the highest number of hexose polymers (Hex_n) and dehydrated Hex_n (Hex_n-18) were observed in the cellulase-treated fragment (Fig. 2A, S1, and Table S5). Further, the types and contents of [Unknown + Hex_n] attained the maximum when cellulase was applied (Fig. 2B and Table S6). Based on these data, we expected cellulase to be the most suitable enzyme for identifying the neutral and acidic RGPs.

3.3. Advantages of the SPE method for purifying and enriching RGPs

LLE is generally utilized to extract and isolate the active components from RG [11,34]. This refining process is practically employed to obtain saponins, which are the marker compounds of the RG extract. Researchers have focused on recovering only the organic solvent layers, e.g., butanol, containing saponins through LLE [15,16,34]. The physiological effects of RGPs were recently highlighted rather than the effects of saponins [6–8]. Therefore, many studies have been channeled toward obtaining and analyzing RGPs. Accordingly, LLE was re-examined as a method for recovering the inorganic solvent layer, e.g., water, containing RGPs.

Although LLE has been mainly utilized to purify and concentrate the substances [13,24], these refinement and enrichment processes have encountered the following setbacks: 1) large amounts of solvents, which can cause environmental pollution, are utilized for the experiment, and 2) since LLE exhibits a small enrichment factor and low selectivity for RGPs, it is challenging to obtain a sufficient amount of the corresponding compounds [13,35,36]. Pectin-like polysaccharides were obtained as a fraction of the acidic RGPs,

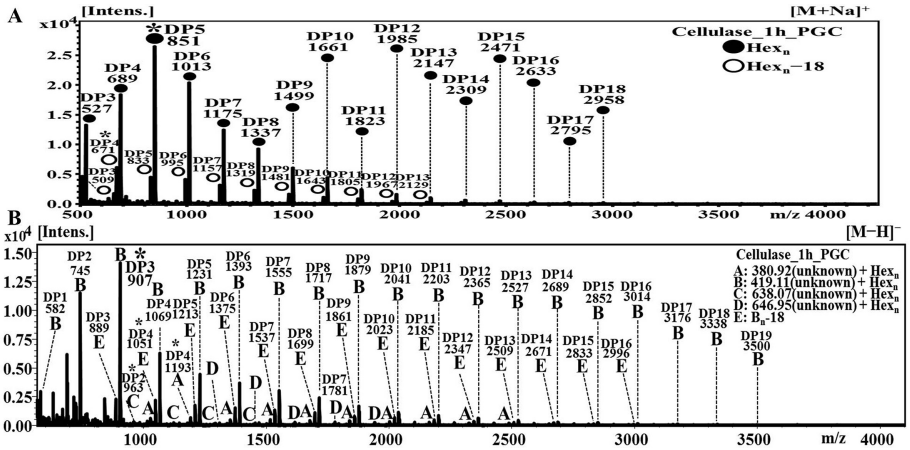

Fig. 2. MS profiling of the whole cellulase-treated RGPs from PGC-SPE (RG-SPEP). (A) Neutral RGPs from PGC-SPE (RG-SPEP). (B) Acidic RGPs from PGC-SPE (RG-SPEP).

Table 1
Comparison of SPE Methods for Enriching Cellulase-Treated Neutral RGPs (RG-SPEP)

SPE methods	PGC	HILIC
Hex_n	+++ DP 3-18	+++ DP 3-26
Hex_n-18	++ DP 3-13	++ DP 3-22

+++: major, ++: minor, +: trace amount, -: not detected.
RG-SPEP: saponin pre-excluded powder of red ginseng.

although with a much lower yield compared with that of the existing content [10,13,33]. Here, SPE was selected as a new strategy for recovering more RGPs in higher quantities than with LLE, as well as for separating, refining, and enriching RGPs.

SPE is majorly employed to isolate, purify, and enrich the glycoconjugates [25]. PGC and HILIC are typical tools for the selective enrichment of oligomers before the MS analysis [26,27], although they are different. PGC can selectively adsorb and separate saccharides based on their sizes and polarities, whereas HILIC can isolate and concentrate glycoconjugates based on their polarities [37]. Therefore, PGC-based SPE (PGC-SPE) and HILIC-based SPE (HILIC-SPE) were employed in this study to establish their respective optimal conditions for the selective isolation, purification, and enrichment of RGPs.

Firstly, we confirmed that many neutral RGPs (Hex_n and Hex_n-18) were obtained by the [Cellulase + HILIC] method (Table 1). Further, a larger number of [Unknown + Hex_n] were detected by the [Cellulase + HILIC] method compared with the [Cellulase + PGC] method, and it also detected a new species of acidic RGPs (H; $HexA_n$) (Fig. 3A and B and Table 2). Based on these results, [Cellulase + HILIC] was considered to be more beneficial for the fractionation and concentration of neutral and acidic RGPs.

3.4. MS-based profiling and detection of RGPs by MALDI-TOF/TOF

Conventional studies depend on spectroscopic analytical instruments to analyze the structures of RGPs [23]. Such equipment are for refractive index, evaporative light scattering, and other detections. However, conventional studies can only analyze RGPs partially because of their limitation in overcoming the two structural setbacks of RGPs, namely complexity and heterogeneity [31,32,38,39], which are due to the multiple glycosidic linkages between the different monosaccharides [9]. Thus, only a few RGPs, such as galacturonan and mannan, have been discovered [10].

LC-MS was recently introduced to investigate the structures of RGPs' [23,40]. Polysaccharide-specific colorimetric quantification has been conducted to facilitate the analyses of RGPs by instruments that are equipped with ultraviolet-visible light (UV-Vis) detector. Related studies are still insufficient because the mass values of RGPs' are much higher than the acceptable mass ranges of such devices [40,41]. Additionally, the polysaccharide-specific colorimetric quantification method of analyzing RGPs is accompanied by further complexities that are due to the inevitable destruction and modification of the RGP-constitutive monosaccharides [2,10]. Owing to this setback, the number and amounts of acidic RGPs, such as heteropolysaccharides, were observed to be lesser than expected.

To overcome the limitations of LC-MS, a new MS technique was necessarily introduced to qualitatively and quantitatively analyze RGPs. Among the many MS techniques, MALDI-TOF/TOF MS is a specialized one for the rapid profiling of large biomolecules, such as peptides, glycans, glycopeptides, and proteins [28]. It is known that the technique exhibits much better performance for analyzing high-molecular substances than LC-MS. Particularly, MALDI-TOF/TOF MS was recently employed to characterize specific RGPs without destroying and modifying their original structures [42]. Although different matrices are utilized depending on the analyte, DHB is known to effectively analyze glycoconjugates [41]. Thus, this study adopted MALDI-TOF/TOF MS and utilized DHB to analyze RGPs more accurately. Consequently, the mass spectra of RGPs were observed in a wide mass range (Figs. 2–4).

The mass spectra obtained from the MALDI-TOF/TOF mass spectrometer afforded detailed information, such as the base peak, composition, content, and distribution, which were necessary for the characterization of various polysaccharides [43]. Tables S5-6, and 1-2, as well as Figs. 2–4 show mass values and peak intensities that were obtained from each mass spectrum in the repeated experiments. Each base peak could be utilized as a marker for certain RGPs considering that fragments of the original RGPs were produced by the enzyme-induced cleavages of specific linkages. When adopting MALDI-TOF/TOF MS to analyze RGPs, the following key factors can be evaluated: the type, composition, degree of polymerization (DP), and DP ranges. Particularly, the peak intensities

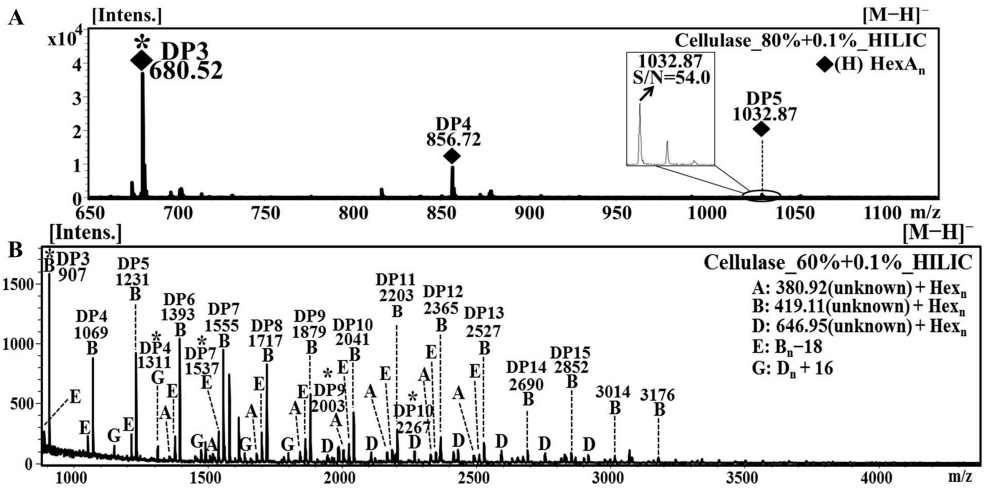

Fig. 3. MS profiling of the whole cellulase-treated acidic RGPs from HILIC-SPE (RG-SPEP). (A) Cellulase-treated acidic RGPs (80% + 0.1%). (B) Cellulase-treated acidic RGPs (60% + 0.1%).

are required for the determination of the overall distribution and the quantification of the relative proportions of RGPs.

As shown in Table S5, Hex_n were obtained upon each enzymatic treatment of the major neutral RGPs. Hex_n were largely distributed over wide DP ranges (Fig. S1). Hex_n-18 were detected in trace amounts only upon the cellulase treatment (Fig. 2A, S1). These neutral RGPs were presumed to be cellulose analogs and derivatives.

As shown in Fig. 2A, the major neutral RGPs were Hex_n based on the difference in the peaks at 162 Da. The base peak (DP5 and, m/z 851) could be a candidate marker for a more accurate determination of the mass values and structures of the original Hex_n. Considering the peak-to-peak mass value differences, the minor species are determined to be Hex_n-18 in which the water molecule in Hex_n (18 Da) had been removed. When assessing the total contents of the neutral RGPs, Hex_n were significantly abundant compared with Hex_n-18 (Fig. 2A).

These cellulase-treated neutral RGPs were more detailedly observed during the purification and enrichment by the two SPE methods (Table 1). Hex_n and Hex_n-18 were detected in similar DP ranges by the two treatment methods ([Cellulase + PGC] and [Cellulase + HILIC]). However, the patterns and distributions were different, depending on the employed methodology. The two cellulose-like polysaccharides, Hex_n and Hex_n-18, were observed more by [Cellulase + HILIC], than by [Cellulase + PGC] (Table 1).

Conversely, new RGPs [Unknown + Hex_n], which were acidic, were obtained upon all the enzymatic treatments and PGC-SPE. These species are most likely different from the previously identified RG acidic polysaccharides (RGAPs) [2,8,9,14]. Notably, six acidic RGPs were detected. Three species (A, B, and C) were discovered in all enzymatic treatments, and three other species (D, E, and F) were limitedly discovered (Table S6). Although A, B and C were detected during all the enzymatic treatments, the highest peak intensities of the acidic RGPs were obtained by the cellulase treatment (Fig. 2B). Therefore, cellulase is the most suitable enzyme for detecting neutral and acidic RGPs.

Since [Unknown + Hex_n] possessed similar characteristics (size and polarity), they are detected in all the fractions (10% ACN, 20% ACN, and [40% ACN + 0.05% trifluoroacetic acid (TFA)]) obtained by PGC-SPE. Meanwhile, HILIC-SPE could additionally separate more acidic RGPs, G (D_n + 16) and H. H was obtained in large quantities from [80% ACN + 0.1% TFA] (Fig. 3A and Table 2). H is suspected to be a hexuronic acid polymer that is linked by β-1,4-glycosidic bonds. However, various [Unknown + Hex_n], i.e., A, B, C, D, E, F, and G, were obtained in trace amounts from only [60% ACN + 0.1% TFA] utilizing the same concentration (Fig. 3B and Table 2). This was due to the nature of HILIC, which isolated the substances based on their polarities only. Thus, HILIC-SPE could be more beneficial in fractionating and concentrating acidic RGPs.

As observed in Fig. 3A–B, H with a large amount is the major acidic RGPs. The observed base peak (DP3; m/z 681) could be an identifying factor of the original H. Compared with H, various [Unknown + Hex_n] were also recognized as minor acidic RGPs with small amounts. Among the minor species, B (419.11 + Hex_n) is the most dominant (Fig. 3B). E (B_n-18) was derived from B, whereas C (D_n-8) and G (D_n + 16) are closely related to D (646.95 + Hex_n). The quantities of these three species were much lower than those of B and E (Fig. 3B).

Table 2
Comparison of SPE Methods for Enriching Cellulase-Treated Acidic RGPs (RG-SPEP)

SPE methods	PGC	HILIC
A: 380.92 + Hex_n	+++ DP 2-15	+
B: 419.11 + Hex_n	+++ DP 1-18	+++ DP 3-17
C: 638.07 + Hex_n	+	-
D: 646.95 + Hex_n	+	+
E: B_n-H_2O	++ DP 1-18	+
F: $B_n + H_2O$	-	-
G: D_n + 16	-	+
H: $HexA_n$	-	+++ DP 3-5

+++: major, ++: minor, +: trace amount, -: not detected.
RG-SPEP: saponin pre-excluded powder of red ginseng.

3.5. Partial structural analysis of RGPs by MALDI-TOF/TOF MS/MS

MALDI-TOF/TOF MS/MS is beneficial to the structural analysis of glycoconjugates [28,42]. The structural information can be obtained from the tandem mass spectra of the fragment ions. Collision-induced dissociation (CID) was applied to separate fragment ions from the parent ones in the analyzer part of the instrument. Particularly, high-energy CID (HCD) enabled the decomposition of large biomolecules into various units that were suitable for MS analysis to obtain information about the original structure. MS/MS also possesses the advantage of accurately characterizing various compounds even with a small amount of the original sample. Therefore, we proved that HCD-based MALDI-TOF/TOF MS/MS is a beneficial analytical tool for the rapid elucidation of RGP structures.

The MS/MS signals effectively detected the A-series in the negative ion mode, $[M-H]^-$, because of the clear fragmentation of A due to the decomposition of the hexose units. m/z 1193.81, one of the A-series, was determined to be five hexose units (hexose: 162.01 Da) that were linked to the acidic head group of m/z 380.92 (Fig. 4A).

The MS/MS signals in $[M-H]^-$ are also beneficial to the structural elucidation of the H-series. m/z 680.74, an H-series, was determined to be three hexuronic acid units (hexuronic acid: 176.11 Da) (Fig. 4B), which were probably linked to m/z 152.42 obtained from DHB. The corresponding peak (m/z 152.42) was partially enlarged and displayed in Fig. 4B. To determine whether m/z 152.42 originated from the MALDI matrix or acidic head group, it was necessary to perform the analysis in $[M-H]^-$ of MS. By applying such experimental conditions, m/z 152.42 was observed with a large peak intensity and high signal-to-ratio (S/N) in the mass range of 0-1000 (Fig. 4B). Thus, H is most probably a polysaccharide comprising only hexuronic acid units.

4. Conclusion

In this study, we developed a novel analysis procedure for the effective fractionation and characterization of various RGPs, including new species that have not been discovered. Some RGPs, categorized as FPs, were successfully extracted from RG-SPEP. Particularly, the cellulase treatment for 1 h at 37 °C was optimal for obtaining the neutral and acidic RGPs with high yields from the RG-SPEP content. This novel HILIC-SPE method employing MALDI-TOF/TOF MS is appropriate for screening and isolating many new RGPs. With the aid of tandem MS, the structures of the two acidic RGPs are determined. For additional in-depth characterizations and detailed structural analyses of acidic RGPs, it is essential to further analyze the other unknown head groups therein by nuclear magnetic resonance (NMR) and LC-MS. This new procedure might be broadly applied to screen and characterize other plant-derived FPs.

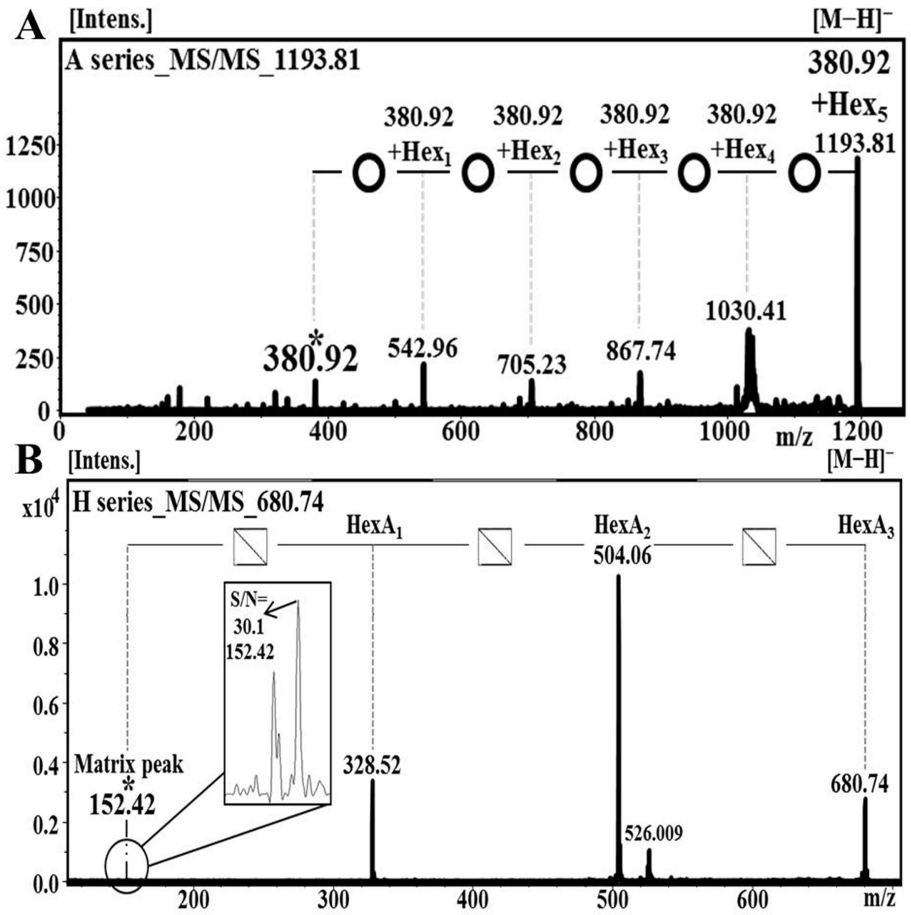

Fig. 4. Tandem MS spectra of the two cellulase-treated acidic RGPs. (A) A (380.02+Hex_n) series; m/z 1193.81. (B) H ($HexA_n$) series; m/z 680.74.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.02.005.

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