The Core Structure of Ginsenan PA, a Phagocytosis-Activating Polysaccharide from the Root of Panax ginseng

Masashi Tomoda,* Keiko Hirabayashi, Noriko Shimizu, Ryoko Gonda, and Naoko Ohara

Kyoritsu College of Pharmacy, Shibakoen, Minato-ku, Tokyo 105, Japan.
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Controlled Smith degradation and limited hydrolysis of ginsenan PA, the main phagocytosis-activating polysaccharide isolated from the root of Panax ginseng C. A. Meyer, were performed. The reticuloendothelial system-potentiating and anti-complementary activities of the degradation products were investigated. Methylation analysis of the primary and secondary Smith degradation products indicated that the core structural features of ginsenan PA include a backbone chain mainly composed of β-1,3-linked d-galactose. Almost half of the galactose units in the backbone carry side-chains composed of β-1,6-linked d-galactosyl residues at position 6. Further 3,6-branching of d-galactose units was observed in a part of the side-chains. α-L-Arabinose units are connected mainly to the core galactose moieties via position 6. Removal of most of the arabinose units had a considerable effect on immunological activity.

Keywords ginseng; polysaccharide; core structure; Smith degradation; de-arabinosylation; immunological activity

We have recently obtained from the root of Panax ginseng C. A. Meyer the main acidic polysaccharide having remarkable activity on the reticuloendothelial system (RES).1) The material root is a well-known Chinese crude drug under the name of ginseng. This polysaccharide, called ginsenan PA, is composed of L-arabinose, D-galactose, L-rhamnose, D-galacturonic acid and D-glucuronic acid in the molar ratio of 11:22:1:6:1, and in addition 1.3% O-acetyl groups. The results of structural studies indicated that the polysaccharide consists mainly of an α-L-arabinono-β-D-galactan type structure with additional rhamnogalacturonan type units and terminal β-D-glucuronic acid residues.

The present paper describes the controlled Smith degradation, limited acid hydrolysis and methylation analysis of the products, and presents the core structural features of ginsenan PA. This paper also describes the immunological effects of the degradation products on RES-potentiating and anti-complementary activities.

MATERIALS AND METHODS

Isolation of Polysaccharide This was performed as described in a previous report.1)

Deacetylation Followed by Periodate Oxidation Ginsenan PA (200 mg) was dissolved in water (20 ml) followed by the addition of 0.1 N sodium hydroxide (20 ml). After standing at room temperature for 30 min, the solution was neutralized with 5 M acetic acid. The solution was adjusted to 50 ml with water, then 0.1 N sodium metaperiodate (50 ml) was added and the solution was kept at 5°C in the dark. The periodate consumption was measured by a spectrophotometric method.2) Oxidation was complete after 5 d. A part (15 ml) of the reaction mixture was applied to a column (2.6 × 95 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected and analyzed using the phenol–sulfuric acid method.3) The eluates obtained from tubes 23 to 28 were combined, concentrated and lyophilized. The yield of the product (POP, periodate oxidation product) was 27.4 mg. The residual reaction mixture (85 ml) was successively treated with ethylene glycol (0.8 ml) at 5°C for 1 h and sodium borohydride (0.8 g) at 5°C for 16 h, then the pH adjusted to 5.0 by the addition of acetic acid. The solution was concentrated and applied to a column (5 × 81 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected and analyzed using the phenol–sulfuric acid method. The eluates obtained from tubes 31 to 38 were combined, concentrated and lyophilized. The yield of the product (PORP, periodate oxidation-reduction product) was 120 mg.

Controlled Smith Degradation PORP (120 mg) was dissolved in 0.5 N sulfuric acid (12 ml). After standing at 25°C for 16 h, the solution was neutralized with barium carbonate. The filtrate was concentrated and passed through a column (1 × 10 cm) of Dowex 50W-X8 (H+). The eluate with water was concentrated and applied to a column (5 × 86 cm) of Sephadex G-25. The eluates obtained from tubes 33 to 38 were combined, concentrated and lyophilized. The yield of this controlled Smith degradation product (SDP) was 40 mg.

Secondary Smith Degradation SDP (7.0 mg) was oxidized with 0.05 M sodium metaperiodate (4 ml) at 5°C for 4 d in the dark. The reaction mixture was successively treated with ethylene glycol (0.04 ml) and sodium borohydride (35 mg) as described above. After the addition of acetic acid up to pH 5.0, the solution was applied to a column (2.6 × 96 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 22 to 26 were combined and lyophilized. This product was treated with 0.5 N sulfuric acid as described above and, after neutralization, the solution was applied to a column (2.6 × 94 cm) of Sephadex G-25. The secondary Smith degradation product was obtained from the eluates in tubes 21 to 22 of fractions of 10 ml each. Yield, 2.6 mg.

Determination of Components Neutral sugars were analyzed by gas chromatography (GC) after conversion
of the hydrolyzate into alditol acetates as described previously.\(^4\) Hexuronic acid was determined by the m-hydroxybiphenyl method.\(^5\)

**Glass-Fiber Paper Electrophoresis** This was carried out as described previously\(^6\) on Whatman GF83 glass-fiber paper at 570 V for 1 h with 0.025 M Na\(_2\)B\(_4\)O\(_7\)-10H\(_2\)O-0.1 M NaOH (10:1, pH 9.3). SDP and the de-arabino-ylsated product (DAraP) gave single spots at distances of 75 and 24 mm from the origin toward the cathode, respectively.

**Gel Chromatography** The sample (2 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (2.6 × 93 cm) of Toyopearl HW-55F, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed using the phenol–sulfuric acid method. Standard pullulans (Showa Denko Co.) having known molecular masses were run on the column to obtain a calibration curve.

**Limited Acid Hydrolysis** The polysaccharide (110 mg) was dissolved in 0.05 M trifluoroacetic acid (11 ml), and the solution was heated at 100°C for 2 h. The acid was removed by evaporation, then the residue was dissolved in a 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (5 × 80 cm) of Toyopearl HW-55F, pre-equilibrated and developed with the same buffer. Fractions of 20 ml were collected, and the eluates obtained from tubes 34 to 48 were combined, concentrated and applied to a column (5 × 85 cm) of Sephadex G-25. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 32 to 37 were combined, concentrated and lyophilized. The yield of this DAraP was 37 mg.

**Reduction of Carboxyl Groups** This was carried out with 1-cyclohexyl-3-(2-morpholinoloxy)carbodiimide metho-p-toluene sulphonate and sodium borohydride as described previously.\(^6\) The reduction was repeated three times under the same conditions. The yield was 12.4 from 20.9 mg of DAraP.

**Methylation Analysis** Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously.\(^7\) The yields were 1.5 from 5.1 mg of SDP, 0.6 from 1.9 mg of the secondary Smith degradation product, 2.5 from 6.2 mg of DAraP, and 5.0 from 4.9 mg of the carboxyl-reduced DAraP. The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described previously.\(^8\) The partially methylated alditol acetates obtained were analyzed by gas chromatography–mass spectrometry (GC-MS) using a fused silica capillary column (0.32 mm i.d. × 30 m) of SP-2330 (Supelco Co.) with a programmed temperature increase of 4°C per min from 160 to 220°C at a helium flow of 1 ml per min. GC-MS was performed with a JEOL JMS-DX 303 mass spectrometer.

**Phagocytic Activity** This was measured by in vivo carbon clearance test as described previously.\(^4\) The samples and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and administered i.p. (20 mg/kg body weight) to male mice (ICR-SPF) once a day for 5 d.

**Anti-complementary Activity** This was measured as described in a previous report.\(^9\) Gelatin-veronal-buffered saline (pH 7.4) containing 500 μM Mg\(^{2+}\) and 150 μM Ca\(^{2+}\) (GVB\(^{2+}\)) was prepared, and normal human serum (NHS) was obtained from a healthy adult. Various dilutions of the samples in water were incubated and the residual total hemolytic complement (TCH\(_{50}\)) was determined using immunoglobulin M (Ig M)-hemolysis-sensitized sheep erythrocytes. NHS was incubated with water and GVB\(^{2+}\) to provide a control, and the activities of the samples were expressed as the percentage inhibition of the TCH\(_{50}\) of the control. Plantago-mucilage A from the seed of Plantago asiatica L.\(^10\) was used as a positive control.

**RESULTS**

Ginsenpan PA, the major polysaccharide obtained from ginseng, was deacetylated and subjected to periodate oxidation. The oxidation product (POP) was reduced, and the reduction product (PORP) was treated with dilute sulfuric acid at room temperature overnight.\(^11\) The controlled SDP thus obtained gave a single spot on glass-fiber paper electrophoresis, and gave a single peak having a value of 4.7 × 10\(^4\) for the molecular mass on gel chromatography. It had [z]\(^{20}\) ≈ -3.0° (H\(_2\)O, c = 0.1). Component sugar analysis showed that SDP was composed of d-galactose alone.

SDP was methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.\(^12\) The methylated product was hydrolyzed, then converted into the partially methylated alditol acetates. Analysis by GC-MS revealed derivatives of 2,3,4,6-tetra-O-methyl-d-galactose, 2,4,6-tri-O-methyl-d-galactose, 2,3,4-tri-O-methyl-d-galactose and 2,3,4-di-O-methyl-d-galactose as the products from the methylated SDP in the molar ratio of 3:3:1:3. In order to elucidate the backbone structure, SDP was further subjected to periodate oxidation followed by reduction. Then the product was treated with dilute sulfuric acid under the same conditions used for the isolation of SDP. Methylation analysis of the secondary Smith degradation product thus obtained revealed derivatives of 2,3,4,6-tetra-O-methyl-d-galactose, 2,4,6-tri-O-methyl-d-galactose, 2,3,4-tri-O-methyl-d-galactose and 2,3,4-di-O-methyl-d-galactose as the products in the molar ratio of 1:8:2:1. In addition, the secondary Smith degradation product showed a value of 1.1 × 10\(^4\) for the molecular mass on gel chromatography. These results suggest that ginsenpan PA has the β-1,3-linked d-galactan backbone structure shown in Chart 1.

Limited hydrolysis of ginsenpan PA with very dilute trifluoroacetic acid resulted in removal of the majority of L-arabinose moieties with partial loss of d-galactose and L-rhamnose units. The DAraP obtained was composed of d-galactose, L-arabinose, L-rhamnose, d-galacturonic acid and d-glucuronic acid in the molar ratio of 36:4:1:12:2. Methylation analysis of DAraP showed the presence of 2,3,4,6-tetra-O-methyl-d-galactose, 2,4,6-tri-O-methyl-d-galactose, 2,3,4-tri-O-methyl-p-galactose, 2,4-di-O-methyl-p-galactose, 2,3,5,6-tetra-O-methyl-L-arabinose and 3,4-di-O-methyl-L-rhamnose as the products in the molar ratio of 2:6:4:8:2:1. In this case, hexuronic acid methyl ethers were removed from the products by treatment with anion-exchange resin. Methylation analysis of DAraP gave an unsatisfactory result with regard to the molar ratio of the products. Therefore, the carboxyl groups of
hexuronic acid residues in DAraP were reduced to give the corresponding neutral sugar residues.\textsuperscript{13} Methylation analysis of the carboxyl-reduced DAraP revealed derivatives of 2,3,4,6-tetra-O-methyl-D-galactose, 2,4,6-tri-O-methyl-D-galactose, 2,3,6-tri-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-galactose, 2,4-di-O-methyl-D-galactose, 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,5-tri-O-methyl-L-arabinose and 3,4-di-O-methyl-L-rhamnose as the products in the molar ratio of 4:12:8:10:2:4:1.

The minimal unit in the structure of ginsenan PA is composed of terminal 2,1-arabinose, 2,1,5-linked L-arabinose, terminal \( \beta \)-D-galactose, \( \beta \)-1,3-linked \( \beta \)-galactose, 2,1,6-linked \( \beta \)-galactose, \( \beta \)-3,6-branched D-galactose, 2,1,2-linked L-rhamnose, 2,1,4-linked D-galacturonic acid and terminal \( \beta \)-D-glucuronic acid residues in the ratio of 9:2:1:4:6:11:1:6:1.\textsuperscript{14} Thus, the methylation analysis of DAraP revealed a pronounced increase in terminal and 1,3-linked D-galactose units and a marked decrease in 1,6-linked and 3,6-branched D-galactose units. These results indicate that the arabinose units are connected mainly to the core galactose moieties via position 6 of the intermediate and terminal of side-chains in ginsenan PA.

The effects of PORP, SDP and DAraP on the RES were demonstrated by a modification\textsuperscript{11} of the \textit{in vivo} carbon clearance test,\textsuperscript{14} using zymosan as a positive control. As
shown in Fig. 1, the phagocytic index of ginsenan PA was clearly decreased by both periodate oxidation followed by reduction and removal of the majority of L-arabinose units, and DPA showed no activity. However, both PORP and D AraP still showed significant activation of the RES at a low dose at which no effect on the positive control, zymosan, was evident.\textsuperscript{15)}

The anti-complementary activities of PORP, SDP and D AraP are shown in Fig. 2. Ginsenan PA showed remarkably active as described previously.\textsuperscript{1) Both de-arabinosylation and the controlled Smith degradation decreased the activity. However, the activities of these products were a little higher than that of the positive control, Plantago-mucilage A. On the other hand, the activity of PORP was lower than that of the other products.

DISCUSSION

Four RES-activating polysaccharides, ginsenan PA, PB, S-I and S-IIA, have been obtained from ginseng.\textsuperscript{1,16) Ginsenan PA, the major one of these, is a typical acidic arabino-3,6-galactan. To date, we have already identified thirty-four polysaccharides as RES-activating substances in crude drugs obtained from various plant sources. Among them, the acidic arabino-3,6-galactan group is very common. It is present in saponshikovan A,\textsuperscript{4) MVS-IIIa, -IVA and -VI,\textsuperscript{17–19) ukonan A, B and C,\textsuperscript{20–22) glycyrrhizans UA, UB and \textit{G}A\textsubscript{15,23) eucomman A,\textsuperscript{24) AMon-S,\textsuperscript{25) cnidirhan AG,\textsuperscript{26) ginsenans PA, PB, S-I and S-IIA,\textsuperscript{1,15) peonan SB,\textsuperscript{27) and alismian PII.\textsuperscript{28)}

We have elucidated the 1-1,3-linked D-galactan backbone structure as the core of polysaccharides in ukonan A, B and C,\textsuperscript{29–31) glycyrrhizans UA and \textit{G}A,\textsuperscript{32,33) cnidirhan AG,\textsuperscript{26) and alismian \textit{P}II\textsuperscript{28) in common. They possess side-chains composed of 1-6-and/or 1-3-linked D-galactose residues via position 6 of the galactan backbone. In general, chemical modifications of these polysaccharides such as periodate oxidation, Smith degradation and de-arabinosylation caused a remarkable decrease in, or disappearance of, the RES-potentiating activity.\textsuperscript{29–33) Similar effects were also observed with the degradation products of ginsenan PA. So the presence of a complicated branching involving arabinosyl residues may contribute to the high phagocytic activity of these arabino-3,6-galactans.

On the other hand, the effects of the products obtained by chemical modification of these polysaccharides on anti-complementary activity differ. The features of the anti-complementary activities of ginsenan PA and its chemical modification products were similar to those of ukonan A and its products.\textsuperscript{29) Both original polysaccharides exhibited remarkable activity.

The activity of SDP obtained from ukonan C was higher than that of the original polysaccharide.\textsuperscript{33}) Ukonan C is a characteristic glucose-rich acidic polysaccharide, and this result shows the contribution made by the arabinogalactan core structure of the polysaccharide to the anti-complementary activity.

In the case of glycyrrhizian \textit{GA}, the activity of the SDP was restored to its original level,\textsuperscript{33)} while the SDP obtained from glycyrrhizian \textit{UA} reduced the activity.\textsuperscript{32)} These results indicate that the presence of complicated side-chains may also contribute to the anti-complementary activity.

REFERENCES


