

# A Novel Neutral Polysaccharide Having Activity on the Reticuloendothelial System from the Root of *Glycyrrhiza uralensis*

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A neutral polysaccharide, named glycyrrhizan UC, was isolated from the root of *Glycyrrhiza uralensis* FISCHER. It was homogeneous on electrophoresis and gel chromatography, and its molecular mass was estimated to be 69000. Glycyrrhizan UC is composed of L-arabinose:D-galactose:D-glucose:L-rhamnose in the molar ratio of 10:30 27:1. Methylation analysis, carbon-13 nuclear magnetic resonance and periodate oxidation studies indicated its structural feature as an arabino-3,6-galacto-glucan type polysaccharide.

**Keywords** *Glycyrrhiza uralensis*; root; licorice; glycyrrhizan UC; polysaccharide structure; reticuloendothelial system; immunological activity; arabinogalactoglucan

Recently, we isolated and elucidated the structural features of two acidic polysaccharides having activity on the reticuloendothelial system (RES), named glycyrrhizans UA and UB, from the root of *Glycyrrhiza uralensis* FISCHER.<sup>1)</sup> The root of this plant is a representative Chinese licorice, which is a very important crude drug in China and Japan. Glycyrrhizans UA and UB were obtained as major acidic polysaccharides having immunological activity. The present paper describes the isolation and structural features of a novel RES-activating neutral polysaccharide from this crude drug.

## Materials and Methods

**Isolation of Polysaccharide** The material was imported from China. The sliced roots (2 kg) were extracted with hot water (20 l) by stirring for 1 h in a boiling water bath. After centrifugation, the residue was extracted with hot water (10 l) for 30 min. The combined supernatant was poured into two volumes of ethanol. The precipitate (37.1 g) obtained was extracted with hot water (7.4 l) by stirring for 30 min in a boiling water bath. After centrifugation, the supernatant was added to 1/9 volume of 0.1% sodium sulfate; 5% cetyltrimethylammonium bromide (1.1 l) was then added to the solution (7.3 l). The supernatant obtained was poured into two volumes of ethanol. The precipitate was dissolved in water, then dialyzed, centrifuged, concentrated and lyophilized. The yield of this fraction (CTAB-Sup) was 3.11 g. Fraction CTAB-Sup (311 mg) was dissolved in water and applied to a column (5 × 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.<sup>2)</sup> The eluates obtained from tubes 30 to 38 were combined, dialyzed and rechromatographed using the same column of Sephacryl S-300. After dialysis and gel chromatography using a column (5 × 84 cm) of Sephadex G-25 with water, fr. A (67 mg) was obtained. Fraction A (170 mg) was dissolved in 1/15 M phosphate buffer (pH 7.0) containing 0.15 M NaCl, 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>, and applied to a column (1.5 × 40 cm) of Con A-Sepharose (Pharmacia Co.). The column was equilibrated and eluted with the same buffer at 4°C. After elution with the same buffer (140 ml), the column was eluted with 10 mM methyl- $\alpha$ -D-mannopyranoside in the same buffer solution. Fractions of 10 ml were collected, and the eluates obtained from tubes 8 and 9 were combined, dialyzed and concentrated. The solution was applied to a column (5 × 83 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. The eluates obtained from tubes 29 to 39 were combined, dialyzed and concentrated. The solution was 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 30 to 33 were combined, concentrated and lyophilized. Glycyrrhizan UC was obtained as white powder. Yield, 9.6 mg.

**Polycrylamide Gel Electrophoresis (PAGE)** This was carried out in an apparatus with gel tubes (4 × 140 mm each) and a 5 mM Tris-glycine buffer (pH 8.3) at 5 mA/tube for 40 min. Gels were stained by the periodate-Schiff (PAS) procedure. Glycyrrhizan UC produced a distinct band at a distance of 72 mm from the origin.

**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 × 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

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

	Relative retention time <sup>a)</sup>	Main fragments (m/z)
1,4-Ac-2,3,5-Me-L-arabinitol	0.69	43, 45, 71, 87, 101, 117, 129, 161
1,5-Ac-2,3,4-Me-L-arabinitol	0.79	43, 101, 117, 161
1,3,5-Ac-2,4-Me-L-arabinitol	1.04	43, 87, 113, 117, 233
1,4,5-Ac-2,3-Me-L-arabinitol	1.13	43, 87, 101, 117, 129, 189
1,5-Ac-2,3,4-Me-L-rhamnitol	0.64	43, 101, 117, 131, 175
1,2,5-Ac-3,4-Me-L-rhamnitol	0.95	43, 89, 129, 131, 189
1,5-Ac-2,3,4,6-Me-D-glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac-2,4,6-Me-D-glucitol	1.38	43, 45, 87, 101, 117, 129, 161
1,4,5-Ac-2,3,6-Me-D-glucitol	1.48	43, 45, 87, 99, 101, 113, 117, 233
1,3,5,6-Ac-2,4-Me-D-glucitol	1.84	43, 87, 117, 129, 189
1,4,5,6-Ac-2,3-Me-D-glucitol	1.92	43, 101, 117, 261
1,5-Ac-2,3,4,6-Me-D-galactitol	1.09	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac-2,4,6-Me-D-galactitol	1.35	43, 45, 87, 101, 117, 129, 161
1,4,5-Ac-2,3,6-Me-D-galactitol	1.44	43, 45, 87, 99, 101, 113, 117, 233
1,5,6-Ac-2,3,4-Me-D-galactitol	1.59	43, 87, 99, 101, 117, 129, 161, 189
1,2,4,5-Ac-3,6-Me-D-galactitol	1.75	43, 45, 87, 99, 113, 129, 189, 233
1,4,5,6-Ac-2,3-Me-D-galactitol	1.97	43, 101, 117, 261
1,3,5,6-Ac-2,4-Me-D-galactitol	2.01	43, 87, 117, 129, 189

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-).

method. Standard pullulans (Shōwa Denkō Co.) having known molecular weights were run on the column to obtain a calibration curve.

**Qualitative Analysis of Component Sugars** Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described in a previous report.<sup>3)</sup> The configurations of component sugars were identified by gas chromatography (GC) of trimethylsilylated  $\alpha$ -methylbenzylaminoalditol derivatives.<sup>4)</sup> GC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector.

**Determination of Components** Component sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described in a previous report.<sup>5)</sup>

**Nuclear Magnetic Resonance (NMR)** NMR spectrum was recorded on a JEOL 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.

**Methylation Analysis** Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described in a previous report.<sup>6)</sup> The yield was 8 mg from 10 mg of glycyrrhizan UC. The product was hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described in a previous report.<sup>7)</sup> 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.  $\times$  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-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** Periodate oxidation followed by reduction with sodium borohydride was performed as described in a previous report.<sup>1)</sup> Yield of the product was 3 mg from 4 mg of glycyrrhizan UC. Determination of the surviving components was carried out as described above.

**Phagocytic Activity** This was measured as described in a previous report.<sup>5)</sup> The sample 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).

## Results

The crude precipitate was obtained from the root of *G. uralensis* by hot water extraction followed by the addition of ethanol. The precipitate was again extracted with hot water, then prepared in a dilute sodium sulfate solution. After treatment with cetyltrimethylammonium bromide, the supernatant obtained was poured into ethanol. The precipitate was purified by gel chromatography with Sephacryl S-300. The solution of high molecular weight fraction obtained was subjected to affinity chromatography on Con A-Sepharose. Glycyrrhizan UA was not retained in the column,<sup>1)</sup> and after elution with a phosphate buffer, a new fraction was obtained from the eluate with a phosphate buffer containing methyl- $\alpha$ -D-mannopyranoside. The eluate was purified by gel chromatography with Sephacryl S-300 and Sephadex G-25, and a pure polysaccharide named glycyrrhizan UC was obtained. The ratio of yields on glycyrrhizans UA, UB and UC was 6.1 : 3.0 : 1.0.

Glycyrrhizan UC produced a single band on PAGE, and a single peak on gel chromatography. It had  $[\alpha]_D^{24} +9.5^\circ$  ( $H_2O$ ,  $c=0.1$ ). The gel chromatography yielded a value of  $6.9 \times 10^4$  for the molecular mass.

Glycyrrhizan UC is composed of L-arabinose, D-ga-

lactose, D-glucose and L-rhamnose. Quantitative analyses showed that it contained 12.3% arabinose, 45.5% galactose, 40.9% glucose and 1.3% rhamnose. The molar ratio of these components was 10:30:27:1. The substance showed reddish violet with an iodine test.

The carbon-13 NMR ( $^{13}C$ -NMR) spectrum of glycyrrhizan UC showed five signals due to anomeric carbons at  $\delta$  101.34, 102.39, 105.85, 106.84 and 110.11 ppm. The signals at  $\delta$  101.34, 102.39 and 105.85 ppm were attributable to anomeric carbons of  $\alpha$ -D-glucopyranose,  $\alpha$ -L-rhamnopyranose and  $\beta$ -D-galactopyranose,<sup>8,9)</sup> and those at  $\delta$  106.84 and 110.11 ppm to anomeric carbons of  $\alpha$ -L-arabinopyranose and  $\alpha$ -L-arabinofuranose.<sup>10)</sup>

The polysaccharide was methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.<sup>11)</sup> The methylated product was hydrolyzed, then converted into the partially methylated alditol acetates. Analysis by GC-MS<sup>12)</sup> showed the presence of 2,3,5-tri-*O*-methyl-L-arabinose, 2,3,4-tri-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2,3,4-tri-*O*-methyl-L-rhamnose, 3,4-di-*O*-methyl-L-rhamnose, 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, 3,6-di-*O*-methyl-D-galactose, 2,3-di-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose and 2,4-di-*O*-methyl-D-glucose in a molar ratio of 7:2:4:7:1:1:4:10:18:10:1:

(seven) <sup>a)</sup>	$\alpha$ -L-Araf	1 $\rightarrow$	(one) <sup>a)</sup>	$\alpha$ -L-Rhap	1 $\rightarrow$
(seven) <sup>a)</sup>	$\rightarrow$ 5 $\alpha$ -L-Araf	1 $\rightarrow$	(one) <sup>a)</sup>	$\rightarrow$ 2 $\alpha$ -L-Rhap	1 $\rightarrow$
(two) <sup>a)</sup>	$\alpha$ -L-Arap	1 $\rightarrow$	(four) <sup>a)</sup>	$\beta$ -D-Galp	1 $\rightarrow$
(four) <sup>a)</sup>	$\rightarrow$ 3 $\alpha$ -L-Arap	1 $\rightarrow$	(ten) <sup>a)</sup>	$\rightarrow$ 3 $\beta$ -D-Galp	1 $\rightarrow$
(twelve) <sup>a)</sup>	$\alpha$ -D-Glcp	1 $\rightarrow$	(eighteen) <sup>a)</sup>	$\rightarrow$ 4 $\beta$ -D-Galp	1 $\rightarrow$
(eight) <sup>a)</sup>	$\rightarrow$ 3 $\alpha$ -D-Glcp	1 $\rightarrow$	(ten) <sup>a)</sup>	$\rightarrow$ 6 $\beta$ -D-Galp	1 $\rightarrow$
(twenty-six) <sup>a)</sup>	$\rightarrow$ 4 $\alpha$ -D-Glcp	1 $\rightarrow$			
	$\downarrow$			$\downarrow$	
(one) <sup>a)</sup>	$\rightarrow$ 3 $\alpha$ -D-Glcp	1 $\rightarrow$	(twelve) <sup>a)</sup>	$\rightarrow$ 3 $\beta$ -D-Galp	1 $\rightarrow$
	$\downarrow$			$\downarrow$	
(seven) <sup>a)</sup>	$\rightarrow$ 4 $\alpha$ -D-Glcp	1 $\rightarrow$	(five) <sup>a)</sup>	$\rightarrow$ 4 $\beta$ -D-Galp	1 $\rightarrow$
			(one) <sup>a)</sup>	$\rightarrow$ 4 $\beta$ -D-Galp	1 $\rightarrow$
				$\uparrow$	

Chart 1. Component Sugar Residues in the Minimal Unit in the Structure of Glycyrrhizan UC

a) Number of residues. Araf, arabinofuranose; Arap, arabinopyranose; Rhap, rhamnopyranose; Glcp, glucopyranose; Galp, galactopyranose.

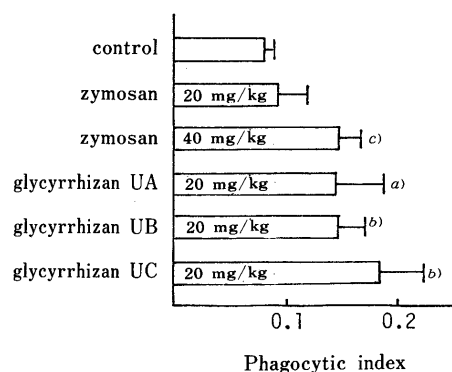


Fig. 1. Effect of Glycyrrhizans UA, UB and UC on Carbon Clearance Index in ICR Mice

Significantly different from the control, a)  $p < 0.05$ , b)  $p < 0.01$ , c)  $p < 0.001$ .

5:12:12:8:26:7:1.

The polysaccharide was subjected to periodate oxidation followed by reduction with sodium borohydride. The product contained 2.5% arabinose, 17.4% galactose, 6.8% glucose and no rhamnose. The accumulated evidence described above indicates that the minimal unit of glycyrrhizan UC is composed of 18 kinds of component sugar units, as shown in Chart 1.

The effect of glycyrrhizan UC on the RES was demonstrated by a modification<sup>5)</sup> of the *in vivo* carbon clearance test<sup>13)</sup> using zymosan as a positive control. As shown in Fig. 1, the phagocytic index was significantly increased, suggesting the activation of RES by i.p. injection of glycyrrhizan UC.

## Discussion

We have already reported the RES activities of glycyrrhizans UA and UB,<sup>1)</sup> the two acidic polysaccharides obtained from the root of *G. uralensis*. These substances are made up of an arabino-3,6-galactan type structure with  $\alpha$ -1,3-linked L-arabinopyranosyl,  $\beta$ -1,4-linked D-galactosyl and 2,4-branched rhamnogalacturonan units.

Further, we have found the RES activities of two polysaccharides obtained from the root and rhizome of *Saposhnikovia divaricata*,<sup>5,14)</sup> three polysaccharides obtained from the seed of *Malva verticillata*,<sup>6,15,16)</sup> a polysaccharide obtained from the barks of *Cinnamomum cassia*<sup>17)</sup> and two polysaccharides obtained from the rhizome of *Curcuma longa*.<sup>18,19)</sup> Among them, cinnaman AX,<sup>17)</sup> the RES-activating arabinoxylan from Cinnamomi Cortex, has the unique  $\beta$ -1,4-linked D-xylan backbone mostly bearing  $\alpha$ -L-arabinofuranosyl-(1 $\rightarrow$ 3)- $\beta$ -L-arabinopyranose side chains at position 3. However, most of the other RES-activating polysaccharides obtained by us possess  $\alpha$ -1,5-linked L-arabino- $\beta$ -3,6-branched D-galactan and  $\alpha$ -1,4-linked D-galacturonan units as their major parts, though saposhnikovan C, an active polysaccharide from *Saposhnikovia Radix*, has  $\beta$ -3,4-branched D-galactan structure instead of the usual 3,6-branched D-galactose residues.

Other examples of plant polysaccharides having a phagocytosis-enhancing effect, have been reported, including: sanchinan-A isolated from the root of *Panax notoginseng*,<sup>20)</sup> tochibanan-A and -B from the rhizome of *Panax japonicus*,<sup>21)</sup> PI and PII from the herbal part of *Eupatorium cannabinum* and *E. perfoliatum*,<sup>22)</sup> polysaccharide F from *Echinacea purpurea* cell culture,<sup>23)</sup> polysaccharide Fb from *Viscum album* berry,<sup>24)</sup> and PS-I to -III from the flower of *Calendula officinalis*.<sup>25)</sup> Sanchinan-A belongs to an arabino-3,6-galactan type substance, and tochibanan-A and -B belong to  $\beta$ -1,4-linked D-galactan and partially 2,3,6-branched  $\beta$ -1,4-linked D-galactan, respectively. PI and PII were identified as 4-methylglucuronoxylans. Polysaccharide F is mostly made up of  $\beta$ -1,3-linked D-galactan backbone bearing  $\beta$ -1,6-linked D-galactose side chains with terminal L-arabinose residues. Fb possesses a rhamnogalacturonan backbone and arabino-3,6-galactan type side chains. PS-I to -III contain

a  $\beta$ -1,3-linked D-galactan backbone with  $\alpha$ -1,3-linked arabinosyl-arabinose and rhamnosyl-arabinose side chains.

In analogy with glycyrrhizan UA, glycyrrhizan UC possesses many  $\beta$ -1,3- and  $\beta$ -1,6-linked and 3,6-branched D-galactose units, terminal and  $\alpha$ -1,5-linked L-arabinose residues. Besides these structural factors, this substance is characteristically rich in  $\beta$ -1,4-linked and 4,6-branched D-galactose units, and in terminal,  $\alpha$ -1,3-linked,  $\alpha$ -1,4-linked and 4,6-branched D-glucose units. The RES activity of glycyrrhizan UC is superior to that of glycyrrhizans UA and UB. In addition to the presence of 1,3-linked L-arabinopyranosyl units,<sup>1,15-18)</sup> its complicated structure may contribute to the activity.

**Acknowledgement** This work was supported in part by the Nishi Cancer Research Fund.

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