Characterization of Two Polysaccharides Having Activity on the Reticuloendothelial System from the Root of Glycyrrhiza uralensis

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Two polysaccharides, called glycyrrhizans UA and UB, were isolated from the root of Glycyrrhiza uralensis FISCHER. They were homogenous on electrophoresis and gel chromatography, and showed reticuloendothelial system-potentiating activity in a carbon clearance test. Glycyrrhizan UA is composed of L-arabinose: D-galactose: L-ramnose: D-galacturonic acid in the molar ratio of 20:14:1:5:3, and glycyrrhizan UB is composed of L-arabinose: D-galactose: D-glucose: L-ramnose: D-galacturonic acid in the molar ratio of 12:10:1:10:20, in addition to small amounts of O-acetyl groups and peptide moiety, respectively. About 10% (glycyrrhizan UA) and 35% (glycyrrhizan UB) of the D-galacturonic acid residues exist as the methyl esters. Methylation analysis, carbon-13 nuclear magnetic resonance and periodate oxidation studies indicated their structural features.

Keywords Glycyrrhiza uralensis; root; licorice; polysaccharide structure; reticuloendothelial system; immunological activity; glycyrrhizan UA; glycyrrhizan UB; acidic arabino-galactan

The root of Glycyrrhiza uralensis FISCHER is a representative Chinese licorice with the trade name Tongpei licorice (Japanese name, Tohoku kanzo). The licorice root is a very important crude drug used in China and Japan as well as in European countries. Many components in this crude drug have been reported, but no pure polysaccharide with biological activity has so far been obtained. We have now isolated two polysaccharides from the root of G. uralensis, and these polysaccharides show significant activity on the reticuloendothelial system (RES). The present paper describes their isolation from this crude drug, their structural analysis and immunological activity.

Material and Methods
Isolation of Polysaccharides The material was imported from China. The sliced roots (1.5 kg) were extracted with hot water (15 l) under stirring for 1 h in a boiling water bath. After centrifugation, the supernatant was poured into two volumes of ethanol. After centrifugation and drying, the precipitate (28.3 g) was dissolved in 0.01% sodium sulfite (631 l) and centrifuged; 5% cetyltrimethylammonium bromide (850 ml) was then added to the supernatant. The precipitate was separated by centrifugation, then dissolved in 0.2 M sodium chloride (1420 ml). After centrifugation, the supernatant was poured into two volumes of ethanol. The resulting precipitate was dissolved in water, then dialyzed and lyophilized. The yield of this fraction (CTAB-ppt) was 841.5 mg. The supernatant obtained after addition of cetyltrimethylammonium bromide was poured into two volumes of ethanol, and after centrifugation, the precipitate was dissolved in water, then dialyzed and lyophilized. The yield of this fraction (CTAB-sup) was 3.5 g. Fraction CTAB-sup (300 mg) was dissolved in water and applied to a column (5 x 85 cm) of Sephacryl S-300. The column was equilibrated and eluted with 0.1 M Tris-HCl buffer (pH 7.0), and fractions of 20 ml were collected and analyzed by the phenol-sulfuric acid method.11 Fraction I was obtained from tubes 27 to 29, fr. 2 from tubes 30 to 33, fr. 3 from tubes 34 to 44, and fr. 4 from tubes 45 to 61. Fractions 3 and 4 were dialyzed and rechromatographed using the same column of Sephacryl S-300. After dialysis and gel chromatography using a column (5 x 79 cm) of Sephadex G-25 with water, frs. A and B were obtained from frs. 3 and 4, respectively; their yields were 42 and 46 mg.

Fraction A (100 mg) was dissolved in 1/15 M phosphate buffer (pH 7.0) containing 0.15 M NaCl, 1 mM MgCl2, and 1 mM CaCl2, and applied to a column (1.5 x 40.5 cm) of Con A-Sepharose (Pharmacia Co.). The column was equilibrated and eluted with the same buffer at 4°C, and fractions of 10 ml were collected. The eluates obtained from tubes 6 to 9 were combined, dialyzed and then concentrated. The solution from 250 mg of fr. A was applied to a column (5 x 77 cm) of Sephadex G-25. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 29 to 33 were combined, concentrated and lyophilized. Glycyrrhizan UA was obtained as a white powder. The yield from 3.5 g of fr. CTAB-sup was 261 mg. Fraction B was applied to affinity chromatography with Con A-Sepharose under the same conditions as those of fr. A, followed by dialysis and gel chromatography with Sephadex G-25. After concentration of the sugar-containing fraction, the solution obtained was treated with one-third volume of 10% trichloroacetic acid. Following centrifugation, the acid was removed from the supernatant by extraction with ether, then the aqueous layer was applied to a column (5 x 87 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 30 to 32 were combined, concentrated and lyophilized. Glycyrrhizan UB was obtained as a white powder from fr. B. The yield from 3.5 g of fr. CTAB-sup was 129 mg.

Polyacrylamide Gel Electrophoresis (PAGE) This was carried out in an apparatus with gel tubes (4 x 140 mm each) and 5 mM Tris–glycine buffer (pH 8.3) at 5 mA/tube for 40 min. Gels were stained by the periodate–Schiff (PAS) procedure and with Coomassie blue reagent. Glycyrrhizans UA and UB gave distinct bands at distances of 66 and 62 mm from the origin, respectively.

Gel Chromatography The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (2.6 x 98 cm) of Sephacryl S-300, 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 Denko Co.) having known molecular weights were run on the column to obtain a calibration curve.

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 in physiological saline and dosed i.p. (20 mg/kg body weight) once a day. The phagocytic index, $A$, 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 ± S.D. of five male mice (ICR-SPF).

Qualitative Analysis of Component Sugars Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described in a previous report.25 Galacturonic acid was determined by a modification of the carbazole method.51 GC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector.

Determination of Components Neutral sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described in a previous report.51 Galacturonic acid was determined by a modification of the carbazole method.51 Peptide determination was performed by the method of Lowry et al.60

Determination of O-Acetyl Groups The sample was hydrolyzed with 0.2 N hydrochloric acid and analyzed by GC using propionic acid as an internal standard as described in a previous report.51

Determination of O-Methyl Groups in Methyl Esters This was performed by GC after saponification using ethanol as an internal standard as described in a previous report.8

Nuclear Magnetic Resonance (NMR) NMR spectra were recorded on

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TABLE I. Relative Retention Times on GC and Main Fragments in MS of Partially Methylated Alditoll Acetates

<table>
<thead>
<tr>
<th>Relative retention time</th>
<th>Main fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4-Ac-2,3,5-Me-α-arabinitol</td>
<td>0.69 43, 45, 71, 87, 101, 117, 129, 161</td>
</tr>
<tr>
<td>1.5-Ac-2,3,4-Me-α-arabinitol</td>
<td>0.79 43, 101, 117, 161</td>
</tr>
<tr>
<td>1.3,5-Ac-2,4,6-Me-α-arabinitol</td>
<td>1.04 43, 87, 113, 127, 233</td>
</tr>
<tr>
<td>1.4,5-Ac-2,3,6-Me-α-arabinitol</td>
<td>1.13 43, 87, 101, 117, 129, 189</td>
</tr>
<tr>
<td>1.2,5-Ac-3,4-Me-α-rhamnitol</td>
<td>0.95 43, 89, 129, 131, 189</td>
</tr>
<tr>
<td>1.2,4,5-Ac-3,5-Me-α-rhamnitol</td>
<td>1.28 43, 87, 109, 129, 143, 189, 203</td>
</tr>
<tr>
<td>1.5-Ac-2,3,4,6-Me-α-glucitol</td>
<td>1.00 43, 45, 71, 87, 101, 117, 129, 145, 161, 205</td>
</tr>
<tr>
<td>1.5-Ac-2,3,4,5-Me-α-galactitol</td>
<td>1.09 43, 45, 71, 87, 101, 117, 129, 145, 161, 205</td>
</tr>
<tr>
<td>1.3,5-Ac-2,4,6,Me-α-galactitol</td>
<td>1.36 43, 45, 71, 87, 101, 117, 129, 161</td>
</tr>
<tr>
<td>1.4,5-Ac-2,3,5-Me-α-galactitol</td>
<td>1.44 43, 45, 87, 99, 101, 113, 117, 233</td>
</tr>
<tr>
<td>1.5,6-Ac-2,3,4-Me-α-galactitol</td>
<td>1.58 43, 87, 99, 101, 117, 129, 161, 189</td>
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<tr>
<td>1.2,4,5-Ac-3,6-Me-α-galactitol</td>
<td>1.75 43, 45, 87, 99, 113, 129, 189, 233</td>
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<tr>
<td>1.3,5,6-Ac-2,4-Me-α-galactitol</td>
<td>2.01 43, 87, 117, 129, 189</td>
</tr>
</tbody>
</table>

a) Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol. Abbreviations: Ac = acetyl; Me = methyl (e.g., 1.4-Ac-2,3,5-Me = 1,4-di-O-acetyl-2,3,5-tri-O-methyl-). a)

A JOEL JNM-GX 270 FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 30°C.

Reduction of Carboxyl Groups This was carried out with 1-cyclohexyl-3(2-morpholinooxy)carbodiimide metho-p-toluene sulfonate and sodium borohydride as described in a previous report. The reduction was repeated four times under the same conditions. Yields were 27 mg from 52 mg of glycyrrhizic acid A (21 mg from 50 mg of glycyrrhizic acid B (21 mg from 50 mg of glycyrrhizic acid U). The reaction was repeated three times under the same conditions. Yields were 4 mg from 10 mg of glycyrrhizic acid A (21 mg from 50 mg of glycyrrhizic acid B (21 mg from 50 mg of glycyrrhizic acid U).

Methylation Analysis Methylation was performed with methylsulfonic carbanion and methyl iodide in dimethyl sulfoxide as described in a previous report. The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described in a previous report. 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. x 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 JOEL JMS-GX mass spectrometer. The relative retention times of the products with respect to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol in GC and the main fragments in MS are listed in Table I.

Periodate Oxidation The sample (5.0 mg) was oxidized with 0.05 M sodium periodate (2.5 ml) at 5°C in the dark. The periodate consumption was measured by a spectrophotometric method. Oxidation was continued after 2.5 d. The reaction mixture was successively treated with ethylene glycol (0.04 ml) at 5°C for 1 h and sodium borohydride (25 mg) at 5°C for 1 h, then distilled to pH 5.0 by addition of acetic acid. The solution was concentrated and applied to a column (2.6 x 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 24 were combined, concentrated, and lyophillized. Yields were 3.1 mg from glycyrrhizic acid A and 2.6 mg from glycyrrhizic acid B. Determination of the components was carried out as described above.

Results The crude polysaccharide fraction was isolated from the root of Glycyrrhiza uraleensis by hot water extraction followed by precipitation with ethanol, then dissolved in dilute sodium sulfate. After treatment with cetyltrimethylammonium bromide, the supernatant obtained was poured into ethanol. The resulting precipitate was purified by gel chromatography with Sephadex S-300 and Sephadex G-25, and the solution obtained was subjected to affinity chromatography on A-Sepharose. A pure polysaccharide designated as glycyrrhizic acid A (21 mg from 50 mg of glycyrrhizic acid U) was obtained from the passed-through fraction with a phosphate buffer, followed by dialysis and gel chromatography with Sephadex G-25. The solution of fr. B was also subjected to affinity chromatography under the same conditions as those of fr. A. The passed-through fraction was dialyzed, concentrated, and purified by gel chromatography with Sephadex G-25. The fraction obtained was treated with trichloroacetic acid followed by gel chromatography with Sephadex G-25, and another pure polysaccharide designated as glycyrrhizic acid B was obtained. The isolation method of the two polysaccharides is summarized in Table I. The Con A-adsorbed fractions obtained from frs. A and B by the affinity chromatography were dialyzed with the other RES-activating polysaccharides called glycyrrhizins UC and UE, respectively. Their properties and structural features will be reported in the near future.

Each polysaccharide gave a single band on PAGE, and gave a single peak on gel chromatography. Glycyrrhizin...
UA had $\Delta_{2}^{24} = 56.3^\circ$ (H$_2$O, c = 0.1) and glycyrrhizic acid UB had $\Delta_{2}^{24} = 61.0^\circ$ (H$_2$O, c = 0.1). Gel chromatography gave values of $69.0 \times 10^3$ and $10.7 \times 10^3$ for the molecular masses of glycyrrhizics UA and UB, respectively.

The effects of the two polysaccharides and fr. CTAB-ppt on the RES were demonstrated by a modification$^3$ of the in vivo carbon clearance test$^3$ using zymosan as a positive control. As shown in Fig. 2, the phagocytic indices were significantly increased, suggesting the activation of RES by i.p. injection of the two polysaccharides. Fraction CTAB-ppt showed no RES activity.

Glycyrrhizic acid UA is composed of L-arabinose, D-galactose, L-rhamnose, D-galacturonic acid, and a peptide moiety. Quantitative analyses showed that it contained 45.0% arabinose, 37.8% galactose, 2.5% rhamnose, 9.0% galacturonic acid and 3.2% peptide moiety. The molar ratio of these component sugars was 20:14:1:3. Glycyrrhizic acid UB is composed of L-arabinose, D-galactose, D-glucose, L-rhamnose, D-galacturonic acid and a peptide moiety. It contained 16.9% arabinose, 16.7% galactose, 1.9% glucose, 14.1% rhamnose, 37.3% galacturonic acid and 7.5% peptide moiety. The molar ratio of these component sugars was 12:10:1:10:20.

The carbon-13 NMR ($^{13}$C-NMR) spectrum of glycyrrhizic acid UA showed signals at $\delta$ 21.75 and 178.26 ppm, suggesting the presence of O-acetyl groups. In addition, the $^{13}$C-NMR spectrum showed a signal at $\delta$ 57.01 ppm, suggesting the presence of O-methyl groups as carboxylic acid methyl esters. The presence of these groups was confirmed by GC of the hydrolyzate, and the polysaccharide contained 2.9% acetyl and 0.2% methoxyl groups. Thus, about 10% of the galacturonic acid residues in the polysaccharide exist as methyl esters.

Further, the $^{13}$C-NMR spectrum showed six signals due to anomic carbons at $\delta$ 102.09, 102.44, 105.31, 105.85, 110.13 and 111.94 ppm. The first and the second were assigned to the anomeric carbons of $\alpha$-D-galactopyranosyluronic acid and $\alpha$-L-rhamnopyranose, respectively.$^{14}$ The signals at $\delta$ 105.31 and 105.85 ppm were assigned to the anomic carbons of $\beta$-D-galactopyranosyluronic acid and $\alpha$-L-rhamnopyranose, respectively.$^{15,16}$ The signals at $\delta$ 110.13 and 111.94 ppm were assigned to the anomic carbons of $\alpha$-L-arabinofuranose.$^{16}$

In the case of glycyrrhizic acid UB, its $^{13}$C-NMR spectrum showed the signals of O-acetyl groups at $\delta$ 21.75 and 177.34 ppm and the signal of O-methyl groups as carboxylic acid methyl esters at $\delta$ 57.01 ppm. The presence of these groups was also confirmed by GC of the hydrolyzate, and quantitative analysis showed that the polysaccharide contained 2.5% acetyl and 2.3% methoxyl groups. About 35% of the galacturonic acid residues exist as methyl esters.

In addition, the $^{13}$C-NMR spectrum of glycyrrhizic acid UB showed seven signals due to anomeric carbons at $\delta$ 100.31, 101.37, 102.18, 106.08, 107.06, 110.14 and 111.94 ppm. The first, the second and the third signals were assigned to the anomeric carbons of $\alpha$-D-glucopyranose,$^{13}$ $\alpha$-D-galactopyranosyluronic acid and $\alpha$-L-rhamnopyranose, respectively. The signals at $\delta$ 106.08 and 107.06 ppm were assigned to the anomeric carbons of $\beta$-D-galactopyranose and $\alpha$-L-arabinopyranose, respectively. The last two signals were assigned to the anomeric carbons of $\alpha$-L-arabinofuranose.

The carboxyl groups of galacturonic acid residues in each polysaccharide were reduced to give the corresponding neutral sugar residues.$^{17}$ Both the original polysaccharides and the carboxyl-reduced derivatives were methylated with methyldimethyl carbamion and methyl iodide in dimethyl sulfide.$^{18}$ The methylated products were hydrolyzed, then converted into the partially methylated aldotic acids. Hexuronic acid methyl ethers from the original samples were removed from the hydrolyzate by treatment with an anion-exchange resin. Analysis by GC-MS$^{19}$ gave the results shown in Tables II and III.
and an arabinoxylan called cinnaminal AX from the bark of *Cinnamomum cassia*).²⁶³ Saposhnikov A has an α-, 1,4-linked β-D-galacturonan backbone bearing arabinono-3,6-galactan type side chains, and saposhnikov C possesses a rhamnogalacturonan backbone bearing α-3,5-branched l-arabinan and β-3,4-branched D-galactan side chains. MVS-III A has mainly arabinono-3,6-galactan structure with α-1,3-linked l-arabinopyranosyl, β-1,4-linked D-xylansyl and α-1,4-linked D-galactan units. MVS-IVA belongs to a structural type similar to MVS-III A with additional α-1,2-linked l-rhamnose units. Further, ukonans A and B are basically arabinono-3,6-galactans having a rhamnogalacturonan backbone. They belong to a type of polysaccharide similar to MVS-IVA. However, ukonans A and B have additional 3,4-branched D-xylansyl, 2,4-branched l-rhamnosyl and terminal α-1,4-linked D-glucosyl residues. Cinnaminal AX is a new structural type of RES-activating polysaccharide. It has a β-1,4-linked D-xylan backbone bearing terminal β-1-arabinopyranosyl units and α-L-arabinofuranosyl-(1→3)-β-D-arabinopyranose side chains.

As the other examples of acidic arabinogalactans having a phagocytosis-enhancing effect, polysaccharide F isolated from *Echinacea purpurea* cell culture²⁷ and polysaccharide Fb isolated from *Vaccinium album* berry²⁸ have been reported. Recently, the effect of the former polysaccharide in activating macrophages to cytotoxicity against tumor cells has been revealed.²⁹ Polysaccharide F has a β-1,3-linked D-galactan backbone with β-1,6-linked D-galactose side chains carrying terminal L-arabinose, and it also contains α-1,4-linked D-galacturonic residues. Fb possesses a rhamnogalacturonan backbone and arabinono-3,6-galactan type side chains.

Evidently the main part of glycyrrhizin UA is occupied by the components of arabinono-3,6-galactan type polysaccharide units. In addition, it has terminal α-1,3-linked L-arabinopyranosyl, β-1,4-linked D-galactosyl residues and branched rhamnogalacturonan units. Glycyrrhizin UB is a basically similar type of polysaccharide to glycyrrhin UA, though branched rhamnogalacturonan units occupy the major part of it. Further, glycyrrhizin UB possesses additional β-2,4-branched D-galactosyl and terminal α-D-glucosyl units. The presence of 1,3-linked l-arabinopyranosyl units in these polysaccharides may contribute to the RES activity.²²²⁻²⁶³

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**References**