Characterization of a Neutral Polysaccharide Having Activity on the Reticuloendothelial System from the Rhizome of *Curcuma longa*

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A neutral polysaccharide, named ukonan D, was isolated from the rhizome of *Curcuma longa* L. It produced a single band on electrophoresis and a single peak on gel chromatography, and its molecular mass was estimated to be 28000. It showed remarkable reticuloendothelial system-potentiating activity in a carbon clearance test. Ukonan D is composed of L-arabinose : D-galactose : D-glucose : D-mannose in the molar ratio of 1 : 1 : 12 : 0.2, in addition to small amounts of peptide moiety. Methylation analysis, carbon-13 nuclear magnetic resonance and enzymic degradation studies indicated that its structural features include mainly both α-1,5-linked L-arabino-β-3,6-branched D-galactan type and α-4,6-branched D-glucan type structural units. The influence of degradation with α-amylase followed by the elimination of glucan side chains on its immunological activity was discussed.

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

The dried rhizome of *Curcuma longa* L. is a well-known crude drug under the name of turmeric. Recently, we isolated and characterized three polysaccharides, ukonan A, ukonan B, and ukonan C, from this crude drug.[1−3] They are acidic polysaccharides composed of L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose and D-galacturonic acid. These polysaccharides show remarkable activity on the reticuloendothelial system (RES). 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 Formosa. The powdered rhizomes (1.2 kg) were extracted with hot water (18 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 (53.1 g) was dissolved in water (800 ml). One eighth of this solution was applied to a column (5 × 80 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 (Pharmacia Co.) which had been pretreated as described previously.[4] After elution with water (1600 ml), the column was eluted with 0.2 M ammonium carbonate (2400 ml). Fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[5] The eluates obtained from tubes 39 to 63 were combined, dialyzed, concentrated and applied to a column (5 × 85 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 41 were combined, concentrated and lyophilized. The yield of this fraction (fr. II) obtained by the eight chromatographies with DEAE-Sephadex A-25 and Sephadex G-25 was 1.55 g.

Fraction II (150 mg) was dissolved in 3 ml of 0.067 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 × 39 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 (180 ml), the column was eluted with 10 mM methyl-α-D-mannopyranoside in the same buffer solution. Fractions of 10 ml were collected and analyzed by the phenol–sulfuric acid method, and the eluates obtained from tubes 8 to 17 were combined, dialyzed, concentrated and lyophilized. The yield of this fraction (fr. IId) was 531 mg from 1.55 g of fr. II. Fraction IId (177 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (5 × 85 cm) of Toyopearl HW-60F. The column was equilibrated and eluted with the same buffer, and fractions of 20 ml were collected. The eluates obtained from tubes 50 to 63 (fr. d2) were combined, dialyzed, concentrated and applied to a column (5 × 85 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 37 were combined, concentrated and lyophilized. Ukonan D was obtained as white powder. Yield, 49 mg.

Polyacrylamide Gel Electrophoresis (PAGE) This was carried out in

an apparatus with gel tubes (4 × 130 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 and with Coomassie blue reagent. Ukonan D produced a clear band at a distance of 54 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 × 95 cm) of Sephacryl S-200, 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.

Determination of Components Component sugars were analyzed by gas chromatography (GC) after conversion of the hydrolyzate into alditol acetates as described previously.[6] The configurations of component sugars were identified by GC of trimethylsilylated α-methylbenzylaminoalditol derivatives.[7] Peptide determination was performed by the method of Lowry *et al.*[8] using bovine serum albumin (Tokyo Kasei Co.) as a standard.

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.

Enzymic Degradation and Isolation of the Products Ukonan D (103 mg) was dissolved in 0.05 M acetate buffer (pH 5.0, 5 ml) and α-amylase preparation (25 μl, Sigma Co.) was added. The solution was incubated with a few drops of toluene at 37 °C for 7 h. After heating in a boiling water bath for 5 min, it was applied to a column (5 × 81 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 the column were divided into five groups: Fraction 1, tubes 30 to 35; fr. 2, tubes 46 to 51; fr. 3, tubes 53 to 55; fr. 4, tubes 56 to 57; fr. 5, tubes 58 to 60. The yields were 35.5 mg for fr. 1, 13.6 mg for fr. 2, 15.3 mg for fr. 3, 19.2 mg for fr. 4, and 19.4 mg for fr. 5. Fraction 1 was dissolved in water and applied to a column (2.6 × 85 cm) of Sephacryl S-200. The column was equilibrated and eluted with 0.1 M Tris–HCl buffer (pH 7.0), and fractions of 10 ml were collected and analyzed as described above. After dialysis and concentration, fr. 1a and fr. 1b were obtained from tubes 19 to 24 and tubes 25 to 38, respectively. Each fraction was rechromatographed using the same column and the same buffer. The two fractions were each dialyzed, concentrated and applied to a column (2.6 × 87 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected and analyzed as described above. The eluates obtained from tubes 20 to 22 were combined, concentrated and lyophilized. DUD-I was obtained from fr. 1a, and DUD-II from fr. 1b; their yields were 4.4 mg and 7.4 mg.

Analysis of Degradation Products Molecular masses were estimated by gel chromatography as described above. 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. *Rf* values of maltotriose, maltose and glucose were 0.34, 0.43 and 0.54. The high-performance liquid chromatography (HPLC) system used consisted of a Hitachi L-6200 intelligent pump, a JASCO Uvidec-100 spectrophotometer and a Hitachi D-2000 chromato-

integrator. HPLC of p-aminobenzoyl derivatives purified using a column (6 × 22 mm) of PBA-60 (Amicon Co.)[9] was performed with a column (4.6 × 250 mm) of TSK-gel Amide-80 (Tosoh Co.) and detected by absorption at 304 nm. Elution was done at a flow rate of 1 ml per min at room temperature using a linear gradient of acetonitrile–water (75 : 25 to 50 : 50) for 20 min after sample injection. Retention times of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were 4.2, 5.4, 8.5, 12.5, 14.9, 16.9 and 18.6 min.

Methylation This was performed with powdered sodium hydroxide and methyl iodide as described in a previous report.[10] Yields were 4.0 mg from 5.2 mg of ukonan D, 1.5 mg form 2.2 mg of DUD-I, and 3.2 mg from 5.4 mg of DUD-II.

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.[11] 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-GX 303 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 are listed in Table I. The main mass fragments of the products were described previously.[3]

Phagocytic Activity This was measured as described in a previous report.[6] The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (5 and 50 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 ± 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 was obtained from the rhizome of *Curcuma longa* by hot water extraction followed by the addition of ethanol. Its aqueous solution was applied to a column of DEAE-Sephadex A-25 (carbonate form). After elution with water, the eluate with 0.2 M ammonium carbonate was dialyzed and purified using a column of Sephadex G-25, then subjected to affinity chromatography on Con A-Sepharose. Ukonans A, B and C were not retained in the column,[2] and after elution with a phosphate buffer, a new fraction was obtained from the eluate with a phosphate buffer containing methyl-α-D-mannopyranoside. The eluate was purified by gel chromatography with Toyopearl HW-60F and Sephadex G-25, and a pure polysaccharide named ukonan D was isolated. The isolation method of the polysaccharides is summarized in Fig. 1. Fraction I also contains RES-activating polysaccharides, though the value was much lower than those of ukonans A, B, C and D as described previously.[2]

Ukonan D gave a single band on PAGE, and a single peak on gel chromatography. It has $[\alpha]_D^{23} + 81.7°$ (H₂O, $c = 0.1$). The gel chromatography yielded a value of 2.8 × 10⁴ for the molecular mass.

Ukonan D is composed of L-arabinose, D-galactose, D-glucose, D-mannose and a peptide moiety. Quantitative analyses showed that it contained 5.8% arabinose, 6.9% galactose, 81.9% glucose, 1.6% of mannose and 3.8% peptide moiety. The molar ratio of these component sugars was 1 : 1 : 12 : 0.2. The substance showed reddish violet with an iodine test.

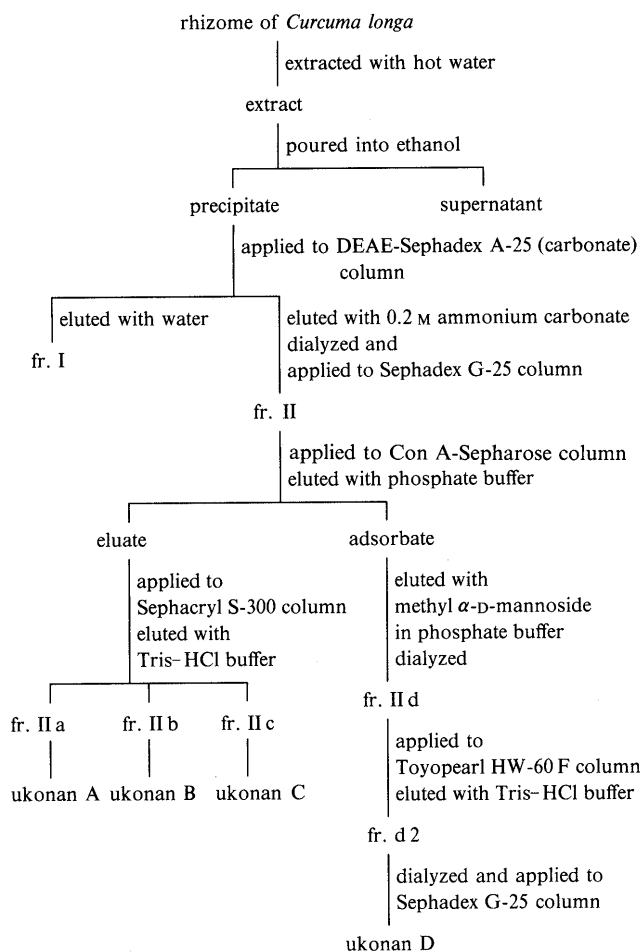The glucan moiety in ukonan D was degraded by

Fig. 1. Isolation of Ukonan D

treatment with α-amylase followed by gel chromatography with Sephadex G-25. Five fractions (*i.e.* frs. 1 to 5) were obtained, and three (frs. 3 to 5) of them were identified as maltotriose, maltose and glucose by TLC and HPLC analysis. Maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were identified in fr. 2 by HPLC of p-aminobenzoyl derivatives and by TLC. Fraction 1 was separated by gel chromatography with Sephacryl S-200 into two products. They are tentatively designated as DUD-I and DUD-II. DUD-I had $[\alpha]_D^{23} - 40.8°$ (H₂O, $c = 0.1$) and DUD-II showed $[\alpha]_D^{23} + 33.1°$ (H₂O, $c = 0.1$). The gel chromatography gave values of 9000 and 7000 for the molecular masses of DUD-I and DUD-II, respectively. DUD-I was composed of L-arabinose, D-galactose, D-glucose and D-mannose in the molar ratio of 12 : 12 : 2 : 1, and DUD-II had the same component sugars in the molar ratio of 3 : 8 : 2 : 5. DUD-I and DUD-II contained 0.8% and 6.8% peptide moieties, respectively.

The ¹³C-NMR spectrum of ukonan D showed four signals due to anomeric carbons at δ 98.40, 102.28, 106.79 and 111.84 ppm. The signals at δ 98.40, 102.28 and 106.79 ppm were assigned to the anomeric carbons of α-D-mannopyranose, α-D-glucopyranose and β-D-galactopyranose, respectively.[12] The signal at δ 111.84 ppm was assigned to the anomeric carbon of α-L-arabinofuranose.[13]

Ukonan D, DUD-I and DUD-II were methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide,[14] respectively. The methylated products were

TABLE I. Methylation Analysis of Ukonan D and Its Enzymic Degradation Products

Methylated sugars (as alditol acetates)	Relative retention time[a]	Molar ratios			Structural features
		Ukonan D	DUD-I	DUD-II	
2,3,5-Me$_3$-L-arabinose	0.69	3	4	1	Ara$f$ 1→
2,3-Me$_2$-L-arabinose	1.13	9	8	2	→5 Ara$f$ 1→
2,3,4,6-Me$_4$-D-galactose	1.10	1	1	—	Gal$p$ 1→
2,4,6-Me$_3$-D-galactose	1.37	3	3	2	→3 Gal$p$ 1→
2,3,4-Me$_3$-D-galactose	1.61	2	2	—	→6 Gal$p$ 1→
2,4-Me$_2$-D-galactose	2.01	6	6	6	⇉3,6 Gal$p$ 1→
2,3,4,6-Me$_4$-D-glucose	1.00	12	—	—	Glc$p$ 1→
2,3,6-Me$_3$-D-glucose	1.49	120	2	2	→4 Glc$p$ 1→
2,3-Me$_2$-D-glucose	1.92	12	—	—	⇉4,6 Glc$p$ 1→
2,3,4,6-Me$_4$-D-mannose	0.99	+[b]	1	5	Man$p$ 1→

a) Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl D-glucitol. Abbreviation: Me = methyl. b) Baseline separation was not achieved. The value in "text" was calculated from the result of component sugar analysis. Ara$f$, arabinofuranose; Gal$p$, galactopyranose; Glc$p$, glucopyranose; Man$p$, mannopyranose.
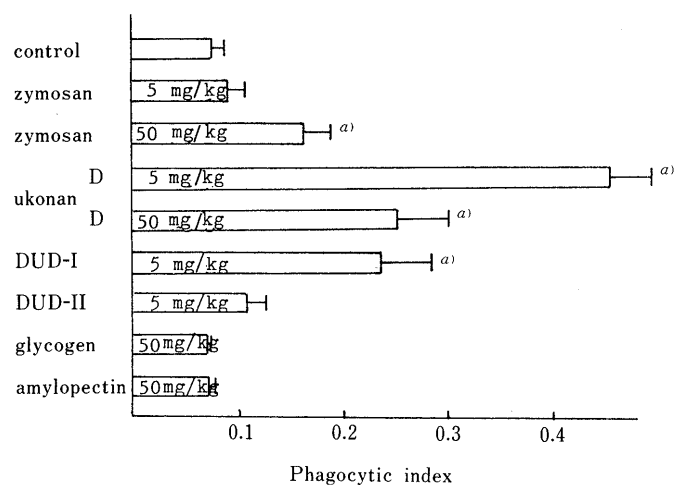

Fig. 2. Effects of Ukonan D, DUD-I and DUD-II on Carbon Clearance Index in ICR Mice

Significantly different from the control, a) $p < 0.001$.

hydrolyzed, then converted into the partially methylated alditol acetates. Analysis by GC-MS gave the results shown in Table I.

These results indicated that the minimal unit of ukonan D is composed of ten kinds of component sugar units. Those are terminal and 1,5-linked α-L-arabinofuranosyl residues, terminal, 1,3-linked, 1,6-linked and 3,6-branched β-D-galactopyranosyl residues, terminal, 1,4-linked and 4,6-branched α-D-glucopyranosyl residues, and terminal α-D-mannopyranosyl residues in a molar ratio of 3:9:1:3:2:6:12:120:12:2. On the other hand, DUD-I lost most of the glucose units. In addition to these glucose units, DUD-II did not possess terminal and β-1,6-linked D-galactosyl residues. The increase of terminal mannose and the decrease of terminal arabinose units were also observed in this degradation product.

The effects of ukonan D, DUD-I and DUD-II on the RES were demonstrated by a modification[6] of the in vivo carbon clearance test[15] using zymosan as a positive control. As shown in Fig. 2, the phagocytic indices of ukonan D and DUD-I increased remarkably, suggesting powerful activation of RES by i.p. injection of these substances. However, DUD-II showed no significant activity in a low dose.

Discussion

Three acidic polysaccharides, named ukonan A, ukonan B and ukonan C, have been obtained from the rhizome of Curcuma longa,[2] and have been reported by us to have remarkable RES activities. 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 are mainly made up of α-L-arabino-β-3,6-branched D-galactan type structure and α-2,4-branched L-rhamno-α-1,4-linked D-galacturonan type structure with α-1,3-linked L-arabinosyl, β-3,4-branched D-xylosyl and α-1,4-linked D-glucosyl units.[1,2] Ukonan C has all the kinds of component sugar units in ukonan A and ukonan B with the other additional five units.[3]

In both ukonan A and ukonan B, glucose is a minor component. In contrast to these substances, glucose is the major component sugar in both ukonan C and ukonan D. However, the structural features of ukonan D are not so complicated as those of ukonan C. Ukonan C has nineteen kinds of component sugar units, while ukonan D possesses ten kinds. Neither xylose nor rhamnogalacturonan units are found in ukonan D, and on the contrary, the presence of terminal mannosyl residues is characteristic of this polysaccharide. The ratio of glucose in component sugars of ukonan D is much higher than that of ukonan C, although both polysaccharides possess the same structural type of glucan moieties having α-1,4-linked chains with partially 4,6-branching. The difference in the affinity to a Con A-Sepharose column between ukonan D and ukonan C must be due to the presence of terminal α-D-mannopyranosyl units in ukonan D.

As a result of the treatment with α-amylase, one of the degradation products, DUD-I, composed of the residual component sugar moieties in ukonan D after removal of most of the glucose units, showed remarkable RES activity. However, another degradation product, DUD-II, having α-D-mannosyl residues as its main terminal units showed no significant RES activity. The remarkable decrease of arabinose residues in DUD-II may affect this variation of activity. Methylation analyses of DUD-I and DUD-II indicated that side chains of branched glucose units in ukonan D were lost by the enzymic degradation. From the comparison of the phagocytosis-enhancing effects of DUD-I and DUD-II, it is conceivable that arabino-3,6-galactan type structure contributes to the RES activities of ukonan

D and DUD-I.

In addition to ukonan A, ukonan B and ukonan C, we have already obtained saposhnikovan A from the root and rhizome of *Saposhnikovia divaricata*,[6] MVS-IIIA, -IVA and -VI from the seed of *Malva verticillata*,[16–18] glycyrrhizans UA, UB and UC from the root of *Glycyrrhiza uralensis*,[19,20] glycyrrhizan GA from the stolon of *G. glabra* var. *glandulifera*,[21] and AMon-S from the root of *Astragalus mongholicus*[22] as other examples of RES-activating polysaccharides having mainly α-1,5-linked L-arabino-β-3,6-branched D-galactan moieties. Another example of RES-activating arabino-3,6-galactan type polysaccharide from Oriental crude drug was sanchinan-A isolated from the root of *Panax notoginseng* by Ohtani *et al.*[23]

Glycyrrhizan UC, ukonan C and ukonan D are characteristically rich in α-1,4-linked D-glucose units with partially 4,6-branching. This is the most remarkable feature among the known RES-activating polysaccharides. Both amylopectin and glycogen as the ordinary 4,6-branched glucans are completely inactive of RES (Fig. 2). In the present work, however, the difference of values of phagocytic indices between ukonan D and DUD-I suggested the participation of glucan moieties in ukonan D in the RES activity as well as ukonan C.[3]

It is quite interesting that the activities of ukonans A, B, C and D are not in a dose dependent manner in contrast to most of the RES-activating polysaccharides including zymosan, a positive control used. Ukonans A, B, C and D gave high values of the activity even in a very low dose. In particular, ukonan D may have an optimum dose of 5 mg/kg.

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