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# Influence of the chain length of chitosan on complement activation

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**Abstract**

Non-water soluble chito-oligosaccharides (COSs) (molecular weights: 8, 800; 14, 200; 18, 200; and 33, 000) were investigated for complement activation by the single radial immuno-diffusion method. C3 activation was increased in a chain length-dependent manner. On regression analysis, there was significant correlation ($P < 0.01$) between the number of $NH_2$ group of COSs and the extent of C3 activation after 20 min of incubation. The binding of C3b to chitosan (82% deacetylated chitin) was also investigated by an immunofluorescence method, and binding of C3b to chitosan particles was clearly observed. From these results, number of amino groups, and trapping of C3b are important evidence of complement activation via alternative pathway by chitosan and non-water soluble COSs.


*Keywords:* Chitosan; Immunofluorescence; Chito-oligosaccharides; Complement activation

## 1. Introduction

We have investigated the mechanisms of accelerated wound healing by chitin and chitosan for over 10 years. We have focused on complement activation by these materials as their major biological effect (Minami, Suzuki, Okamoto, Fujinaga, & Sigemasa, 1998). Regarding the activation of complement by these materials, we have assessed the influence of molecular weight (M.W.) and water solubility of the amino-polysaccharides (Suzuki et al., 2000). A water-soluble mixture of a monomer (D-glucosamine, M.W. 216) and chito-oligosaccharides (COSs) (2–14 residues, M.W. 432-3, 024) did not activate complement component 3 (C3). In addition, 50% homogenously acetylated chitosan (M.W. 80, 000), which was soluble in water, also did not activate C3, whereas, insoluble 50% heterogeneously acetylated chitosan (M.W. 80, 000) caused C3 activation (Suzuki et al., 2000). From these results, the most important characteristic of amino-saccharides inducing complement activation seemed to be insolubility. However, chito-oligosaccharides with a higher M.W. (over 3, 024) were not tested previously.

In the process of complement activation, formation of C3b complexes on the surface of cells and/or other materials is essential (Müller-Eberhard, 1975). The alternative pathway is involved in complement activation (Minami et al., 1998) and is known to require the binding of C3b. When C3 is activated by C3 convertase, nascent complement fragment C3b can bind to surfaces by a covalent bond that has been suggested to be an ester (Law, Lichtenberg, & Levine, 1979). Zymosan is another complement activator via alternative pathway that also binds C3b (Nicholson et al., 1975).

In the present study, the objectives were to test whether higher M.W. (over 3, 024) COSs could activate complement or not, and to confirm the binding of C3b to chitosan like zymosan.

## 2. Materials

### 2.1. Reagents

Chito-oligosaccharides (COSs): COSs were prepared by a modified version of the method described by Muraki,

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Yaku, and Kojima (1993). In brief, 100% deacetylated chitosan (DAC 100) was purchased from Koyo Chemical Co. (Osaka, Japan) and COSs were separated from a chitosan hydrolyzate prepared with cellulase. Prefractionation of chitosan hydrolyzate was performed with MeOH–water (9:1).

Separation of COSs by column chromatography. A column (15 × 450 mm) was packed with BIO-GEL (P-4, P-6, P-10, P-30). An oligosaccharide sample (100 mg) was dissolved in a minimal volume of water and applied to the column, which was then eluted with 0.1 M HAc–NaAc buffer (pH 4.2). COSs were separated from each fraction by 1 N NaOH.

HPLC: COSs were analyzed on a column of TSK-GEL G3000PW (Tosoh Co. Ltd). The eluent was 0.5 M HAc–NaAc buffer (pH 4.2) and peaks were detected by changes in refractive index. Peaks were identified by performing chromatography after the addition of standard oligosaccharides (pullulan).

DPs 44, 71, 91 and 165 were recovered and selected for the study. These were insoluble in water, so each sample was suspended in saline at 10 mg/ml before use.

Chitosan: chitosan powder was used, which was 82% deacetylated chitin purified from crab shell and had an average molecular weight of 80,000 (maximum 1.2% ash; 5 ppm of heavy metals, including Pb, Cd, and As). The preparation was pulverized into several grades (mean particle size: 5 μm) with a mill (Ube Industries, Ltd, CF-400) by Sunfive Co. (Tottori, Japan). The distribution of granule size was measured with a SK Laser Micron Sizer 7000S (Seisin K K., Japan). The chitosan powder was sterilized with ethylene oxide gas, and was suspended in sterile saline (pH 6.5–7.8). The molecular weight and the extent of deacetylation were determined by the viscosity method (Tokura & Nishi, 1995) and the IR method (Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996), respectively. Endotoxin was undetectable in a hot water exact (70 °C) tested by the specific colorimetric method (Endospecy, Seikagaku-kogyo, Japan). The sample was resuspended in sterile saline at 10 mg/ml before use and was designated as DAC 82.

Zymosan A (Lot. 49H0557) was purchased from Sigma Chemical Co. (St. Louis, USA); 10 mg of zymosan was suspended in 1 ml saline and was activated by boiling for 30 min. After this treatment, the zymosan suspension was stocked in micro tubes at −80 °C and was resuspended in sterile saline at 10 mg/ml before use.

Antibody for single radial immuno-diffusion (SRID): Goat Anti-Dog C3 was purchased from the ICN Pharmaceuticals, Inc. (Ohio, USA).

Antibodies for immunofluorescence analysis: Mouse monoclonal anti-human C3b-alpha and FITC-rabbit anti-mouse IgG (H + L) were purchased from Progen Biotechnik GmbH. (Heidelberg, Germany) and Zymed Laboratories, Inc. (California, USA), respectively.

## 3. Methods

### 3.1. Effects of COSs on C3

#### 3.1.1. Blood collection

Three healthy beagle dogs were used for the collection of normal plasma. Blood samples were collected from the jugular vein into a heparinized sterile syringe, were portioned into sample tubes on ice, and were centrifuged at 4 °C and 1200*g* for 5 min. Plasma was separated in micro tubes and was frozen at −80 °C until use.

#### 3.1.2. C3 assay

The sample used for the C3 assay was the supernatant separated by centrifugation at 1500 rpm for 5 min at 4 °C after incubation with 100 μl of dog plasma and 100 μl of COSs or chitosan for 20 min or 40 min at 37 °C. Each assay was performed in triplicate. The supernatant was separated into micro tubes and was frozen at −80 °C until use.

The details of the SRID method were described previously (Suzuki et al., 2000). In brief, to made a gel plate, purified agar dissolved in phosphate buffer by microwave treatment and about 250 μl of anti-dog C3 serum was added to 6 ml of the gel. The liquid gel was poured into a glass box and immediately cooled in an ice bath. Then the solid gel plate was cut into pieces (1 × 1 $cm^2$) and a hole was made in the center of each piece. Next, each supernatant was added to a gel and incubated at 4 °C for 48 h. The control (100 μl of non-incubated plasma) was diluted with 100 μl of saline and was also processed by SRID. The area of the ring produced by diffusion of immunoglobulin (Ig) into the antiserum-containing gel was proportional to the amount of Ig present, so the diameter of the ring was measured under a stereoscopic microscope. Then the C3 value was calculated as follows: Ring size = $\pi(Ra^2 - Rb^{2)}$, where Ra is the radius of the ring diameter and Rb is the radius of the central hole diameter. C3 value (%) = (diffusion ring size for serum incubated with each reagent/diffusion ring size of a serum incubated for saline) × 100.

### 3.2. Immunofluorescence analysis

#### 3.2.1. Blood collection

Peripheral blood was obtained from healthy human volunteers. The blood was added to heparinized sample tubes and centrifuged at 1500 rpm for 5 min at 4 °C. The plasma thus obtained was portioned into the micro tubes and was frozen at −80 °C until use.

#### 3.2.2. Fluorescent antibody technique

Chitosan and zymosan suspensions (100 μl) were incubated with 100 μl of human plasma (positive sample) or saline (negative sample) in a water bath at 37 °C for 20 min. Then each sample was centrifuged 1500 rpm for 5 min at 4 °C, the supernatant was discarded, and the pellet

was washed three times with saline. Next, the pellet was incubated with mouse monoclonal anti-human C3b-alpha for 1 h on ice and washed by the same procedure. Subsequently, the pellet was incubated with FITC-rabbit anti-mouse IgG (H + L) and washed again by the same procedure. Finally, the pellets were resuspended in 20 μl of saline and scanned by a Confocal Laser Scanning Microscope (CLSM).

### 3.2.3. Image analysis of fluorescence intensity

The digital images taken by CLSM were analyzed using computer image analyzer software (Adobe Photoshop 3.0 for Macintosh, Adobe Systems, Tokyo). Each image was analyzed on the green setting. The fluorescence intensity (FI) was obtained by subtracting FI of the negative sample from FI of the positive sample. FI values of 200,000 pixels were randomly collected from 10 fields in a CLSM image and image analysis was performed using Adobe Photoshop 3.0 for Macintosh.

## 4. Results and discussion

### 4.1. C3 activation

C3 activation by COSs is shown in Fig. 1. C3 was less activated by all COSs compared with the chitosan positive control. C3 activation by COSs was increased in a chain length-dependent manner after 20 min of incubation. By regression analysis, the correlation between the number of $NH_2$ group of COSs and the % C3 activation after 20 min of incubation (Fig. 2) gave the following equation ($y = 0.0865x + 10.751$, $R^2 = 0.9916$, $P < 0.01$).

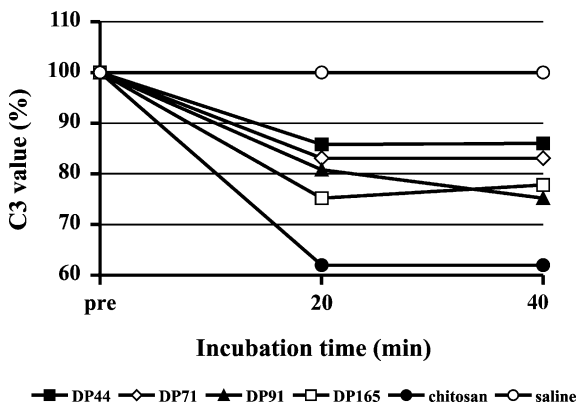


Fig. 1. *Effect of COSs on C3*. Concentration of each suspension (COSs: DPs 44, 71, 91, and 165, chitosan: 82%-deacetylated chitin) was 10 mg/ml. Hundred micro litre of COSs or chitosan were mixed with 100 μl of dog plasma and incubated at 37 °C for 20 and 40 min. The control (100 μl of non-incubated plasma) was diluted with 100 μl of saline and was also processed by SRID. C3 value (%) = (diffusion ring size for serum incubated with each reagent/diffusion ring size of a serum incubated for saline) × 100.

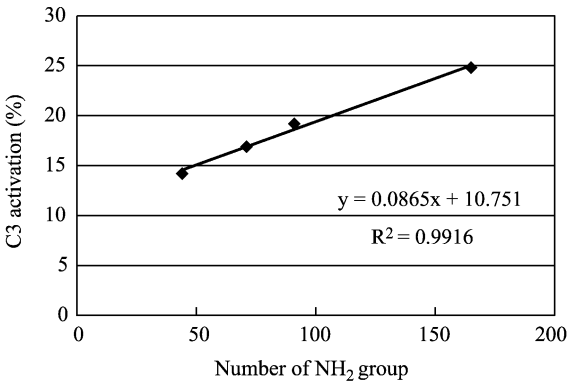


Fig. 2. *Simple linear regression between C3 activation value and number of $NH_2$ group*. C3 activation by COSs was increased in a chain length-dependent manner after 20 min of incubation. By regression analysis, the correlation between the number of $NH_2$ group of COSs and the % C3 activation after 20 min of incubation gave the following equation ($y = 0.0865x + 10.751$, $R^2 = 0.9916$, $P < 0.01$).

Since chitosan has obtained about 328 $NH_2$ groups in its chain, the calculated intensity of C3 activation using the equation is about 39%. C3 activation actually detected by SRID was 38%. Moreover, natural chitin (M.W. 300, 000) was obtained with 8% $NH_2$ groups (about 110 $NH_2$ groups). Therefore, $y = 20.3\%$ and C3 activation was about 23% in our previous report (Minami et al., 1998). The extent of C3 activation by the reagents was significantly correlated with the number of $NH_2$ groups.

### 4.2. C3b binding

The results of immunofluorescence analysis are shown in Fig. 3. Binding of C3b was clearly detected on chitosan (DAC 82) particles (Fig. 3(A)) as well as on zymosan particles (Fig. 3(C)). The FI of chitosan and zymosan was 19.897 and 17.523, respectively. From these results, chitosan binds C3b in the same way same as zymosan. The nascent complement fragment C3b can bind to the surface of zymosan (Nicholson, Brade, Lee, Shin, & Mayer, 1974) and zymosan-C3b has been used for much research into the adsorption of component (Brade, Bentley, Kossorotow, & Bitter-Suermann, 1977; Discipio, 1981; Law et al., 1979). The initial activation of the alternative pathway occurs as follows. C3 undergoes transformation to C3 ($H_2O$) by spontaneous hydrolysis. C3 ($H_2O$) acts as a 'C3b-like' C3 molecule (Pangburn, Schreiber, & Müller-Eberhard, 1981), and provides a subunit for the initial C3 convertase, C3 ($H_2O$) Bb, which cleaves fluid-phase C3 to generate C3b and C3a. C3b combines by an ester bond or amide bond to hydroxyl and amino groups on the cell membrane and stabilizes C3bB by having an effect on factor D, an amplifier of the alternative pathway (Law & Levine, 1977; Vogt, Dames, Schmidt, & Dieminger, 1977). Zymosan is well known as an activator of the alternative pathway (Brade et al., 1977; Nicholson et al.,

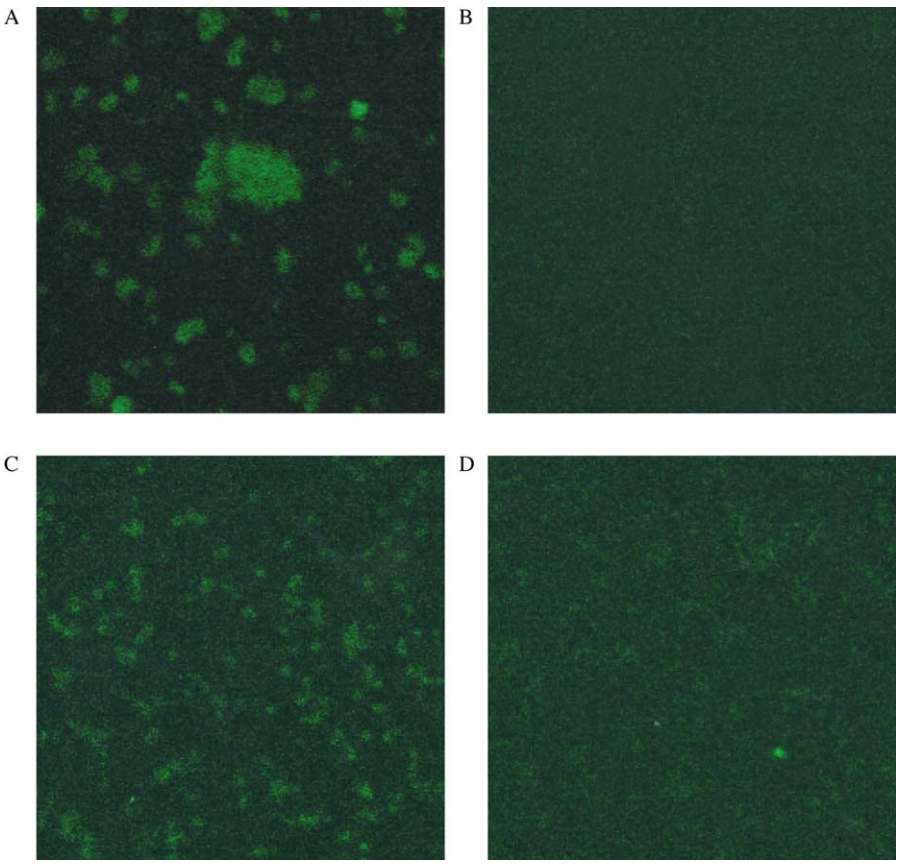


Fig. 3. *Immunofluorescence analysis of C3b on the particle of chitosan and zymosan.* Chitosan and zymosan were incubated with human plasma (positive sample) or saline (negative sample) in water bath at 37 °C for 20 min. After washed each pellets, those were incubated with mouse anti-human C3b-alpha antibodies and FITC-labeled rabbit anti-mouse IgG antibodies Finally, the pellets were re-suspended in 20 μl of saline and scanned by a confocal laser scanning microscope (CLSM). (A): Positive sample of chitosan, (B): negative sample of chitosan, (C): positive sample of zymosan, (D): negative sample of zymosan.

1974). C3b can bind to various surfaces by a covalent bond that has been proposed to be an ester (Law et al., 1979). Zymosan was used for competitive binding studies of factor B, β1H, C5, and properdin by utilizing its special binding profile (Discipio, 1981).

Discipio (1981) reported that zymosan has four distinct binding sites for C3b on its surface. Recently, studies on the binding sites for complement have been progressing. Lambris et al. (1996) reported that factor B binds to at least the N-terminal part of the C3b α′-chain, and Jokiranta et al. (2001) also found (using the surface plasmon resonance technique) that binding of factor B to C3b occurred at the C3d-fraction, which was a component of C3b.

Chitosan is also an activator of the alternative pathway (Minami et al., 1998), but there have been no reports previously about its complement binding during activation of this pathway.

In the present study, C3 activation was correlated with the number of $NH_2$ groups, so C3 ($H_2O$) would bind to the amino group of D-glucosamine.

## 5. Conclusion

Chitosan activates complement in an $NH_2$ group-dependent fashion. After activation of C3, C3b is produced and effectively binds to chitosan, while stabilized C3b acts as a binder for factor B.

The mechanism of complement activation by chitosan seems to closely resemble that for zymosan.

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