

Arabinogalactan Core Structure and Immunological Activities of Ukonan C, an Acidic Polysaccharide from the Rhizome of *Curcuma longa*

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Controlled Smith degradation of ukonan C, a phagocytosis-activating polysaccharide isolated from the rhizome of *Curcuma longa* L., was performed. The reticuloendothelial system-potentiating, anti-complementary and alkaline phosphatase-inducing activities of ukonan C and its degradation products were investigated. Methylation analyses of the primary and secondary Smith degradation products and of a de-arabinosylated product indicated that structural features of the arabinogalactan core of ukonan C include a backbone chain composed of β -1,3-linked D-galactose and β -1,4-linked D-xylose. All of the galactose units in the backbone carry side chains composed of β -1,6-linked D-galactosyl residues with or without terminal α -L-arabinose units at position 3.

Ukonan C showed remarkable effects on both reticuloendothelial system-potentiating and alkaline phosphatase-inducing activities. Periodate oxidation caused a decrease in or disappearance of the immunological activities, but the controlled Smith degradation product having the arabinogalactan core structure of polysaccharide showed a pronounced effect on anti-complementary activity.

Keywords acidic polysaccharide; core structure; immunological activity; *Curcuma longa*; ukonan C; Smith degradation

We have so far obtained and characterized three acidic polysaccharides from the rhizome of *Curcuma longa* L.¹⁾ This material is a well-known crude drug under the name of turmeric, and it is noteworthy that these three polysaccharides, ukonan A, ukonan B and ukonan C, show especially remarkable activity on the reticuloendothelial system (RES) even in a low level of dose.¹⁾ These polysaccharides are commonly composed of L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose and D-galacturonic acid, in addition to small amounts of peptide moiety. The molar ratios of these component sugars were 12:4:12:1:4:10 in ukonan A, 12:4:12:1:2:4 in ukonan B and 8:3:6:14:2:3 in ukonan C. Thus, ukonan C is a characteristic glucose-rich acidic polysaccharide among them. The structural features of ukonan C are considerably complicated. The polysaccharide has 19 different component sugar units.²⁾ Recently, the core structures of both ukonan A and ukonan B have been elucidated.^{3,4)} In addition, variations in immunological activity with the chemical modification of these two substances were also reported.^{3,4)}

The present paper describes the controlled Smith degradation and limited acid hydrolysis of ukonan C, and affords the results of methylation analysis of the products for the purpose of elucidating its core structural features. This paper also describes the immunological effects of ukonan C 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 previously.¹⁾

Periodate Oxidation Ukonan C (200 mg) was oxidized with 0.05 M sodium metaperiodate (100 ml) at 5°C in the dark. The periodate consumption was measured by a spectrophotometric method.⁵⁾ Oxidation was completed after 8 d. A part (10 ml) of the reaction mixture was treated with ethylene glycol (0.2 ml) at 5°C for 1 h, then 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.⁶⁾ The eluates obtained from tubes 24 to 26 were combined, concentrated and lyophilized. The yield of the periodate oxidation product

(POP) was 8 mg. The residual reaction mixture (90 ml) was successively treated with ethylene glycol (1 ml) at 5°C for 1 h and sodium borohydride (540 mg) at 5°C for 18 h, then adjusted to pH 5.0 by the addition of acetic acid. The solution 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 34 to 41 were combined, concentrated and lyophilized. The yield of this periodate oxidation-reduction product (PORP) was 155 mg.

Controlled Smith Degradation PORP (153 mg) was dissolved in 0.5 N sulfuric acid (15.3 ml). After standing at 22°C for 18 h, the solution was neutralized with Dowex 2 (OH⁻). The filtrate 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 34 to 42 were combined, concentrated and lyophilized. The yield of the Smith degradation product (SDP) was 23.1 mg.

Secondary Smith Degradation SDP (15.2 mg) was oxidized with 0.05 M sodium metaperiodate (6 ml) at 5°C for 5 d in the dark. The reaction mixture was successively treated with ethylene glycol (0.2 ml) and sodium borohydride (30 mg) as described above. After neutralization with acetic acid, the solution 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. The eluates obtained from tubes 23 to 27 were combined and lyophilized. Yield, 12.5 mg. This was treated with 0.5 N sulfuric acid as described above, and after neutralization, the solution was applied to a column (2.6 × 95 cm) of Sephadex G-25. The secondary Smith degradation product was obtained from the eluates in tubes 22 to 24. Yield, 1.9 mg.

Determination of Components Neutral sugars were analyzed by gas chromatography (GC) after conversion of the hydrolyzate into alditol acetates as described previously.⁷⁾ Peptide determination was performed by the method of Lowry *et al.*⁸⁾ using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis (PAGE) This was carried out in an apparatus equipped with gel tubes (4 × 135 mm) and 5 mM Tris-glycine buffer (pH 8.3) at 5 mA/tube for 35 min. Gels were stained by the periodate-Schiff (PAS) procedure and with Coomassie blue reagent. SDP gave a clear band at a distance of 56 mm from the origin.

Gel Chromatography SDP (2 mg) was dissolved in a 0.1 M Tris-HCl buffer (pH 7.0) and applied to a column (2.6 × 90 cm) of Toyopearl HW-55F, 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 carried out with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously.⁹⁾ The yields were 2.6 mg from 2.9 mg of SDP, 0.4 mg from 0.9 mg of the secondary Smith degradation product, and 3.3 mg from 2.8 mg of the limited hydrolysis product. The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described previously.¹⁰⁾ 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 200 °C at a helium flow of 1 ml per min. GC-MS was performed with a JEOL JMS-GX303 mass spectrometer.

Limited Acid Hydrolysis Most of the glucan moiety in ukonan C was degraded by treatment with α -amylase, then DUC-I was obtained as described previously.²⁾ DUC-I (10.8 mg) was dissolved in 0.05 M trifluoroacetic acid (1 ml), and the solution was heated at 100 °C for 90 min. The acid was removed by evaporation, then the residue was dissolved in water and applied to a column (1.9 \times 41 cm) of Sephadex G-25. The column was eluted with water and fractions of 4 ml were collected. The eluates obtained from tubes 13 to 15 were combined, concentrated and lyophilized. Yield, 2.8 mg.

Phagocytic Activity This was measured as described in a previous report.⁷⁾ The samples and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (5 mg and 50 mg/kg body weight on zymosan, ukonan C and DUC-I; 5 mg/kg body weight on the others) to male mice (ICR-SPF) once a day.

Anti-complementary Activity This was measured as described previously.¹¹⁾ Gelatin-veronal-buffered saline (pH 7.4) containing 500 μ M Mg^{2+} and 150 μ M Ca^{2+} (GVB²⁺) was prepared, and normal human serum (NHS) was obtained from a healthy adult. Various dilutions of the samples in water were incubated and the residual total hemolytic complement (TCH₅₀) was determined using immunoglobulin M (IgM)-hemolysin-sensitized sheep erythrocytes. NHS was incubated with water and GVB²⁺ to provide a control, and the activities of the samples were expressed as the percentage inhibition of the TCH₅₀ of the control. Plantago-mucilage A^{11,12)} from the seed of *Plantago asiatica* L. was used as a positive control.

Alkaline Phosphatase Assay This was measured as described previously.¹³⁾ Each sample solution and the cell suspensions obtained from the spleen of male mice (ICR-SPF) were mixed and incubated. Each of the resultant cell suspensions was added to 10% diethanolamine-HCl buffer (pH 9.8) containing 0.1% *p*-nitrophenylphosphate. After incubation of the reaction mixture, 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

Ukonan C is a glucose-rich polysaccharide composed of terminal α -L-arabinose, α -1,3-linked L-arabinose, α -1,5-linked L-arabinose, α -2,5-branched L-arabinose, β -1,4-linked D-xylose, β -3,4-branched D-xylose, terminal β -D-galactose, β -1,3-linked D-galactose, β -1,4-linked D-galactose, β -1,6-linked D-galactose, β -2,4-branched D-galactose, β -3,6-branched D-galactose, terminal α -D-glucose, α -1,4-linked D-glucose, α -4,6-branched D-glucose, terminal α -L-rhamnose, α -1,2-linked L-rhamnose, α -2,4-branched L-rhamnose and α -1,4-linked D-galacturonic acid residues in the ratio of 9:5:1:1:2:4:2:1:1:2:1:5:2:24:2:1:2:1:6.²⁾

In previous studies on ukonan C, we have obtained a degradation product designated as DUC-I from ukonan C by treatment with α -amylase followed by gel chromatography.²⁾ After this enzymatic treatment, ukonan C lost about 80% of its glucose units; DUC-I was composed of a residual acidic polysaccharide moiety. Thus, DUC-I was composed of L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose and D-galacturonic acid in the molar ratio of 8:3:6:3:2:3.²⁾ The effects of ukonan C and DUC-I on the RES were demonstrated by a modification⁷⁾ of the *in vivo* carbon clearance test¹⁶⁾ using zymosan as a positive control. As shown in Fig. 1, DUC-I having an arabinogalactan moiety as its major part showed remarkable activity, especially at a high dose.

In order to elucidate the arabinogalactan core structure of the polysaccharide, ukonan C was subjected to periodate oxidation, and most of the product (POP) was reduced. The

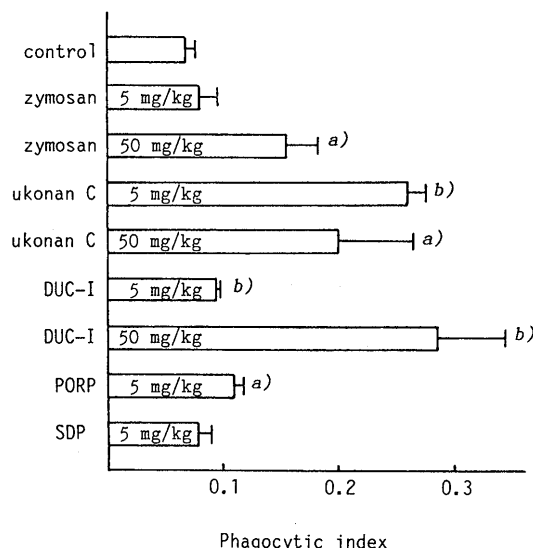


Fig. 1. Effects of Ukonan C and Its Degradation Products on Phagocytosis

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

reduction product (PORP) was treated with dilute sulfuric acid under a mild condition, then the controlled Smith degradation product (SDP) was isolated. SDP was obtained in reasonable yield. It was about 60% of the non-degradation part on treatment with periodate.

SDP gave a single band on PAGE, and gave a single peak on gel chromatography. It had $[\alpha]_D^{24} - 10.0^\circ$ (H_2O , $c = 0.1$), and gel chromatography gave a value of 6.8×10^3 for the molecular mass. The result of analysis showed that SDP was composed of L-arabinose, D-xylose and D-galactose in the molar ratio of 2:1:5, and it contained 3.2% peptide moiety.

SDP was methylated with sodium hydroxide and methyl iodide in dimethyl sulfoxide.¹⁴⁾ The product was hydrolyzed, then converted into the partially methylated alditol acetates. Analysis by GC-MS¹⁵⁾ revealed derivatives of 2,3,5-tri-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-D-xylose, 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 as the products in the molar ratio of 6:3:2:2:3:8. The terminal arabinose only appeared in a furanose form. It is therefore confirmed that 1,3-linked L-arabinose units must be present as arabinofuranose in ukonan C.

SDP was subjected to periodate oxidation followed by reduction. The controlled hydrolysis of the product was carried out under the same conditions as the isolation of SDP. The secondary Smith degradation product thus obtained was composed of D-galactose alone. Methylation analysis revealed derivatives of 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 as the products from the secondary Smith degradation product in the molar ratio of 1:1:2:1. This result suggests that terminal arabinosyl units in SDP must be combined to position 3 of some of the galactosyl residues in 1,6-linked galactosyl side chains.

Limited hydrolysis of DUC-I with dilute trifluoroacetic acid resulted in a complete removal of L-arabinose moiety with some D-xylose, D-glucose and L-rhamnose residues.

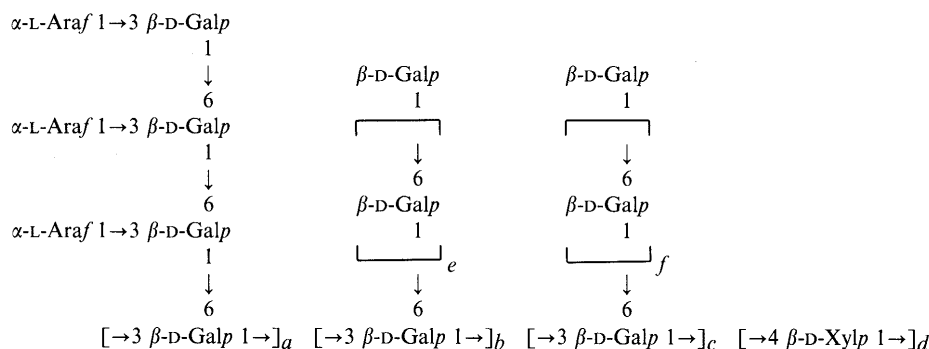


Chart 1. Possible Structural Units of the Smith Degradation Product of Ukonan C

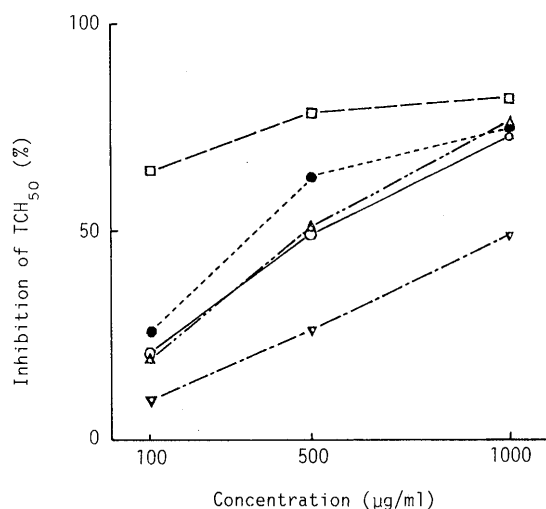
$$a:b:c:d:(e+f)=2:1:1:3:3.$$


Fig. 2. Anti-complementary Activity of Ukonan C and Its Degradation Products

—○—, ukonan C; ----△----, POP; ----▽----, PORP; ----□----, SDP; ----●----, Plantago-mucilage A.

The molar ratio of xylose, galactose, glucose and rhamnose in the degradation product obtained was 1.0:6.0:0.6:0.8. Methylation analysis of the degradation product revealed derivatives of 2,3-di-*O*-methyl-D-xylose, 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, 2,4-di-*O*-methyl-D-galactose, 2,3,4,6-tetra-*O*-methyl-D-glucose, 3,4-di-*O*-methyl-L-rhamnose and 3-*O*-methyl-L-rhamnose as the products in the molar ratio of 1.5:2.0:1.5:4.0:2.5:1.0:0.8:0.5. In this case, the hexuronic acid methyl ether was removed from the products by treatment with anion-exchange resin. Thus, the methylation analysis of the limited hydrolysis product of DUC-I revealed an increase in 1,6-linked D-galactose and a decrease in 3,6-branched D-galactose, and a disappearance of 3,4-branched D-xylose. These results indicate that the arabinose units are mainly connected to both galactose residues *via* position 3 and xylose residues *via* position 3 in ukonan C.

The accumulated evidence described above indicated that SDP, the arabinogalactan core part of ukonan C, has the structural features shown in Chart 1.

The effects of PORP and SDP on the RES are also shown in Fig. 1. The phagocytic index of ukonan C was markedly decreased by periodate oxidation. SDP showed no effect in a low level of dose; however, PORP afforded weak activity.

The anti-complementary activities of ukonan C, POP,

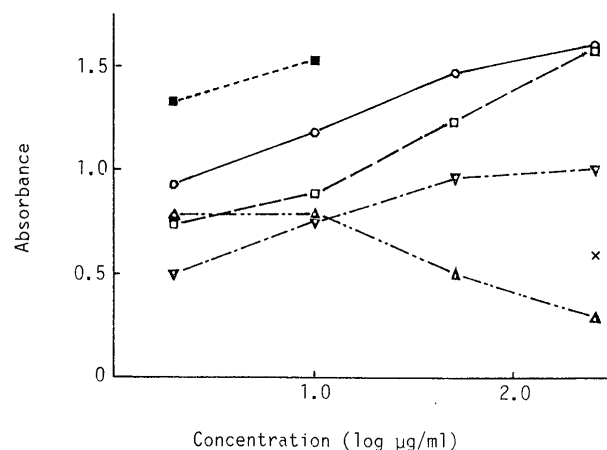


Fig. 3. Alkaline Phosphatase-Inducing Activity of Ukonan C and Its Degradation Products

—○—, ukonan C; —△—, POP; —▽—, PORP; —□—, SDP; —■—, lipopolysaccharide; ×, nil.

PORP and SDP are shown in Fig. 2. Ukonan C showed a little lower activity than the positive control. The activity of POP was almost at the same level as that of the original polysaccharide. The activity was decreased by periodate oxidation followed by reduction (PORP). In contrast to these products, the activity of SDP was markedly increased.

The measurements of alkaline phosphatase-inducing activity with ukonan C, POP, PORP and SDP were performed by *in vitro* murine spleen cell assay.¹⁷⁾ As shown in Fig. 3, ukonan C showed considerable activity. POP showed no effect, but subsequent reduction to give PORP raised the activity to some extent. SDP restored the activity in a similar manner to the product from ukonan A.³⁾

Discussion

Three acidic polysaccharides, ukonan A, ukonan B and ukonan C, obtained from the rhizome of *Curcuma longa* by us, showed remarkable RES-potentiating activities in a carbon clearance test, even at a very low dose.¹⁾ Among these three acidic polysaccharides from turmeric, both ukonan A and ukonan B are mainly made up of arabino-3,6-galactan type and rhamno-galacturonan type structures with β -3,4-branched D-xylosyl and small α -1,4-linked D-glucosyl units. Glucose is a very minor component in these two acidic polysaccharides, while ukonan C contains about 40% glucose as the component with long side chains.²⁾ The enzymatic removal of the

glucosyl side chains from ukonan C caused a remarkable lowering of its RES-potentiating activity at a low level of dose.²⁾ However, we have now observed the pronounced activity of DUC-I, the de-glucosylated product from ukonan C, in a relatively high dose (Fig. 1). Thus, the presence of glucose moieties in side chains of ukonan C contributes to the RES-activity in a very low dose, probably owing to steric effects. This result also proved the considerable contribution of the residual polysaccharide moiety having an arabinogalactan type structure as its major part obtained from ukonan C by the removal of glucose side chains to the RES-potentiating activity.

In addition to the RES-potentiating activity, we have now found considerable anti-complementary and alkaline phosphatase-inducing activities in ukonan C. In general, periodate oxidation caused a remarkable decrease in or disappearance of the immunological activities of ukonan C, though PORP had the significant effect on RES-potentiating activity. However, the controlled Smith degradation product, SDP, had significant effects on both anti-complementary and alkaline phosphatase-inducing activities. These results showed the contribution of an arabinogalactan core structure of the polysaccharide to immunological activity. Ukonan C has 19 different component sugar units,²⁾ and 15 of them, *i.e.*, terminal, 1,5-linked and 2,5-branched L-arabinosyl, 1,4-linked D-xylosyl, terminal, 1,4- and 1,6-linked and 2,4-branched D-galactosyl, terminal, 1,4-linked and 4,6-branched D-glucosyl, terminal, 1,2-linked and 2,4-branched L-rhamnosyl and 1,4-linked D-galacturonic acid residues, must have been destroyed or removed by the controlled Smith degradation.

The effects of SDP from ukonan C on both anti-complementary and alkaline phosphatase-inducing activities are at almost the same levels as those of SDP from ukonan A.³⁾ On the other hand, SDP from ukonan A showed significant RES-potentiating activity,³⁾ while SDP from ukonan C had no effect on RES at a low dose.

In addition, SDP from ukonan B afforded no effect on RES, and its effects on anti-complementary and alkaline phosphatase-inducing activities are lower than those of

SDPs from ukonan A and ukonan C.⁴⁾

SDP from ukonan A has a higher value of molecular mass than those from ukonan B and ukonan C. The former SDP possesses more terminal α -1,3-linked L-arabinosyl units in its side chains than the latter two. In addition, the former has α -1,2-linked L-rhamnosyl units but the latter two contain no rhamnose. It can be conceivable that the difference in these factors may have an influence on the RES-potentiating activity.

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