Core Structure of Glycyrrhizan GA, the Main Polysaccharide from the Stolon of *Glycyrrhiza glabra* var. *glandulifera*; Anti-complementary and Alkaline Phosphatase-Inducing Activities of the Polysaccharide and Its Degradation Products

Katsutoshi TAKADA, Masashi TOMODA* and Noriko SHIMIZU

Kyoritsu College of Pharmacy, Shibakōen, Minato-ku, Tokyo 105, Japan.   Received March 24, 1992

The controlled Smith degradation and limited hydrolysis of glycyrrhizan GA, a representative polysaccharide with remarkable phagocytosis-enhancing activity isolated from the stolon of *Glycyrrhiza glabra* L. var. *glandulifera* REG. *et* HERD. were carried out. Methylation analyses of the primary and the secondary Smith degradation products and of the limited hydrolysis product indicated that the core structural features of glycyrrhizan GA include a backbone chain composed of β-1,3-linked D-galactose residues. Three-fifths of the galactose units in the backbone carry side chains composed of β-1,3- and β-1,6-linked D-galactosyl residues at position 6. Anti-complementary and alkaline phosphatase-inducing activities of the polysaccharide, periodate oxidation-reduction and the controlled Smith degradation products were investigated, and the controlled Smith degradation product showed significant activity.

Keywords   *Glycyrrhiza glabra* var. *glandulifera*; licorice; glycyrrhizan GA; polysaccharide structure; Smith degradation; limited hydrolysis; immunological activity; anti-complementary activity; alkaline phosphatase-inducing activity

Glycyrrhizan GA is the representative polysaccharide having immunological activities obtained from the stolon of *Glycyrrhiza glabra* L. var. *glandulifera* REG. *et* HERD.[1] This plant is one of the materials of Chinese licorice in the Japanese market. We have already isolated three polysaccharides, glycyrrhizans UA, UB and UC, having significant activity on the reticuloendothelial system (RES) from the root of *Glycyrrhiza uralensis* FISCHER.[2,3] It is interesting that glycyrrhizan GA showed superior RES-potentiating activity to these three polysaccharides. The results of structural studies indicated that it has mainly an α-1,5-linked L-arabino-β-3,6-branched D-galactan type structure with additional α-1,3-linked and 2,5-branched L-arabinose, terminal β-D-galactose, α-1,2-linked and 2,4-branched L-rhamnose, α-1,4-linked D-galacturonic acid and terminal β-D-glucuronic acid residues.[1]

We now report the controlled Smith degradation and limited acid hydrolysis of glycyrrhizan GA, and methylation analysis of the degradation products for the elucidation of the core structural features. Besides the structure of the polysaccharide, the present paper also describes immunological effects of the degradation products on anti-complementary and alkaline phosphatase-inducing activities in order to prove the contribution of the core structure.

Materials and Methods

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

Deacetylation Followed by Periodate Oxidation   Glycyrrhizan GA (102 mg) was dissolved in 0.1 N sodium hydroxide (20 ml), and after standing at room temperature for 10 min, the solution was neutralized with 10 M acetic acid. The solution was adjusted to 22 ml with water, then 0.1 M sodium metaperiodate (22 ml) was added and the mixture was kept at 4 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[4] Oxidation was completed after 7 d. After successive treatment with ethylene glycol (0.4 ml) at 4 °C for 1 h and sodium borohydride (500 mg) at 4 °C for 16 h, the reaction mixture was adjusted to pH 5.0 by the addition of acetic acid. The solution was concentrated and applied to a column (5 × 80 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[5] The eluates obtained from tubes 29 to 33 were combined, concentrated and lyophilized. The yield of this periodate oxidation-reduction product (PORP) was 81.7 mg.

Controlled Smith Degradation   PORP (70 mg) was dissolved in 0.5 N sulfuric acid (7 ml), and after standing at 24 °C for 16 h, the solution was neutralized with Dowex 2 (OH⁻). The filtrate was concentrated and applied to a column (2.6 × 93 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 20 to 23 were combined, concentrated and lyophilized. The yield of this product (SDP) was 15.1 mg.

Secondary Smith Degradation   SDP (10.1 mg) was oxidized with 0.05 M sodium metaperiodate (5 ml) at 4 °C for 63 h in the dark. After successive treatment with ethylene glycol (0.05 ml) and sodium borohydride (50 mg) as described above, the solution was neutralized with acetic acid and applied to a column (2.6 × 94 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, concentrated and lyophilized. Yield, 5.2 mg. 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 × 93 cm) of Sephadex G-25. The secondary Smith degradation product was obtained from the eluates in tubes 22 to 24. Yield, 2.5 mg.

Determination of Components   Neutral sugars were analyzed by gas chromatography (GC) after conversion of the hydrolyzate into alditol acetates as described previously.[6]

Gel Chromatography   SDP (3 mg) was dissolved in a 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (2.6 × 96 cm) of Sephacryl S-200HR, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collectd and analyzed by the phenol–sulfuric acid method. Standard pullulans (Shōwa Denkō Co.) having known molecular masses were run on the column to obtain a calibration curve.

Methylation Analysis   Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously.[7] The yields were 4.5 mg from 4.0 mg of SDP, 2.8 mg from 2.5 mg of the secondary Smith degradation product, and 2.1 mg from 2.0 mg of the limited hydrolysis product. 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-GX303 mass spectrometer.

Limited Acid Hydrolysis   The polysaccharide (12 mg) was dissolved in 0.05 M trifluoroacetic acid (1.2 ml), and the solution was heated at 100 °C for 2 h. The acid was removed by evaporation, then the residue was dissolved in water and applied to a column (2.6 × 91 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, concentrated and lyophilized. Yield, 3.5 mg.

Anti-complementary Activity   This was measured as described previously.[9] The activities of the samples and a positive control, Plantago-mucilage A from the seed of *Plantago asiatica* L.,[10] were expressed as the percentage inhibition of the residual total hemolytic complement

(TCH$_{50}$) of the control.

Alkaline Phosphatase Assay This was measured as described previously.[11] Each sample solution and the cell suspension obtained from ICR-SPF male mice were mixed and incubated. The results were expressed as the arithmetic mean $\pm$ S.D. of triplicate cultures. Lipopolysaccharide from *E. coli* 0111:B4 (DIFCO Lab.) was used as a positive control. Some modifications of the amounts of reagents and the reaction time were applied in this study. Thus the cell suspensions (25 μl), 10% diethanolamine–HCl buffer (100 μl), 0.25 N sodium hydroxide (100 μl), and 15 min incubation were used, respectively.

Results

Glycyrrhizan GA was deacetylated and subjected to periodate oxidation followed by reduction. The product (PORP) was treated with dilute sulfuric acid at room temperature overnight,[12] then the controlled Smith degradation product (SDP) was obtained. SDP gave a single peak having a value of 3.6×10^4 for the molecular mass on gel chromatography. It had $[\alpha]_D^{24}$ $-9.3°$ (H$_2$O, $c = 0.1$). Quantitative analysis showed that SDP was composed of L-arabinose and D-galactose in the molar ratio of 1:28.

SDP was methylated with sodium hydroxide and methyl iodide in dimethyl sulfoxide.[13] The methylated product was hydrolyzed, then converted into the partially methylated alditol acetates. Analysis by GC-MS[14] gave the result shown in Table I. In order to elucidate the structural features of SDP, the secondary Smith degradation was performed by periodate oxidation followed by successive reduction and mild hydrolysis under the same conditions as the preparation of SDP. The product obtained was composed only of D-galactose. The result of its methylation analysis is also shown in Table I.

From these results, it can be presumed that SDP has a backbone chain composed of β-1,3-linked D-galactose residues, and that three-fifths of the galactose units in the backbone chain must carry both β-1,6- and β-1,3-linked galactosyl side chains at position 6. A part of β-1,6-linked galactose residues in the side chains carries a terminal arabinose at position 3. Possible structural features of SDP are given in Chart 1.

Limited acid hydrolysis of glycyrrhizan GA with very dilute acid afforded nearly complete removal of L-arabinose and L-rhamnose residues. The neutral component sugar observed in the de-arabinosylated product (DAraP) was mostly D-galactose. The result of methylation analysis of DAraP is given in Table I. In this case, hexuronic acid methyl ethers were removed from the products by teatment with anion-exchange resin. The methylation analysis of DAraP revealed a pronounced decrease in 3,6-branched D-galactose units and the disappearance of 1,3-linked

TABLE I. Methylation Analysis of the Limited Degradation Products of Glycyrrhizan GA

Methylated sugar (as alditol acetate)	Relative retention time[a]	Molar ratios			Structural feature
		Primary SDP	Secondary SDP	DAraP	
2,3,5-Me$_3$-L-arabinose	0.69	1.1	—	—	Ara$f$ 1→
2,3,4,6-Me$_4$-D-galactose	1.10	6.0	1.8	1.0	Gal$p$ 1→
2,4,6-Me$_3$-D-galactose	1.38	6.2	8.0	—	→3 Gal$p$ 1→
2,3,4-Me$_3$-D-galactose	1.62	9.3	0.9	3.0	→6 Gal$p$ 1→
2,4-Me$_2$-D-galactose	2.02	6.8	2.1	1.2	→6 Gal$p$ 1→ 3 ↑

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl D-glucitol. Abbreviation: Me = methyl. Ara$f$, L-arabinofuranose; Gal$p$, D-galactopyranose.
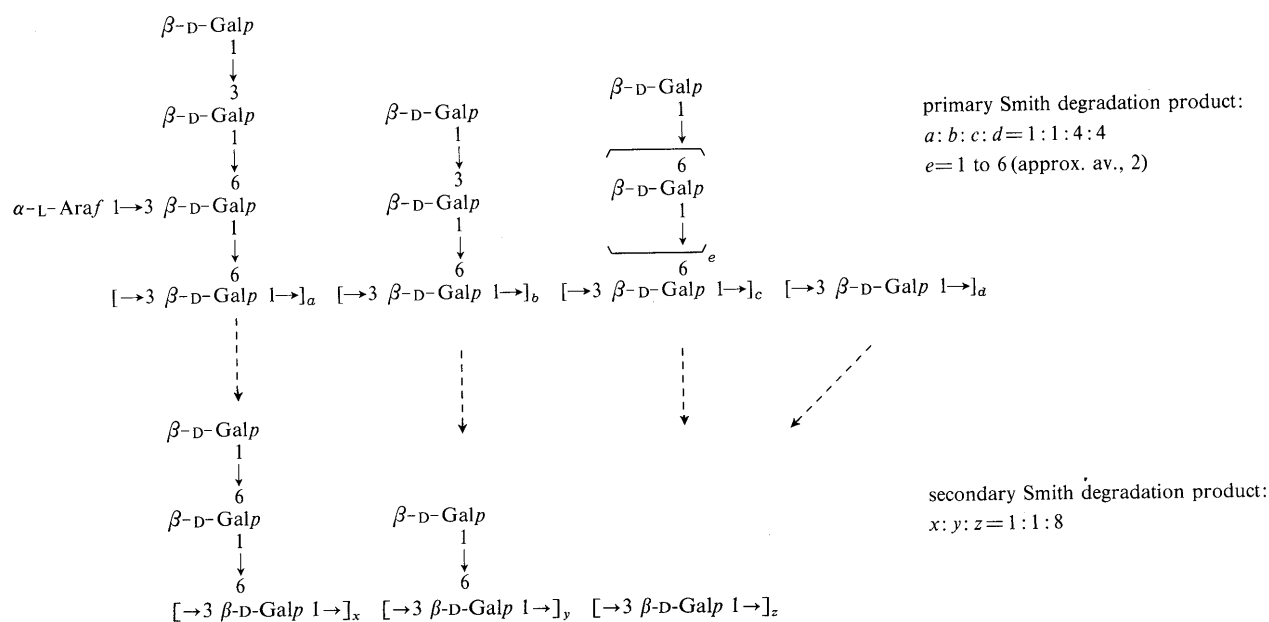

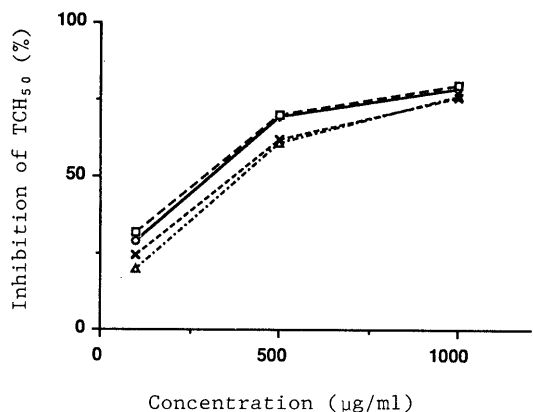Chart 1. Possible Structural Units of the Primary and the Secondary Smith Degradation Products of Glycyrrhizan GA

Fig. 1. Anti-complementary Activity of Glycyrrhizan GA and Its Degradation Products

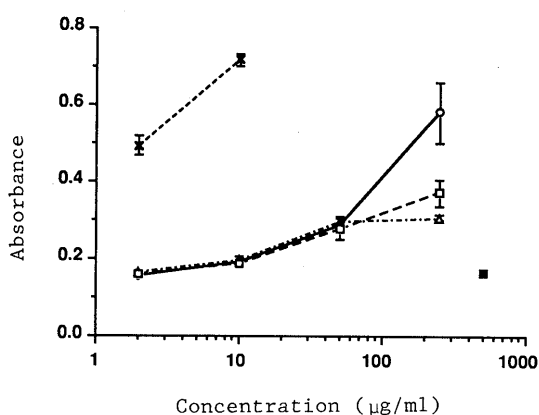—O—, glycrrhizan GA; ---△---, PORP; --□--, SDP; ---×---, Plantago-mucilage A.

Fig. 2. Mitogenic Activity of Glycyrrhizan GA and Its Degradation Products Assessed by Alkaline Phosphatase-Inducing Activity

—O—, glycyrrhizan GA; ---△---, PORP; --□--, SDP; ---×---, lipopolysaccharide; ■, nil.

D-galactose residues. These results indicate that the arabinose units are mainly connected to the galactose residues *via* position 3 of the terminal and the intermediate of galactosyl side chains in glycyrrhizan GA.

The anti-complementary activities of glycyrrhizan GA, PORP and SDP are shown in Fig. 1. Glycyrrhizan GA showed potent activity, which was a little higher than that of the positive control, Plantago-mucilage A. The activity of PORP was a little lower than that of the original polysaccharide, but the activity of SDP was restored to the orignial level.

The alkaline phosphatase activity of murine spleen cell was induced by stimulating directly with B cell mitogen or indirectly *via* lymphokines with T cell mitogen,[15] so the measurements of alkaline phosphatase-inducing activity with glycyrrhizan GA, PORP and SDP were then performed. As shown in Fig. 2, the activity was induced with glycyrrhizan GA in a dose dependent manner. Both SDP and PORP resulted in a lowering of the activity especially at a higher dose.

Discussion

We have obtained two acidic polysaccharides and a neutral polysaccharide, called glycyrrhizans UA, UB and

UC, from the root of *Glycyrrhiza uralensis*,[2,3] and isolated an acidic polysaccharide, called glycyrrhizan GA, from the stolon of *G. glabra* var. *glandulifera*.[1] These substances showed significant RES-potentiating activity. Based on the results of various structural studies,[1] it can be assumed that glycyrrhizan GA belongs to an acidic arabino-3,6-galactan type polysaccharide group. The other RES-active substances obtained by us, glycyrrhizan UA, saposhnikovan A[6] from the root and rhizome of *Saposhnikovia divaricata*, MVS-IIIA,[16] MVS-IVA,[17] and MVS-VI[18] from the seed of *Malva verticillata*, ukonans A and B[19,20] from the rhizome of *Curcuma longa*, and AMon-S[21] from the root of *Astragalus mongholicus* are also classified in the same group.

In addition to RES-potentiating activity,[1] the values of both anti-complementary and alkaline phosphatase-inducing activities of glycyrrhizan GA are superior to those of glycyrrhizan UA.[22] Glycyrrhizan UA, the main polysaccharide from *G. uralensis* root, has mainly α-1,5-linked L-arabino-β-3,6-branched D-galactan structure with α-1,3-linked L-arabinose, β-1,4-linked D-galactose, α-1,2-linked linear and α-2,4-branched L-rhamnose, and α-1,4-linked linear and α-2,4-branched D-galacturonic acid units. Glycyrrhizan GA possesses neither 1,4-linked galactose nor branched galacturonic acid residues, while α-2,5-branched L-arabinose and the unique terminal β-D-glucuronic acid units are present in it.[1]

Quite recently, the core structural features of glycyrrhizan UA were elucidated: it possesses a backbone chain composed of β-1,3-linked D-galactose, and all of the galactose units in the backbone carry side chains composed of α-L-arabino-β-1,3-linked D-galactose or β-1,6-linked D-galactose residues at position 6.[23] The core structure of glycyrrhizan GA was revealed in the present study to have a backbone chain composed of β-1,3-linked D-galactose, and three-fifths of the galactose units in the backbone carry side chains composed of β-1,3-linked and β-1,6-linked D-galactose residues at position 6. Some D-galactose units in the β-1, 6-linked side chains carry an α-L-arabinose residue at position 3.

With regard to the immunological activities of the controlled Smith degradation products having the core structures of original polysaccharides, SDP obtained from glycyrrhizan GA gave obviously higher values of anti-complementary and alkaline phosphatase-inducing activities than that from glycyrrhizan UA. The value of molecular mass of the former SDP is two times as high as the latter. In addition, the side chains of the former SDP are more complicated than those of the latter. These factors could be involved in the difference of these activities.

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