Structural Features of Ukonan C, a Reticuloendothelial System-Activating Polysaccharide from the Rhizome of *Curcuma longa*

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The structural features of ukonan C, the polysaccharide having remarkable reticuloendothelial system-potentiating activity obtained from the rhizome of *Curcuma longa* L., were investigated by methylation analysis, carbon-13 nuclear magnetic resonance, periodate oxidation and enzymic degradation studies. It is mainly made up of arabino-3,6-galactan type and 4,6-branched glucan type structural units. Degradation with α -amylase followed by the elimination of 1,4-linked glucan side chains resulted in a marked decrease of its immunological activity.

Keywords *Curcuma longa*; rhizome; ukonan C; reticuloendothelial system; immunological activity; polysaccharide structure; methylation analysis; enzymic degradation

Recently, we isolated and characterized three polysaccharides, ukonan A, ukonan B and ukonan C, from the rhizome of *Curcuma longa* L.,¹⁾ having remarkable activity on the reticuloendothelial system (RES). The dried rhizome of this plant is a well-known crude drug under the name of turmeric. The structural features of ukonan B and ukonan A have been elucidated by us.^{1,2)} Among these three substances, ukonan C is a characteristic glucose-rich acidic polysaccharide having the value of 9×10^3 for its molecular mass. It contains 94.4% polysaccharide and 5.6% peptide moieties. The polysaccharide is composed of L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose and D-galacturonic acid in the molar ratio of 8:3:6:14:2:3.¹⁾ The present paper describes methylation analysis and partial degradation studies of it, and presents structural features of the polysaccharide.

Materials and Methods

Isolation of Polysaccharide This was performed as described in a previous report.¹⁾ The yield of ukonan C was 315 mg from 1 kg of the material rhizomes.

Determination of Components Neutral sugars were analyzed by gas chromatography (GC) after conversion of the hydrolysate into alditol acetates as described previously.³⁾ Galacturonic acid was determined by the *m*-hydroxybiphenyl method.⁴⁾ Peptide determination was performed

by the method of Lowry *et al.*⁵⁾

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 70 °C.

Reduction of Carboxyl Groups This was carried out with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and sodium borohydride as described previously.⁶⁾ The reduction was repeated three times under the same conditions. Yield was 32.6 mg from 50.3 mg of ukonan C.

Methylation This was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described in a previous report.⁷⁾ Yields were 7.0 mg from 9.7 mg of ukonan C, 7.2 mg from 10.0 mg of its carboxyl-reduced product, 6.1 mg from 10.5 mg of degraded ukonan C (DUC)-I, and 3.2 mg from 4.8 mg of DUC-II and -III each.

Analysis of Methylated Products The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner 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. $\times$ 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 previously.¹⁾ The yield of the product was 6.0 mg from 11.5 mg of ukonan C. Determination of the surviving components was carried out as described above.

Enzymic Degradation and Isolation of the Products Ukonan C (102 mg)

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

	Relative retention time ^{a)}	Main fragments (<i>m/z</i>)
1,4-Ac ₂ -2,3,5-Me ₃ -L-Arabinitol	0.69	43, 45, 71, 87, 101, 117, 129, 161
1,3,5-Ac ₃ -2,4-Me ₂ -L-Arabinitol	1.04	43, 87, 113, 117, 233
1,4,5-Ac ₃ -2,3-Me ₂ -L-Arabinitol	1.13	43, 87, 101, 117, 129, 189
1,2,4,5-Ac ₄ -3-Me-L-Arabinitol	1.51	43, 87, 129, 189
1,4,5-Ac ₃ -2,3-Me ₂ -D-Xylitol	1.21	43, 87, 101, 117, 129, 189
1,3,4,5-Ac ₄ -2-Me-D-Xylitol	1.54	43, 117, 261
1,5-Ac ₂ -2,3,4-Me ₃ -L-Rhamnitil	0.64	43, 101, 117, 131, 175
1,2,5-Ac ₃ -3,4-Me ₂ -L-Rhamnitil	0.95	43, 89, 129, 131, 189
1,2,4,5-Ac ₄ -3-Me-L-Rhamnitil	1.28	43, 87, 101, 129, 143, 189, 203
1,5-Ac ₂ -2,3,4,6-Me ₄ -D-Galactitol	1.09	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac ₃ -2,4,6-Me ₃ -D-Galactitol	1.36	43, 45, 87, 101, 117, 129, 161
1,4,5-Ac ₃ -2,3,6-Me ₃ -D-Galactitol	1.44	43, 45, 87, 99, 101, 113, 117, 233
1,5,6-Ac ₃ -2,3,4-Me ₃ -D-Galactitol	1.60	43, 87, 99, 101, 117, 129, 161, 189
1,2,4,5-Ac ₄ -3,6-Me ₂ -D-Galactitol	1.75	43, 45, 87, 99, 113, 129, 189, 233
1,3,5,6-Ac ₄ -2,4-Me ₂ -D-Galactitol	2.01	43, 87, 117, 129, 189
1,5-Ac ₂ -2,3,4,6-Me ₄ -D-Glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,4,5-Ac ₃ -2,3,6-Me ₃ -D-Glucitol	1.49	43, 45, 87, 99, 101, 113, 117, 233
1,4,5,6-Ac ₄ -2,3-Me ₂ -D-Glucitol	1.92	43, 101, 117, 261

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-Ac₂-2,3,5-Me₃ = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-).

was dissolved in 0.05M acetate buffer (pH 5.0, 5ml) and α -amylase preparation (25 μ l, Sigma Co.) was added. The solution was incubated with a few drops of toluene at 37°C for 6h. After heating in a boiling water bath for 5 min, it was applied to a column (5 $\times$ 85 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.⁹⁾ The eluates obtained from the column were divided into four groups: Frac. 1, tubes 32 to 36; frac. 2, tubes 38 to 41; frac. 3, tubes 60 to 61; frac. 4, tubes 63 to 66. The yields were 38.4 mg for frac. 1, and 14.1 mg for frac. 2. For the removal of a salt in low molecular weight fractions, fracs. 3 and 4 were each treated with a column (1 $\times$ 18 cm) of Dowex 50W-X8 (H⁺) and the eluates with water were evaporated to dryness. Then the yields were 14.6 mg for frac. 3, and 18.0 mg for frac. 4. DUC-I was obtained from frac. 1. Frac. 2 was dissolved in water and applied to a column (2.6 $\times$ 95 cm) of Sephadex S-200. The column was equilibrated and eluted with 0.1M Tris-HCl buffer (pH 7.0), and fractions of 10 ml were collected and analyzed by the phenol-sulfuric acid method. The eluates obtained from tubes 56 to 62 were combined, dialyzed, concentrated and applied to a column (2.6 $\times$ 95 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected and analyzed as described above. DUC-II was obtained from tubes 24 to 28, and DUC-III from tubes 29 to 32; their yields were 4.2 and 4.7 mg.

Analysis of Degradation Products Molecular weights were estimated by the method of Park *et al.*¹⁰⁾ compared with the value of glucose. Thin-layer chromatography (TLC) was performed on Merck precoated Kieselgel 60 plates using *n*-butanol-acetic acid-water (2:1:1, v/v) as a developing solvent. Detection was done by spraying 0.2% orcinol in 20% sulfuric acid followed by heating at 110°C for 5 min. *R_f* values of maltose and glucose were 0.50 and 0.59. The high-performance liquid chromatography (HPLC) system used consisted of a Hitachi L-6200 intelligent pump, a Shodex SE-51 RI monitor and a Hitachi D-2000 chromatointegrator. The analysis was carried out using a column (0.46 $\times$ 25 cm) of Asahipak NH 2P-50 with acetonitrile-water (3:1, v/v) as an eluent at a flow rate of 1 ml per min at room temperature. Retention times of glucose and maltose were 9.14 and 15.37.

Phagocytic Activity This was measured as described in a previous report.³⁾ The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (5 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 Student's *t*-test.

Results

The crude polysaccharide fraction obtained from the rhizome of *Curcuma longa* was applied to a column chromatography of diethylaminoethyl (DEAE)-Sephadex A-25. The eluate with 0.2M ammonium carbonate was subjected to affinity chromatography on Con A-Sepharose. The passed-through fraction was applied to gel chromatography on a Sephadex S-300 column. Ukonan C was isolated from the relatively low molecular weight fraction.¹⁾

The polysaccharide was composed of L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose and D-galacturonic acid in the molar ratio of 8:3:6:14:2:3.¹⁾ The glucan moiety in ukonan C was degraded by treatment with α -amylase followed by gel chromatography with Sephadex G-25. Five products were obtained, and two of them were identified as glucose and maltose by TLC and HPLC analysis. The residual three fractions are tentatively designated as DUC-I, DUC-II and DUC-III. In both DUC-II and DUC-III, glucose was the only component sugar. DUC-I, the highest molecular weight fraction in the enzymic degradation products, contained 26.9% arabinose, 9.8% xylose, 24.7% galactose, 12.2% glucose, 7.0% rhamnose, 13.8% galacturonic acid and 6.4% peptide moiety. The molar ratio of these

component sugars was 8:3:6:3:2:3. DUC-I had $[\alpha]_D^{24} - 10.6^\circ$ (H₂O, *c*=0.1). Determination of the reducing powers gave values of 6000, 1300 and 830 for the molecular weights of DUC-I, DUC-II and DUC-III, respectively.

The carbon-13 NMR (¹³C-NMR) spectrum of ukonan C gave no good result on anomeric signals. However, the ¹³C-NMR spectrum of DUC-I showed seven signals due to anomeric carbons at δ 100.48, 100.94, 102.55, 104.33, 105.36, 105.87 and 111.19 ppm. The signals at δ 100.94 and 102.55 ppm were assigned to the anomeric carbons of α -D-galactopyranosyluronic acid and α -L-rhamnopyranose, respectively.¹¹⁾ The signals at δ 100.48, 104.33 and 105.36 ppm were assigned to the anomeric carbons of α -D-glucopyranose, β -D-xylopyranose and β -D-galactopyranose, respectively.¹²⁾ The signals at δ 105.87 and 111.19 ppm were assigned to the anomeric carbons of α -L-arabinopyranose and α -L-arabinofuranose.¹³⁾

The carboxyl groups of galacturonic acid residues in

TABLE II. Methylation Analysis of Ukonan C and Its Carboxyl-Reduced Derivative

Methylated sugars (as alditol acetates)	Molar ratios	
	Original	Carboxyl-reduced
2,3,5-Me ₃ -L-Arabinose	9	9
2,4-Me ₂ -L-Arabinose	5	5
2,3-Me ₂ -L-Arabinose	1	1
3-Me-L-Arabinose	1	1
2,3-Me ₂ -D-Xylose	2	2
2-Me-D-Xylose	4	4
2,3,4-Me ₃ -L-Rhamnose	1	1
3,4-Me ₂ -L-Rhamnose	2	2
3-Me-L-Rhamnose	1	1
2,3,4,6-Me ₄ -D-Galactose	2	2
2,4,6-Me ₃ -D-Galactose	1	1
2,3,6-Me ₃ -D-Galactose	1	7
2,3,4-Me ₃ -D-Galactose	2	2
3,6-Me ₂ -D-Galactose	1	1
2,4-Me ₂ -D-Galactose	5	5
2,3,4,6-Me ₄ -D-Glucose	2	2
2,3,6-Me ₃ -D-Glucose	24	24
2,3-Me ₂ -D-Glucose	2	2

TABLE III. Methylation Analysis of the Enzymic Degradation Products of Ukonan C

Methylated sugars (as alditol acetates)	Molar ratio		
	DUC-I	DUC-II ^{a)}	DUC-III ^{a)}
2,3,5-Me ₃ -L-Arabinose	9	—	—
2,4-Me ₂ -L-Arabinose	5	—	—
2,3-Me ₂ -L-Arabinose	1	—	—
3-Me-L-Arabinose	1	—	—
2,3-Me ₂ -D-Xylose	2	—	—
2-Me-D-Xylose	4	—	—
2,3,4-Me ₃ -L-Rhamnose	1	—	—
3,4-Me ₂ -L-Rhamnose	2	—	—
3-Me-L-Rhamnose	1	—	—
2,3,4,6-Me ₄ -D-Galactose	2	—	—
2,4,6-Me ₃ -D-Galactose	1	—	—
2,3,6-Me ₃ -D-Galactose	1	—	—
2,3,4-Me ₃ -D-Galactose	2	—	—
3,6-Me ₂ -D-Galactose	1	—	—
2,4-Me ₂ -D-Galactose	5	—	—
2,3,4,6-Me ₄ -D-Glucose	—	+	+
2,3,6-Me ₃ -D-Glucose	6	+	+

a) Precise molar ratios of the products were not obtained.

ukonan C were reduced to give the corresponding neutral sugar residues.¹⁴⁾ Both the original polysaccharide and the carboxyl-reduced derivative were methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.¹⁵⁾ The methylated products were hydrolyzed, then converted into the partially methylated alditol acetates. The hexuronic acid methyl ether was removed from the hydrolysis products of the methylated polysaccharide by treatment with an anion-exchange resin. Analysis by GC-MS¹⁶⁾ gave the results shown in Table II. The methylation analyses of DUC-I, DUC-II and DUC-III were performed in the similar manner to that of ukonan C, and the results were shown in Table III.

Ukonan C was subjected to periodate oxidation followed by reduction. The maximal value of periodate consumption was 0.6 mol per mol of anhydrosugar unit. The component sugar analysis of the product showed that three-eighths of the arabinose units, two-thirds of the xylose units, seven-twelfths of the galactose units and a quarter of the rhamnose units survived after periodate oxidation. All the glucose and the galacturonic acid residues were completely destroyed by this treatment.

These results indicated that the minimal unit of ukonan C is composed of eighteen kinds of component sugar units, as shown in Chart 1.

Chart 1. Component Sugar Residues in the Minimal Unit in the Structure of Ukonan C

a) Number of residues. Araf, arabinofuranose; Arap, arabinopyranose; Xylp, xylopyranose; Galp, galactopyranose; Glcp, glucopyranose; Rhap, rhamnopyranose; GalpA, galactopyranosyluronic acid.

Fig. 1. Effects of Ukonan C, DUC-I and Glucan Fraction on Carbon Clearance Index in ICR Mice

Significantly different from the control, a) $p < 0.001$. b) The glucan fraction is composed of DUC-II and -III in a ratio of 1.0:1.1.

The effects of ukonan C, DUC-I and the fraction containing DUC-II and -III on the RES were demonstrated by a modification³⁾ of the *in vivo* carbon clearance test¹⁷⁾ using zymosan as a positive control. As shown in Fig. 1, DUC-I showed significant activity. However, the value was much lower than that of ukonan C. The fraction containing DUC-II and -III showed no RES activity.

Discussion

We have already reported remarkable RES activities of ukonan A, ukonan B and ukonan C, the three acidic polysaccharides obtained from the rhizome of *Curcuma longa*.¹⁾ These substances are commonly composed of L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose, D-galacturonic acid and a peptide moiety. Both ukonan A and ukonan B possess the same fourteen kinds of component sugar residues, though the two substances are different in the ratios of these sugar units.^{1,2)} Ukonan A and ukonan B are mainly made up of an arabino-3,6-galactan type structure with additional α -1,3-linked L-arabinopyranosyl, β -3,4-branched D-xylosyl, α -1,4-linked D-glucosyl and α -2,4-branched L-rhamno-D-galacturonan units. In both ukonan A and ukonan B, glucose is a quite minor component. In contrast to these substances, glucose is the major component sugar in ukonan C.

The structural features of ukonan C are more complicated than those of ukonan A and ukonan B. The polysaccharide has nineteen kinds of component sugar units. All the kinds of component sugar units in ukonan A and ukonan B are also found in ukonan C with the other additional five units, those are, α -2,5-branched L-arabinosyl, β -1,4-linked D-galactosyl, β -2,4-branched D-galactosyl, α -4,6-branched D-glucosyl and terminal α -L-rhamnosyl residues. After treatment with α -amylase, ukonan C lost about 80% of its glucose units. As a result of this enzymic degradation, RES activity of the main product, DUC-I, composed of the residual acidic polysaccharide moiety was more markedly decreased than that of the original substance. This fact showed that the presence of component glucose units contributes to the activity of ukonan C. The degradation products composed of only glucose showed no RES activity. Methylation analysis of DUC-I revealed only 1,4-linked units as its glucose linkage. So is conceivable that side chains of branched glucose units in ukonan C were lost by the enzymic degradation.

As other examples of RES-activating polysaccharides from Oriental crude drugs, sanchinan-A isolated from the root of *Panax notoginseng*,¹⁸⁾ and tochibanan-A and -B from the rhizome of *Panax japonicus*¹⁹⁾ have been reported. Sanchinan-A possesses an α -L-arabino- β -3,6-branched D-galactan type structure, and tochibanan-A and -B belong to β -1,4-linked D-galactan and partially 2,3,6-branched β -1,4-linked D-galactan, respectively.

In addition, we have reported the RES activities and structural features of saposchnikovans A and C from the root and rhizome of *Saposchnikovia divaricata*,^{3,20)} MVS-III, -IVA and -VI from the seed of *Malva verticillata*,^{7,21,22)} cinnaman AX from the bark of *Cinnamomum cassia*,²³⁾ glycyrrhizans UA, UB and UC from the root of *Glycyrrhiza uralensis*,^{24,25)} and of eucommian A from the bark of *Eucommia ulmoides*.²⁶⁾ Saposchnikovan A has an α -1,4-linked D-galacturonan backbone bearing α -1,5-

linked L-arabino- β -3,6-branched D-galactan side chains, and saposhnikovan C possesses a rhamnogalacturonan type backbone with α -3,5-branched L-arabinan and β -3,4-branched D-galactan side chains. Both MVS-IIIa and MVS-IVa are mainly made up of an α -1,5-linked L-arabino- β -3,6-branched D-galactan structure. MVS-VI, the main acidic polysaccharide in the material seed, has similar arabinogalactan moieties as its main part, though the ratio of these moieties in the components is lower than those of MVS-IIIa and -IVa. The backbone of cinnaman AX is a β -1,4-linked D-xylan mostly bearing α -L-arabinofuranosyl-(1 $\rightarrow$ 3)- β -L-arabinopyranose side chains. The main part of glycyrrhizan UA is occupied by α -1,5-linked L-arabino- β -3,6-branched D-galactan. Glycyrrhizan UB is a polysaccharide basically similar to glycyrrhizan UA, though α -2,4-branched rhamnogalacturonan units occupy its major part. Glycyrrhizan UC also possesses many arabino-3,6-galactan units, and besides these factors, this neutral polysaccharide is rich in β -1,4-linked D-galactan and α -1,4-linked D-glucan moieties. Eucommian A has similar arabinogalactan and branched rhamnogalacturonan units to those in glycyrrhizan UB.

Thus, most of the RES-activating polysaccharides obtained by us possess α -1,5-linked L-arabino- β -3,6-branched D-galactan moieties as their major parts, though cinnaman AX is a new structural type of RES-activating substance. Among them, ukonan C and glycyrrhizan UC are characteristically rich in α -1,4-linked D-glucose units. In the present work, the participation of glucan side chains in the RES activity was found in ukonan C.

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