The Core Structure of Ukonan A, a Phagocytosis-Activating Polysaccharide from the Rhizome of *Curcuma longa*, and Immunological Activities of Degradation Products

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The controlled Smith degradation of ukonan A, 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 A and its degradation products were investigated. Methylation analyses of both the primary and the secondary Smith degradation products indicated that the core structural features of ukonan A include a backbone chain mainly composed of β-1,3-linked D-galactose, β-1,4-linked D-xylose and α-1,2-linked L-rhamnose residues. All of the galactose units in the backbone carry side chains composed of α-L-arabino-β-D-galactosyl or β-D-galactosyl residues at position 6.

Ukonan A has a remarkable effect on each of the three kinds of immunological activities. Periodate oxidation caused pronounced decrease or disappearance of the activities, but the controlled Smith degradation product having the core structure of polysaccharide showed considerable restoration of these activities.

Keywords *Curcuma longa*; rhizome; ukonan A; polysaccharide structure; Smith degradation; partial hydrolysis; immunological activity; reticuloendothelial system; anti-complementary activity; alkaline phosphatase-inducing activity

Ukonan A is one of the representative polysaccharides having phagocytic activity obtained from the rhizome of *Curcuma longa* L.[1] It is composed of 92.7% polysaccharide and 7.3% peptide moiety, and the polysaccharide is composed of L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose and D-galacturonic acid in the molar ratio of 12:4:12:1:4:10. It is noteworthy that ukonan A shows especially remarkable activity on the reticuloendothelial system (RES) even in a low level of dose.[1] The results of structural studies showed that the polysaccharide possesses mainly an α-L-arabino-β-3,6-D-galactan type structure with additional α-1,3-linked L-arabinose, β-3,4-branched D-xylose, α-2,4-branched L-rhamnose, α-1,4-linked D-glucose and α-1,4-linked D-galacturonic acid residues.[2]

The present paper describes the controlled Smith degradation, partial acid hydrolysis and methylation analysis of the products, and presents its core structural features. Because of the importance of macrophages and lymphocytes for the body's defense system against microbial infections and tumors, this paper also describes the immunological effects of ukonan A 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 in a previous report.[1]

Periodate Oxidation Ukonan A (120 mg) was oxidized with 0.05 M sodium metaperiodate (60 ml) at 5 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[3] Oxidation was completed affter 7 d. A part (5 ml) of the reaction mixture 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 and analyzed by the phenol–sulfuric acid method.[4] The eluates obtained from tubes 24 to 27 were combined, concentrated and lyophilized. The yield of the product (POP) was 10 mg.

The residual reaction mixture (55 ml) was successively treated with ethylene glycol (1 ml) at 5 °C for 1 h and sodium borohydride (300 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 × 88 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 32 to 37 were combined, concentrated and lyophilized. The yield of this product (PORP) was 90 mg.

Controlled Smith Degradation PORP (76.6 mg) was dissolved in 0.5 N sulfuric acid (7.6 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 × 88 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 33 to 37 were combined, concentrated and lyophilized. The yield of the product (SDP) was 32.1 mg.

Secondary Smith Degradation SDP (10.7 mg) was oxidized with 0.05 M sodium metaperiodate (5 ml) at 5 °C for 4 d in the dark. The reaction mixture was successively treated with ethylene glycol (0.2 ml) and sodium borohydride (60 mg) as described above. After the addition of acetic acid, the solution was applied to a column (2.6 × 96 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 25 were combined and lyophilized. Yield, 9.0 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 × 96 cm) of Sephadex G-25. The secondary Smith degradation product was obtained from the eluates in tubes 23 to 25. Yield, 4.0 mg.

Determination of Components Neutral sugars were analyzed by gas chromatography (GC) after conversion of the hydrolyzate into alditol acetates as described in a previous report.[5] Hexuronic acid was determined by the *m*-hydroxybiphenyl method.[6] Peptide determination was performed by the method of Lowry *et al.*[7] using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis (PAGE) This was carried out in an apparatus with gel tubes (4 × 132 mm each) and a 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 distinct band at a distance of 57 mm from the origin.

Gel Chromatography SDP (3 mg) was dissolved in a 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (2.6 × 94 cm) of Sephacryl S-300HR, 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 performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as previously described.[8] The yields were 3.4 mg from 5 mg of SDP, 3.4 mg from 3.2 mg of the secondary Smith degradation product, and 3.9 mg from 4.0 mg of the limited hydrolysis product. The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described in a previous report.[9] 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 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 The polysaccharide (12.3 mg) was dissolved in 0.05 M trifluoroacetic acid (1.4 ml), and the solution was heated at

100 °C for 2 h. The acid was removed by evaporation, then the residue was dissolved in water and 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 21 to 23 were combined, concentrated and lyophilized. Yield, 6.3 mg.

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 and suspended in physiological saline and dosed i.p. (5 mg/kg body weight) to male mice (ICR-SRF) once a day.

Anti-complementary Activity Gelatin–veronal-buffered saline (pH 7.4) containing 500 μM Mg^{2+} and 150 μM Ca^{2+} (GVB^{2+}) was prepared,[10] and normal human serum (NHS) was obtained from a healthy adult. Various dilutions of the samples in water (50 μl) were incubated with 50 μl of NHS and 50 μl of GVB^{2+}. The mixtures were incubated at 37 °C for 30 min and the residual total hemolytic complement (TCH_{50}) was determined by a method using immunoglobulin M (Ig M)–hemolysin–sensitized sheep erythrocytes at 1×10^8 cells/ml. NHS was incubated with water and GVB^{2+} to provide a control. The activities of the samples were expressed as the percentage inhibition of the TCH_{50} of the control. Plantago-mucilage A[11,12] was used as a positive control.

Alkaline Phosphatase Assay This was measured as described in a previous report.[13] Cells obtained from the ICR-SPF male mice were treated with ACK buffer, then washed with RPMI-1640 medium (Nissui Seiyaku Co.), resuspended with the medium containing 10% fetal calf serum (FCS, Flow Lab.) and adjusted to 5×10^6 viable cells per ml. Each sample solution (100 μl) and the cell suspension (100 μl) were mixed and incubated at 37 °C for 48 h in a humidified atmosphere of 5% CO_2. Each of the resultant cell suspensions was added to 10% diethanolamine–HCl buffer (pH 9.8) containing 0.1% *p*-nitrophenylphosphate. The reaction mixture was incubated at 37 °C for 1 h and was terminated by the addition of sodium hydroxide. The absorbance at 405 nm was measured and the results were expressed as the arithmetic mean ± S.D. of triplicate cultures. Lipopolysaccharide from *E. coli* 0111:B4 (Difco Lab.) was used as a positive control.

Results

Ukonan A was subjected to periodate oxidation, and the product (POP) was reduced. The reduction product (PORP) was treated with dilute sulfuric acid at room temperature overnight,[14] then the controlled Smith degradation product (SDP) was isolated. SDP gave a single band on PAGE, and gave a single peak on gel chromatography. It had $[\alpha]_D^{21}$ −46.2° (H_2O, $c=0.1$). Gel chromatography gave a value of 1.4×10^4 for the molecular mass. Quantitative analyses showed that SDP was composed of L-arabinose, D-xylose, D-galactose and L-rhamnose in the molar ratio of 11 : 8 : 26 : 1, and it contained 11.2% peptide moiety.

SDP was methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.[15] The methylated product was hydrolyzed, then converted into the partially methylated alditol acetates. Analysis by GC-MS[16] revealed derivatives of 2,3,5-tri-*O*-methyl-L-arabinose, 2,5-di-*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, 2,4-di-*O*-methyl-D-galactose and 3,4-di-*O*-methyl-L-rhamnose as the product from the methylated SDP in the molar ratio of 7 : 4 : 8 : 4 : 3 : 8 : 11 : 1. 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 A.

The result of methylation analysis of SDP revealed that this product still possesses a complicated structure, so SDP was further subjected to periodate oxidation followed by reduction. Then the product was treated with dilute sulfuric acid under the same conditions as the isolation of SDP. The secondary Smith degradation product thus obtained was composed of D-galactose. Methylation analysis revealed derivatives of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-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 3 : 8 : 3. This result suggests that 1,3-linked arabinosyl side chains in SDP must be combined to the galactosyl residues in a backbone chain through 1,6-linked galactosyl units.

Limited hydrolysis of ukonan A with very dilute trifluoroacetic acid resulted in a nearly complete removal of L-arabinose moiety with some D-xylose and L-rhamnose residues. The degradation product obtained was composed of D-xylose, D-galactose, D-glucose, L-rhamnose and D-galacturonic acid in the molar ratio of 2 : 14 : 1 : 3 : 10 with 6.8% peptide moiety. Methylation analysis of the degradation product revealed derivatives of 2,3-di-*O*-methyl-D-xylose, 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, 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, 3,4-di-*O*-methyl-L-rhamnose and 3-*O*-methyl-L-rhamnose as the products in the molar ratio of 4 : 1 : 1 : 8 : 5 : 9 : 6 : 3 : 3. 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 revealed the increase in 1,3-linked D-galactose, 1,6-linked D-galactose and 1,4-linked D-xylose, and the decrease in 3,6-branched D-galactose and 2,4-branched L-rhamnose, and a disappearance of 3,4-branched D-xylose. These results indicate that the arabinose units are connected to galactose residues *via* positions 3 or 6 and to position 3 of xylose residues in ukonan A. In addition, it is conceivable that a part of the rhamnose units carry arabinoxylan type side chains at position 4.

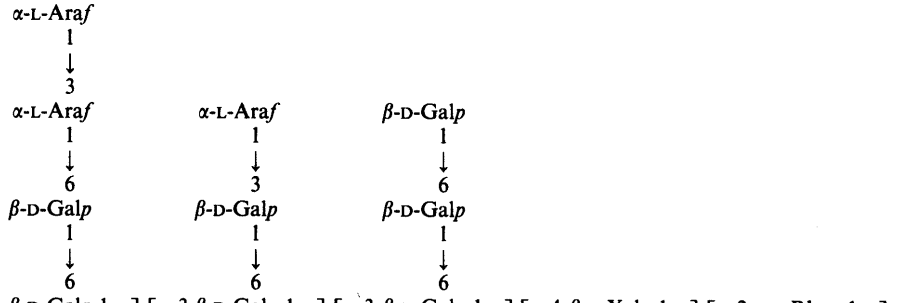

Chart 1. Possible Structural Units of the Smith Degradation Product of Ukonan A

a:*b*:*c*:*d*:*e*=4:3:4:8:1.

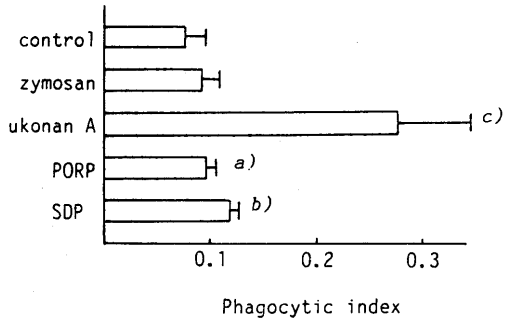

Fig. 1. Effects of Ukonan A and Its Degradation Products on Phagocytosis

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

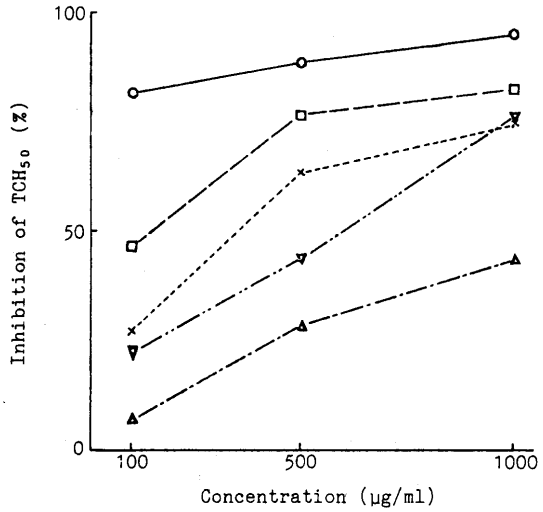

Fig. 2. Anti-complementary Activity of Ukonan A and Its Degradation Products

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

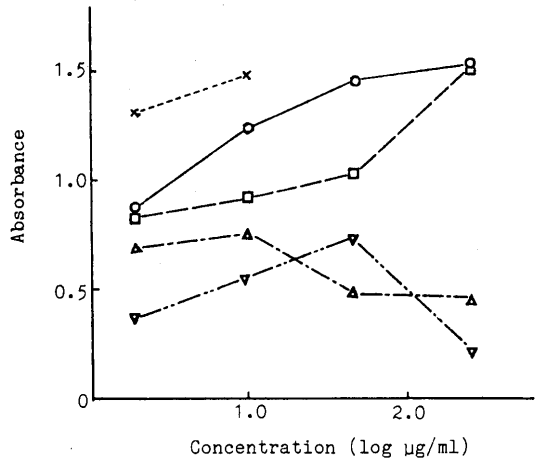

Fig. 3. Mitogenic Activity of Ukonan A and Its Degradation Products Assessed by Alkaline Phosphatase-Inducing Activity

○—○, ukonan A; △—·—△, POP; ▽—··—▽, PORP; □——□, SDP; ×---×, lipopolysaccharide.

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

The effects of PORP and SDP on the RES were demonstrated by a modification[5] of the *in vivo* carbon clearance test[17] using zymosan as a positive control. As shown in Fig. 1, the phagocytic index of ukonan A was markedly decreased by periodate oxidation. However, the activity of SDP was enhanced to some extent, compared with PORP.

The anti-complementary activities of ukonan A, POP, PORP and SDP are shown in Fig. 2. Ukonan A showed especially remarkable activity. Periodate oxidation highly decreased its activity, but subsequent reduction to give PORP raised the activity to some extent. Further pronounced restoration of the activity was observed in SDP.

The measurements of alkaline phosphatase-inducing activity with ukonan A, POP, PORP and SDP were performed by the *in vitro* murine spleen cell assay.[18] As shown in Fig. 3, ukonan A showed pronounced activity. Both POP and PORP showed no effect. However, when the cells were stimulated with SDP, the activity was induced in a dose dependent manner.

Discussion

We have obtained three acidic polysaccharides, ukonan A, ukonan B[1] and ukonan C,[19] and a neutral polysaccharide, ukonan D,[20] from the rhizome of *Curcuma longa*, and have reported their remarkable RES-potentiating activities in the carbon clearance test even in a very low dose. The three acidic glycans are commonly composed of L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose and D-galacturonic acid with a small amount of peptide moiety. Peptide moieties in the glycans must give no effect on the activity.[21] Both ukonan A and ukonan B are mainly made up of arabino-3,6-galactan type and rhamnogalacturonan type structures with α-1,3-linked L-arabinosyl, β-3,4-branched D-xylosyl and α-1,4-linked D-glucosyl units. In addition to the distinguished RES activity, we have now found especially remarkable anti-complementary and pronounced alkaline phosphatase-inducing activities in ukonan A.

We have already isolated and characterized saposhnikovan A[5] from the root and rhizome of *Saposhnikovia divaricata*, MVS-IIIA,[22] -IVA[23] and -VI[24] from the seed of *Malva verticillata*, glycyrrhizans UA[25] and UC[26] from the root of *Glycyrrhiza uralensis*, glycyrrhizan GA[27] from the stolon of *G. glabra* var. *glandulifera*, and AMon-S from the root of *Astragalus mongholicus*[28] as other examples of RES-activating polysaccharides having mainly α-1,5-linked L-arabino-β-3,6-branched D-galactan moieties. Among them, MVS-VI showed remarkable anti-complementary activity, but alkaline phosphatase-inducing and anti-complementary activities in the other substances have not been investigated yet.

Ukonan A possesses fourteen kinds of component sugar units,[2] and nine kinds of them, *i.e.*, terminal and 1,5-linked L-arabinosyl, 1,4-linked D-xylosyl, terminal and 1,6-linked D-galactosyl, terminal and 1,4-linked D-glucosyl, 1,2-linked L-rhamnosyl and 1,4-linked D-galacturonic acid residues, must have been destroyed by periodate oxidation. Two kinds of periodate oxidation products, POP (polyaldehyde form) and PORP (polyol form), showed a marked decrease or disappearance of immunological activities in ukonan A. However, the controlled Smith degradation product, SDP, gave significant effects on these activities. The structural features of SDP are much simpler than those of the original glycan. Thus, the core structure of

the polysaccharide shown in SDP contributes to the immunological activities investigated by us, though it is conceivable that the complicated branching structure in ukonan A may increase the effects on these activities.

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