

The Core Structure and Immunological Activities of Glycyrrhizan UA, the Main Polysaccharide from the Root of *Glycyrrhiza uralensis*

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The controlled Smith degradation and limited hydrolysis of glycyrrhizan UA, the main phagocytosis-activating polysaccharide isolated from the root of *Glycyrrhiza uralensis* FISCHER, was performed. The reticuloendothelial system-potentiating, anti-complementary and alkaline phosphatase-inducing activities of glycyrrhizan UA and its degradation products were investigated. Methylation analyses of primary, secondary and tertiary Smith degradation products and of the limited hydrolysis product indicated that the core structural features of glycyrrhizan UA include a backbone chain composed of β -1,3-linked D-galactose. All of the galactose units in the backbone carry side chains composed of mainly α -1,5-linked L-arabino- β -1,6- or 1,3-linked D-galactose residues at position 6. Removal of the arabinosyl side chains caused a pronounced decrease in immunological activity.

Keywords *Glycyrrhiza uralensis*; root; glycyrrhizan UA; polysaccharide structure; Smith degradation; limited hydrolysis; immunological activity; reticuloendothelial system; anti-complementary activity; alkaline phosphatase-inducing activity

We recently have isolated from the root of *Glycyrrhiza uralensis* FISCHER three polysaccharides having significant activity on the reticuloendothelial system (RES).^{1,2} The root of this plant is a representative Chinese licorice. Among these polysaccharides, glycyrrhizan UA is present in the highest quantity. It is composed of L-arabinose, D-galactose, L-rhamnose and D-galacturonic acid in the molar ratio of 20:14:1:3, in addition to a 3.2% peptide moiety. 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 terminal α -L-arabinopyranose, α -1,3-linked L-arabinose, β -1,4-linked D-galactose, α -2,4-branched L-rhamnose, α -1,4-linked and α -2,4-branched D-galacturonic acid residues.¹

The present paper describes the controlled Smith degradation, limited acid hydrolysis and methylation analysis of the products. Besides the core structure of the polysaccharide, this paper also describes immunological effects of glycyrrhizan UA and its degradation products on RES-potentiating, anti-complementary and alkaline phosphatase-inducing activities.

Materials and Methods

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

Deacetylation Followed by Periodate Oxidation Glycyrrhizan UA (221 mg) was dissolved in water (22 ml), then 0.2 N sodium hydroxide (22 ml) was mixed in. After standing at room temperature for 30 min, the solution was neutralized with 10 M acetic acid. The solution was adjusted to 50 ml with water, then 0.1 M 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.³ Oxidation was completed after 5 d. A part (5 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 by the phenol-sulfuric acid method.⁴ The eluates obtained from tubes 24 to 27 were combined, concentrated and lyophilized. The yield of the product (POP) was 9 mg. The residual reaction mixture (95 ml) was successively treated with ethylene glycol (0.9 ml) at 5°C for 1 h and sodium borohydride (1 g) at 5°C for 16 h, then adjusted to pH 5.0 by the addition of acetic acid. The solution was concentrated and applied to a column (5 × 82 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. The eluates obtained from tubes 31 to 36 were combined, concentrated and lyophilized. The yield of the product (PORP) was 194 mg.

Controlled Smith Degradation The periodate oxidation-reduction

product (PORP; 176.4 mg) was dissolved in 0.5 N sulfuric acid (17 ml). After standing at 24°C for 16 h, the solution was neutralized with barium carbonate. The filtrate was concentrated and passed through a column (0.7 × 4 cm) of Dowex 50W-X8 (H⁺). The eluate with water was concentrated and applied to a column (5 × 84 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 31 to 35 were combined, concentrated and lyophilized. The yield of this product (SDP: controlled Smith degradation product) was 36.8 mg.

Secondary Smith Degradation SDP (10.5 mg) was oxidized with 0.05 M sodium metaperiodate (5 ml) at 5°C for 2 d in the dark. The reaction mixture was successively treated with ethylene glycol (0.05 ml) and sodium borohydride (53 mg) as described above. After the addition of acetic acid, the solution was applied to a column (2.6 × 90 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 24 were combined and lyophilized. Yield, 8.8 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 × 94 cm) of Sephadex G-25. The secondary Smith degradation product was obtained from the eluates in tubes 21 to 22. Yield, 2.9 mg.

Determination of Components Neutral sugars were analyzed by gas chromatography (GC) after conversion of the hydrolyzate into alditol acetates as described previously.⁵ Hexuronic acid was determined by the *m*-hydroxybiphenyl method.⁶

Polyacrylamide Gel Electrophoresis (PAGE) This was carried out in an apparatus with gel tubes (4 × 148 mm each) and a 5 mm Tris-glycine buffer (pH 8.3) at 5 mA/tube for 60 min. Gels were stained by the periodate-Schiff (PAS) procedure. SDP gave a distinct band at a distance of 16 mm from the origin.

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 × 98.5 cm) of Sephacryl S-300HR, pre-equilibrated, and developed with the same buffer. Fractions of 5 ml were collected 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 previously described.⁷ The yields were 2.1 mg from 4.9 mg of SDP, 2.0 mg from 2.3 mg of the secondary Smith degradation product, and 5.1 mg from 5.6 mg of the limited hydrolysis product. The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described in a previous report.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 (23.7 mg) was dissolved in 0.05 M trifluoroacetic acid (2.4 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 × 95 cm) of

Sephadex G-25. The column was eluted with water and fractions of 10 ml were collected. The eluates obtained from tubes 21 to 24 were combined, concentrated and lyophilized. Yield, 9.6 mg.

Tertiary Smith Degradation This was performed under the same conditions as in the secondary degradation described above. The yield of the product was 1.9 mg from the secondary Smith degradation product (4.1 mg).

Phagocytic Activity This was measured as described in a previous report.⁵⁾ The samples and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (20 mg/kg body weight) once a day. The phagocytic index, *K*, was calculated by means of the following equation:

$$K = (\ln OD_1 - \ln OD_2) / (t_2 - t_1)$$

where OD_1 and OD_2 are the optical densities at times t_1 and t_2 , respectively. Results were expressed as the arithmetic mean \pm S.D. of five male mice (ICR-SPF). The comparison of results was performed by means of the Student's *t*-test.

Anti-complementary Activity This activity was measured as described previously.⁹⁾ The sample solutions were incubated with normal human serum and gelatin-veronal-buffered saline containing Mg^{2+} and Ca^{2+} , and the residual total hemolytic complement (TCH_{50}) was determined by a method using immunoglobulin M-hemolysin-sensitized sheep erythrocytes. The activities of the samples were expressed as the percentage inhibition of the TCH_{50} of the control. Plantago-mucilage A¹⁰⁾ was used as a positive control.

Alkaline Phosphatase Assay This was measured as described in a previous report.¹¹⁾ Cells obtained from the ICR-SPF male mice were treated with ACK buffer, then washed with RPMI-1640 medium (Nissui Seiyaku Co.), resuspended with the medium containing 10% fetal calf serum (Flow Lab). Each sample solution and the cell suspension was mixed and incubated in a humidified atmosphere of 5% CO_2 . Each of the resultant cell suspensions was added to 10% diethylamine-HCl buffer (pH 9.8) containing 0.1% *p*-nitrophenyl phosphate. The reaction mixture was incubated at 37°C for 1 h and was terminated by the addition of sodium hydroxide. The absorbance at 405 nm was measured and 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.

Results

The hot water extract obtained from the root of *Glycyrrhiza uralensis* was treated with cetyltrimethylammonium bromide, and the supernatant fraction was purified by successive gel and affinity chromatographies.¹¹⁾ The minimal unit in the structure of glycyrrhizian UA, the major polysaccharide obtained, is composed of terminal α -L-arabinose, α -1,3-linked L-arabinose, α -1,5-linked L-arabinose, terminal β -D-galactose, β -1,3-linked D-galactose, β -1,4-linked D-galactose, β -1,6-linked D-galactose, β -3,6-branched D-galactose, α -1,2-linked L-rhamnose, α -2,4-branched L-rhamnose, α -1,4-linked D-galacturonic acid and α -2,4-branched D-galacturonic acid residues in the ratio of 26:8:26:1:8:8:3:22:1:2:6:3.¹¹⁾

Glycyrrhizian UA was deacetylated, then subjected to periodate oxidation. Most of the product (POP) was reduced, and the reduction product (PORP) was treated with dilute sulfuric acid at room temperature overnight,¹²⁾ then SDP was obtained. SDP gave a single band on PAGE, and gave a single peak having a value of 18.6×10^3 for the molecular mass on gel chromatography. It had $[\alpha]_D^{23} - 5.5^\circ$ (H_2O , $c=0.2$). Quantitative analysis showed that SDP was composed of L-arabinose and D-galactose in the molar ratio of 1:1.9, and it contained neither hexuronic acid nor a peptide moiety.

SDP was methylated with sodium hydroxide and methyl iodide in dimethyl sulfoxide.¹³⁾ The methylated product was hydrolyzed, then converted into the partially methyl-

ated alditol acetates. Analysis by GC-MS¹⁴⁾ showed the presence of 2,3,5-tri-*O*-methyl-L-arabinose, 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 2,4-di-*O*-methyl-D-galactose in the molar ratio of 1.0:3.8:2.0:8.2:5.2.

From the result of methylation analysis of SDP, it can be presumed that four-fifths of the branched galactose residues in the backbone chain of SDP must carry β -1,6-linked galactan type short side chains at position 6. In order to elucidate the structural features of SDP, secondary Smith degradation was performed by periodate oxidation followed by successive reduction and mild acid hydrolysis under the same conditions as the isolation of SDP. The product thus obtained was composed of only D-galactose. Methylation analysis of it revealed derivatives of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose as the products in a molar ratio of 0.9:5.4:1.0. This result shows two possible structural features of SDP as given in Chart 1. Therefore, tertiary Smith degradation was carried out, and methylation analysis of the product obtained gave derivatives of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose as the products in a molar ratio of 1.0:3.8:0.8. This result suggests that SDP possesses a type A structure in Chart 1.

Limited acid hydrolysis of glycyrrhizian UA with very dilute trifluoroacetic acid resulted in a nearly complete removal of an L-arabinose moiety. The de-arabinosylated product (DAraP) obtained was composed of D-galactose, L-rhamnose and D-galacturonic acid in the molar ratio of 18:1:4. 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, 2,3,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, 3,4-di-*O*-methyl-L-rhamnose and 3-*O*-methyl-L-rhamnose as the products in the molar ratio of 7.6:4.2:1.1:22.0:8.2:1.0:1.3. In this case, galacturonic acid methyl ethers were removed from the products by treatment with anion-

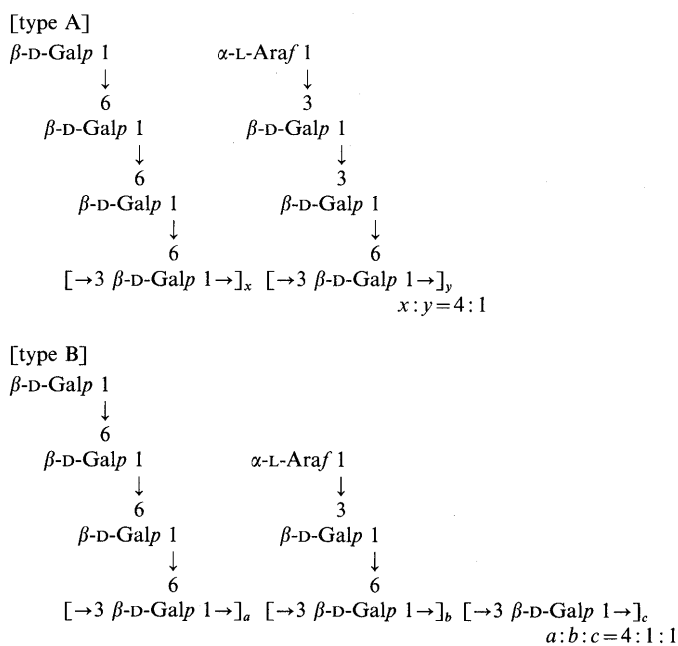


Chart 1. Possible Structural Units of the SDP of Glycyrrhizian UA

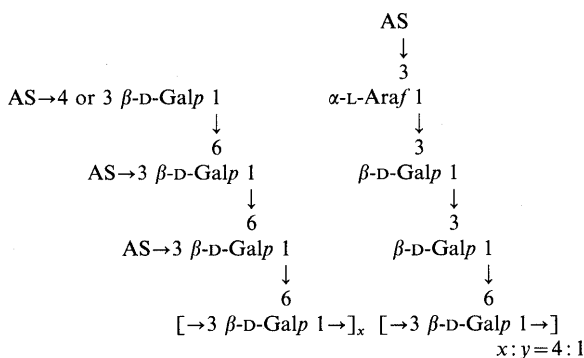


Chart 2. Possible Structural Units of the Arabinogalactan Core in Glycyrrhizan UA

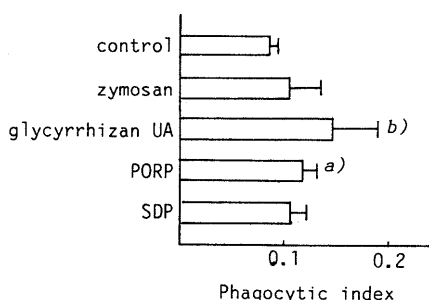


Fig. 1. Effects of Glycyrrhizan UA and Its Degradation Products on Phagocytosis

Significantly different from the control, a) $p < 0.01$, b) $p < 0.05$.

exchange resin. Thus, the methylation analysis of DAraP revealed a pronounced increase in terminal and 1,6-linked D-galactose units, and a marked decrease in 1,3-linked, 1,4-linked and 3,6-branched D-galactose units. These results indicate that the arabinose units are connected mainly to galactose residues *via* position 3 of the intermediate and positions 4 or 3 of the terminal of galactosyl side chains in glycyrrhizan UA.

Based on the accumulated evidence described above, it can be conceivable that glycyrrhizan UA has the core arabinogalactan structure shown in Chart 2. AS means mainly α -1,5-linked L-arabinosylarabinose side chains in Chart 2.

The effects of PORP and SDP on the RES were demonstrated by a modification⁵⁾ of the *in vivo* carbon clearance test¹⁵⁾ using zymosan as a positive control. As shown in Fig. 1, the phagocytic index of glycyrrhizan UA was decreased by periodate oxidation, and SDP showed no activity.

The anti-complementary activities of glycyrrhizan UA, POP, PORP, SDP and DAraP are shown in Fig. 2. Glycyrrhizan UA showed significant activity, though its activity was a little lower than that of the positive control (Plantago-mucilage A, the polysaccharide from the seed of *Plantago asiatica*^{10,16)}). Periodate oxidation had almost no influence on the activity, but the subsequent reduction which gave PORP considerably increased the activity. Both the Smith degradation and the de-arabinylation clearly decreased their activities.

The measurements of alkaline phosphatase-inducing activity with glycyrrhizan UA, POP, PORP, SDP and DAraP were performed by *in vitro* murine spleen cell

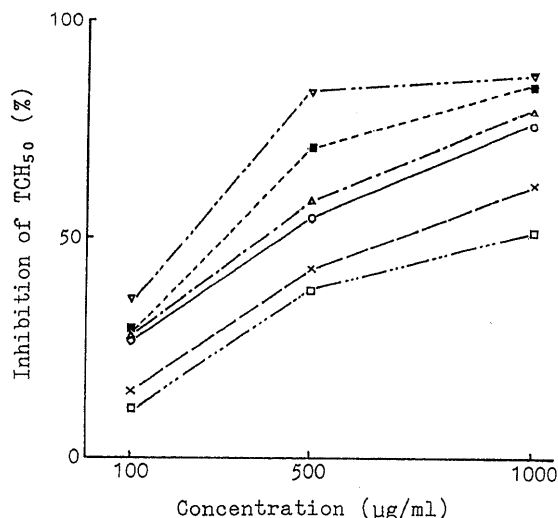


Fig. 2. Anti-complementary Activity of Glycyrrhizan UA and Its Degradation Products

Glycyrrhizan UA, \circ —; POP, \triangle —; PORP, ∇ —; SDP, \square —; DAraP, \times —; Plantago-mucilage A, \blacksquare —.

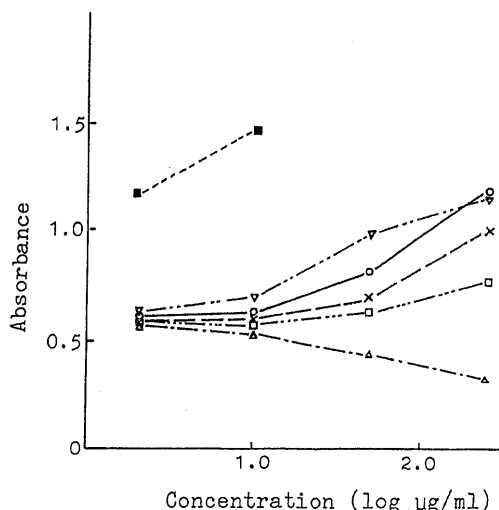


Fig. 3. Mitogenic Activity of Glycyrrhizan UA and Its Degradation Products Assessed by Alkaline Phosphatase-Inducing Activity

Glycyrrhizan UA, \circ —; POP, \triangle —; PORP, ∇ —; SDP, \square —; DAraP, \times —; Lipopolysaccharide, \blacksquare —.

assay.¹⁷⁾ As shown in Fig. 3, when the cells were stimulated with glycyrrhizan UA, the activity was induced in a dose dependent manner. Periodate oxidation produced a negative effect, but subsequent reduction to give PORP increased the activity to some extent, compared with the original polysaccharide. Both the de-arabinylation and the Smith degradations resulted in lowering the activity.

Discussion

Three RES-activating polysaccharides, glycyrrhizans UA, UB and UC, have been obtained from the root of *Glycyrrhiza uralensis*.^{1,2)} Glycyrrhizan UA, the major one among them, belongs to an acidic arabino-3,6-galactan type polysaccharide. We have already isolated and characterized seventeen polysaccharides from nine kinds of crude drugs as the other active substances.^{5,7,11,18-31)}

These polysaccharides are divided into four groups:

acidic arabino-3,6-galactan,^{1,5,19-21,23,24,29,30} arabinogalactoglucan,^{2,7,25,26} arabinoxylan,^{11,22} and rhamnogalacturonan having various arabinogalactan type side chains.^{1,18,27,28,31}

Thus, acidic arabino-3,6-galactan occupies the position of the major group, and glycyrrhizic acid is a typical α -1,5-linked L-arabino- β -3,6-branched D-galactan. The ratio of arabinogalactan moieties in glycyrrhizic acid amounts to nearly 90%. In the present study, we found both anti-complementary and alkaline phosphatase-inducing activities on glycyrrhizic acid, in addition to RES-potentiating activity. The complement system plays an important role in host defence, inflammation or allergic reactions, and various plant polysaccharides and bacterial lipopolysaccharides activate the complement.⁹ The alkaline phosphatase activity of murine spleen cells was induced by direct stimulation with B cell mitogen or indirectly via lymphokines with T cell mitogen.¹⁷ Recently, we found a marked enhancement of this activity by the deacetylated product of *Plantago-mucilage A*.¹¹ In the present study, it was elucidated that arabinose residues form the many short branches linking to the galactan core in glycyrrhizic acid. From the results of a pronounced decrease in activities resulting from controlled Smith degradation and selective removal of arabinose residues in glycyrrhizic acid, it can be presumed that the complicated branching structure in the polysaccharide contributes to these immunological activities.

The polyol form product, PORP, obtained by periodate oxidation followed by reduction, enhanced the anti-complementary activity. This is the first example of such an effect on the activity by a polyol-type degradation product. It may be conceivable that the steric influence of the galactan core supports this effect.

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