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Structure and biological activities of hypochlorite oxidized zymosan

N. Ohno[a,*], T. Miura[a,b], N.N. Miura[a], Y. Adachi[a], T. Yadomae[a]

[a]*School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*
[b]*Department of Pharmacy, Kitasato University Hospital, 1-15-1 Kitasato, Sagamihara, Kanagawa 229, Japan*

Abstract

Zymosan (ZYM), a strong complement activating yeast cell preparation, is also a potent inflammatory substance, which shows immunopharmacological activity. Major component of ZYM is β-glucan but contains other constituents, such as mannan, protein, and nucleic acid. We applied sodium hypochlorite (NaClO) treatment to ZYM to reduce impurities and compared the activity with native/parent ZYM. Oxidized ZYM (OX-ZYM) became a nitrogen-free agent. By NMR analysis of native OX-ZYM and zymolyase (endo-1,3-β-glucanase) digest, OX-ZYM was found to contain 1,3-β-linked and 1,6-β-linked glucan moieties, while the latter degraded by sodium metaperiodate treatment. OX-ZYM also contained small amounts of anionic groups, partly reducible by sodium borohydride. Degree of polymerization (DP) of 1,6-β-glucan moiety was estimated to be about DP10–DP50 by MALDI-TOF-MS analysis. In comparison with ZYM activities, OX-ZYM and derivatives showed strong antitumor activity to solid form of Sarcoma 180 in mice, and showed strong activity on alternative pathway of complement, but lost secondary response to ZYM-immune mice. These facts strongly suggested that a particulate form of β-glucan was prepared by NaClO treatment of ZYM and at least a part of ZYM-mediated biological activity was found unmediated by β-glucan moiety.

Keywords: 1,3-β-Glucan; 1,6-β-Glucan; Zymosan; MALDI-TOF-MS; Immunopharmacological activity

1. Introduction

Beta 1,3-glucans (β-glucan(s)) are widely distributed in nature. Some of the β-glucan preparations have already been applied clinically for cancer immunotherapy (Hamuro & Chihara, 1984; Okamura, Suzuki & Chihara, 1986). Furthermore, β-glucans are implicated as a causative candidate in some allergic pneumonia, such as the maple bark disease, farmer's lung, and sick building syndrome (Fogelmark, Sjostrand, Williams & Rylander, 1997). From the perspective of ultrastructure, β-glucan exists in both soluble and insoluble forms. The soluble form of β-glucan exists as "sol", "single helix" or "triple helix" conformation (Saito, Tabeta, Yoshioka, Hara, Kiho & Ukai, 1987; Saito, Yoshioka, Yokoi & Yamada, 1990). The single and the triple helical conformations were clearly demonstrated by X-ray crystallography (Deslandes, Marchessault & Sarko, 1980). In addition, these forms and conformations are, at least in part, inter-changeable, and strongly influence the biological activity of β-glucans (Miura et al., 1995a; Ohno, Miura, Chiba, Adachi & Yadomae, 1995; Ohno, Hashimoto, Adachi & Yadomae, 1996). Because of these specific properties, purification of β-glucan is sometimes difficult, and thus some of the experiments are limited without special care to the structural background of each β-glucan preparation. We are interested in the relationship between structure and biological activity of β-glucans, and have long been working on characterizing the molecular mechanism of β-glucan mediated biological activity (Yadomae & Ohno, 1996). We, and others, reported that the biological activities of soluble β-glucans and particulate β-glucans are significantly different, even though both show beneficial immunopharmacological activities, such as antitumor activity. The latter can easily induce the production of various inflammatory mediators from phagocytes in vivo and in vitro (Adachi, Okazaki, Ohno & Yadomae, 1997; Okazaki, Chiba, Adachi, Ohno & Yadomae, 1996; Suzuki, Ohno, Chiba, Miura, Adachi & Yadomae, 1996). In addition, activities of the soluble β-glucans were also significantly different depending on the conformation (Miura, Ohno, Suda, Miura, Shimada & Yadomae, 1995b; Ohno et al., 1995, 1996). However, to obtain enough quantity of the particulate form of β-glucan with adequate quality seems to be still difficult.

Zymosan (ZYM) is a well-known reagent prepared from yeast, *Saccharomyces cerevisiae*, to show various biological activities, related to inflammatory and immune responses (DiCarlo & Fiore, 1958). Some of these activities, active oxygen synthesis, prostaglandin synthesis, and inflammatory

* Corresponding author. Tel.: +81-426-76-5570; fax: +81-426-76-5570.
E-mail address: ohnonao@ps.toyaku.ac.jp (N. Ohno).

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cytokine synthesis, are related to the phagocytic response of macrophage and neutrophil against microbes. Thus, ZYM is utilized as a model substance for analyzing acute inflammatory response and development of anti-inflammatory substances (Allen & Aderem, 1995; Au, Williams & Collins, 1994; Janusz, Austen & Czop, 1989; Jiang, Wu & Rubin, 1997; Kusner, Hall & Schlesinger, 1996; Lu, Thiel, Wiedemann, Timpl & Reid, 1990; Lundy, Dowling, Stevens, Kerr, Mackin & Gans, 1990; Ridger, Pettipher, Bryant & Brain, 1997; Sanguedolce, Capo, Bongrand & Mege, 1992; Sanguedolce, Capo, Bouhamdan, Bongrand, Huang & Mege, 1993; Sergeant & McPhail, 1997; Steadman et al., 1990; Veras, de Chastellier & Rabinovitch, 1992; Zaffran, Escallier, Ruta, Capo & Mege, 1995). ZYM is also a useful agonist for the functional β-glucan receptor(s). The main component of ZYM is β-glucan (DiCarlo & Fiore 1958), however, there is no evidence that all of ZYM-mediated biological activity is due to the presence of β-glucan moiety. Recently, we found that ZYM administered mice produced on antibody to ZYM and induced the antigen specific T-cell response (Miura, Ohno, Miura, Adachi, Shimada & Yadome, 1999). In the previous study, we have prepared OX-ZYM by sodium metaperiodate oxidation ($NaIO_4$) and borohydride reduction (I/B-ZYM) and/or limited hydrolysis of oxidized moieties (I/B/H-ZYM). Unexpectedly, I/B/H-ZYM still contained significant proportion of 1,6-β-glucan moiety (Ohno et al., 1998). We have also shown that extensive oxidation of ZYM by sodium hypochlorite (NaClO) solubilized 1,6-β- as well as 1,3-β-glucan moiety (Miura, Ohno, Adachi & Yadome, 1996a). In this paper, we applied NaClO oxidation to prepare the particulate form of pure β-glucan and examined its biological activity. The results clearly showed that some of the activities were mediated by other than β-glucan moiety of ZYM.

2. Materials and methods

2.1. Carbohydrate analyses

Carbohydrate content was determined by the phenol-sulfuric acid method. Component sugars were determined by capillary gas–liquid chromatography (Ohkura Riken Co. Ltd, Tokyo) of alditol acetate derivatives after complete hydrolysis by 2 M trifluoroacetic acid. A capillary column of fused silica (J and W Scientific, Inc., CA, 30 m × 0.262 mm, liquid phase; DB-225, 0.25 mM) was used at 220°C. Molar ratio of glucose and mannose (G/M) was calculated from the peak area of each component (glucose as 100).

2.2. Preparation of OX-ZYM

Preparation of the NaClO-oxidized ZYM was followed by the procedure used in the previous paper (Miura, Ohno, Aketagawa, Tamura, Tanaka & Yadomae, 1996b). Briefly, ZYM (2 g) were suspended in 200 mL of 0.1 M NaOH and oxidized with appropriate volume of NaClO solution (25 ~ 100 ml: final concentration of available chlorine; 0.5 ~ 2%) for 1 day at 4°C. After the reaction was completed, the insoluble fraction was washed extensively by distilled water and dried by ethanol and acetone (yield ca. 55%).

2.3. Preparation of B-, I/B- and I/B-H-OX-ZYM

OX-ZYM, suspended in 50 mM sodium acetate buffer pH 5.0, was mixed with sodium metaperiodate in final concentration of 20 mM and incubated at 4°C in dark for a day. After incubation, excess periodate was destroyed by adding ethyleneglycol. Oxidized OX-ZYM was collected by centrifugation and washed several times with aliquots of distilled water. The resulting oxidized OX-ZYM was suspended in distilled water and reduced with sodium borohydride at room temperature. Particles were collected by centrifugation and washed with dilute acetic acid and distilled water, followed by lyophilization (I/B-OX-ZYM). B-OX-ZYM was prepared from OX-ZYM by direct borohydride reduction. A half part of I/B-OX-ZYM was hydrolyzed partially by 0.1 M trifluoroacetic acid at 20°C for 48 h. The resulting particles were collected by centrifugation, washed with sodium bicarbonate solution and distilled water, followed by lyophilization (I/B/H-OX-ZYM).

2.4. Zymolyase digestion of OX-ZYM and derivatives

OX-ZYM and derivatives (20 mg) suspended in 10 ml of acetate buffer (50 mM, pH 6.0) were mixed with 1 mg of zymolyase 100T (Seikagaku Corp.). After overnight incubation at 45°C, the reaction mixture was boiled for 3 min to inactivate the enzyme. The resulting supernatant was concentrated and applied to a Toyopearl HW-40 column. The elution profile of each fraction was monitored by the phenol-H_2SO_4 method. High molecular weight fraction (fraction A) was applied to a column of DEAE-Sephadex A25 (Cl^-) equilibrated with H_2O. After extensive washing with H_2O, adsorbed fraction was eluted with 1 M NaCl. Passed through (neutral fraction) and adsorbed (acidic fraction) fractions were quantitated by phenol-H_2SO_4 method.

2.5. NMR analysis

OX-ZYM, derivatives, enzyme digests and authentic materials were dissolved in D_2O or Me_2SO-d_6 and the ^1H- and ^{13}C-NMR spectra were measured at 70°C. Bruker DPX400 and DRX500 instruments equipped with a software "XWIN-NMR" were used.

2.6. MALDI-TOF-MS

MALDI-TOF-MS analysis was conducted using a Perceptive Biosystems Voyager RP-DE equipped with delayed extraction and a nitrogen laser. All analyses were conducted using 2,5-dihydrobenzoic acid (DHB, Tokyo

Table 1
Elemental analysis of ZYM and derivatives

	C	H	N
Theoretical value	44.4	6.2	0
ZYM	42.12	6.95	2.28
ZYC	45.5	7.69	0.25
OX-ZYM(25/200)	42.37	6.8	0.17

Kasei Co.) as matrix. We used a linear detection mode with delayed extraction and detected positive ions.

2.7. Antitumor activity

Male ICR mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). These mice were 5 weeks of age and maintained under specific pathogen-free conditions. Antitumor activity against the solid form of Sarcoma 180 tumor was measured by the method previously described (Iino et al., 1985).

2.8. Activation of alternative pathway of complement

Freshly prepared human serum was used as a complement source. Rabbit red blood cells (RRBC) were washed with veronal buffered saline (VBS) containing 0.1%(W/V) EDTA, and 7.5×10^8 of RRBC were prepared with VBS containing 0.1% gelatin, 2 mM EGTA, 10 mM $MgCl_2$, 4.45 mM barbital sodium, 127 mM NaCl, pH 7.4 (VEM). Human plasma (25 μl) and VEM (25 μl) were mixed and placed in a centrifuge tube. To the resulting mixture, OX-ZYM or derivatives (2 mg/ml, 25 μl) suspended in VEM were added and incubated for 15 or 30 min at 37°C. After incubation, the tubes were cooled on ice, RRBC (100 μl) added and incubated for 1 h at 37°C. The tubes were centrifuged and the supernatants collected. Hemolysis was monitored by a microplate reader (MTP-32, Corona electric) at 550 nm.

2.9. Measurement of cytokine production by spleen cells stimulated with ZYM or related substances in vitro

Spleen cells from ZYM administered mice (1 mg of ZYM every week for 1 month) were prepared at 1×10^7 cells/ml and stimulated with ZYM (100 μg/ml) and related substances (100 μg/ml) for 48 h.

2.10. Assay of IL-6 and IFN-γ

IL-6 and IFN-γ levels of spleen cell culture supernatants were measured by using enzyme-linked immunosorbent assay (ELISA). Five μg/ml of anti-mouse IL-6 (Pharmingen, San Diego, CA) or IFN-γ mAb (Pharmingen, San Diego, CA) was bound to the surface of 96-well, flat bottomed plate (Sumitomo Bakelite Co., Tokyo, Japan) by incubation at 4°C overnight with Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS) containing 0.05% Tween20 (PBST) and blocked with 0.5% bovine serum albumin in PBST at 37°C for 40 min. After being washed with PBST, the plate was incubated with culture supernatants, rMu IL-6 (R and D Systems, Minneapolis, MN) or rMu IFN-γ (PharMingen, San Diego, CA), followed by incubation with an adequate dilution of anti-IL-6 rat monoclonal antibody (PharMingen, San Diego, CA) or anti-IFN-γ rabbit polyclonal antibody (Genzyme, Boston, MA). After washing and blocking, the plates were treated with a 1/10 000 dilution of peroxidase-labeled anti-rabbit IgG (Genzyme, Boston, MA). After final washing, peroxidase-conjugated antibody was detected by the addition of a TMB substrate system (KPL Inc., Gaithersburg, MD). Color development was stopped with the help of 1 N phosphoric acid and the absorbance at 450 nm was measured with a micro plate reader (MTP32, Corona Electric Co., Ltd, Tokyo, Japan).

2.11. Statistics

Results are expressed as mean ± standard deviation (S.D.). Statistical evaluations were performed by Student's *t*-test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Preparation of NaClO oxidized zymosan and derivatives

ZYM, suspended in 0.1 M NaOH, was mixed with NaClO (final concentration of available chlorine; 0.5 ~ 2%) and incubated at 4°C for 24 h. After centrifugation and extensive washing, resulting pellet (OX-ZYN) was dried. Yield of OX-ZYM was approximately 55%, which did not change much depending on the concentration of NaClO used in this study.

Previously, we had applied periodate oxidation to ZYM, but the reaction could not degrade a majority of 1,6-β-glucan moieties (Ohno et al., 1998). We again tried to prepare similar derivatives using OX-ZYM, by treatment with sodium metaperiodate, reduction with sodium borohydride, followed by partial hydrolysis by trifluoroacetic acid. Each derivative was designated as I/B-OX-ZYM and I/B/H/-OX-ZYM. Direct reduction of OX-ZYM by borohydride was achieved in parallel experiments for reference purposes (B-OX-ZYM).

3.2. Physicochemical properties of OX-ZYM and derivatives

Nitrogen content of ZYM and OX-ZYM were compared and it was found to be 2.2 and 0.2%, respectively (Table 1). ZYC, a commercially available β-glucan preparation, was used as the reference. Data of OX-ZYM was comparable to ZYC. Nitrogen content clearly shows almost all protein, nucleic acid as well as chitin were degraded and/or solubilized during NaClO treatment. NaClO is a strong oxidant, and thus, these constituents could be removed by non-specific oxidation reactions. It is also suggested that β-glucan is

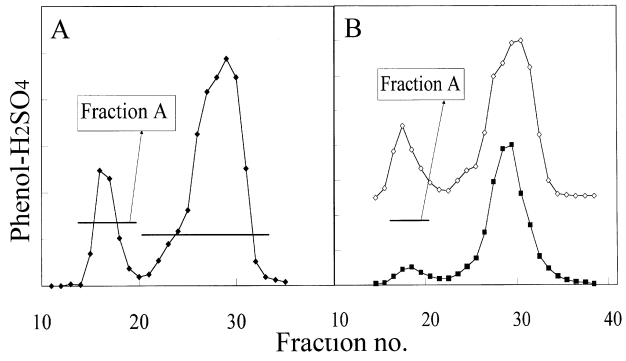

Fig. 1. Gel filtration chromatography of OX-ZYM and derivatives. Gel filtration chromatography of zymolyase digested (A) OX-ZYM, (B, ◇) B-OX-ZYM, and (B, ■) I/B-OX-ZYM. OX-ZYM, B-OX-ZYM, or I/B-OX-ZYM (20 mg each) digested with zymolyase 100T as described in materials and methods, was applied to a column of Toyopearl HW40 (1 × 40 cm) equilibrated with distilled water. Each 0.5 ml fraction was collected and carbohydrate content measured using the phenol–H_2SO_4 method. The fraction eluted around void volume was designated as fraction A.

significantly resistant to such oxidation process. Component sugar of ZYM and derivatives were analyzed by gas–liquid chromatography. Man:Glc ratio of ZYM was 27:73 and only Glc was detected from OX-ZYM. It is reported that the mannan moiety is linked to β-glucan moiety via the protein component (Kollar et al., 1997), thus the mannan moiety might be released during solubilization of the protein moiety.

To demonstrate the structure of OX-ZYM and derivatives, zymolyase, an endo 1,3-β-glucanase, digestion was applied and the products were analyzed in several methods. Fig. 1A shows an elution profile of the zymolyase digest of OX-ZYM. Zymolyase digested OX-ZYM was applied to a column of Toyopearl HW40 equilibrated with H_2O, and separated into high MW (fraction A) and mono/oligosaccharides. Low MW fraction contained glucose and laminaraoligosccharides, corresponding to the 1,3-β-glucan moiety of OX-ZYM. Fraction A was analyzed by NMR and MS spectrometry, and ion-exchange chromatography as described. B-OX-ZYM and I/B-OX-ZYM were also digested by zymolyase and the products separated by Toyopearl HW40 (Fig. 1B). Content of fraction A prepared from I/B-OX-ZYM was significantly reduced.

The proton-NMR spectra of fraction A of OX-ZYM and derivatives were measured (Fig. 2). The spectrum was measured in D_2O at 70°C. Only regions of anomeric signals were shown. OX-ZYM gave strong signals around 4.5 ppm attributable to 1,6-β-glucan moiety, and around 4.7 ppm attributable to the non-reducing terminal, present at 3- and 6-substituted branching points (Kollar et al., 1997). The spectrum of the fraction A prepared from ZYC was quite similar. In contrast, that of I/B-OX-ZYM was significantly different and the signals shown above were almost

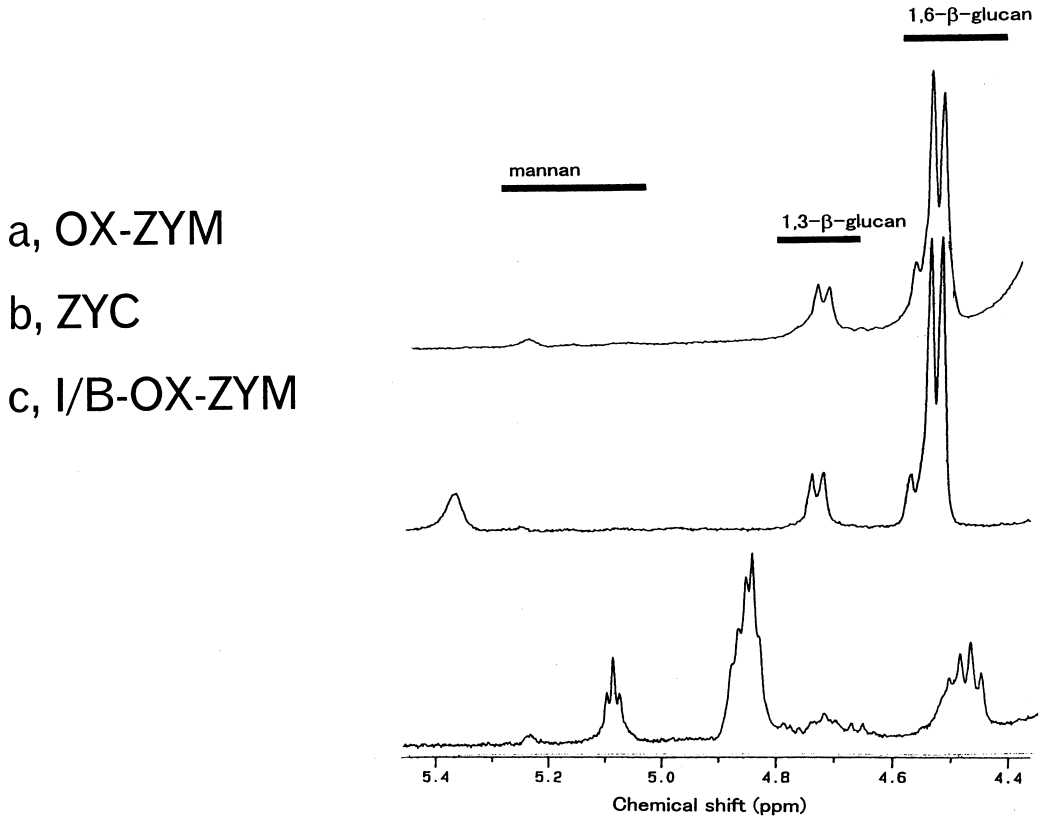

Fig. 2. ^1H NMR spectra of OX-ZYM and derivatives. Proton NMR spectra of fraction A of zymolyase digested OX-ZYM, ZYC, and I/B-OX-ZYM in D_2O (5 mg/ml) at 70°C.

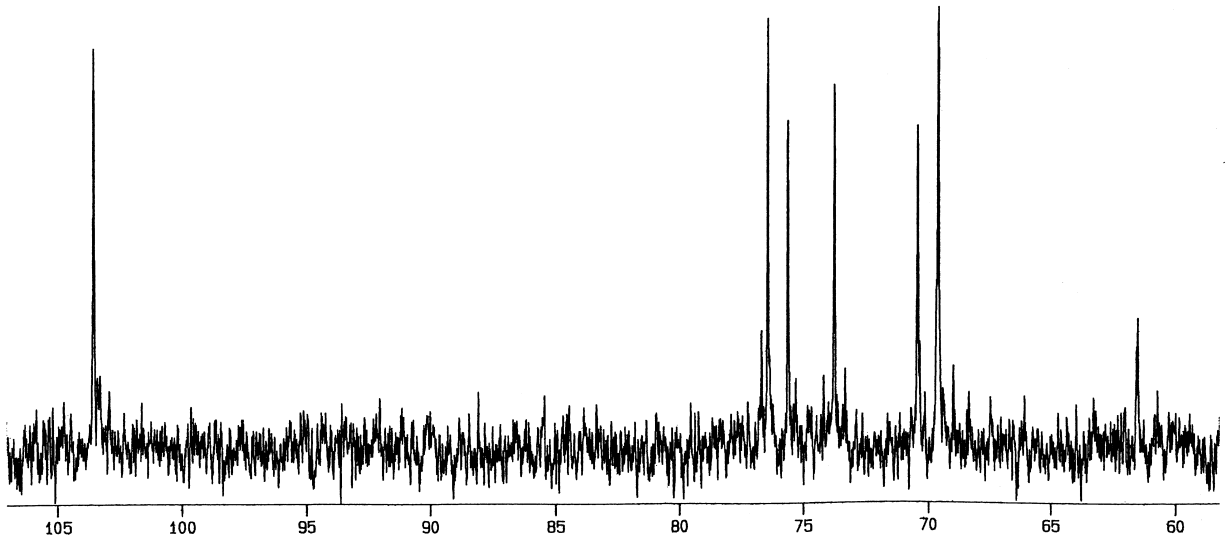

Fig. 3. DEPT45 spectrum of fraction A of OX-ZYM. DEPT45 NMR spectrum of fraction A of zymolyase digested OX-ZYM in D_2O (5 mg/ml) at 70°C.

completely disappeared, suggesting that almost all of 1,6-β-linkage was degraded.

The linkage was also analyzed by DEPT45 spectrum of OX-ZYM-fraction A (Fig. 3). Six major signals were easily assignable as 1,6-β-linked glucan chain (Ohno, Suzuki, Oikawa, Sato, Miyazaki & Yadomae, 1984; Ohno et al., 1999). In addition, the spectrum shows very low intensity of signal around 60 ppm, characteristic for some branching points in 1,6-β-linked glucan chain. Carbon-13 NMR spectra of native OX-ZYM and derivatives were measured in

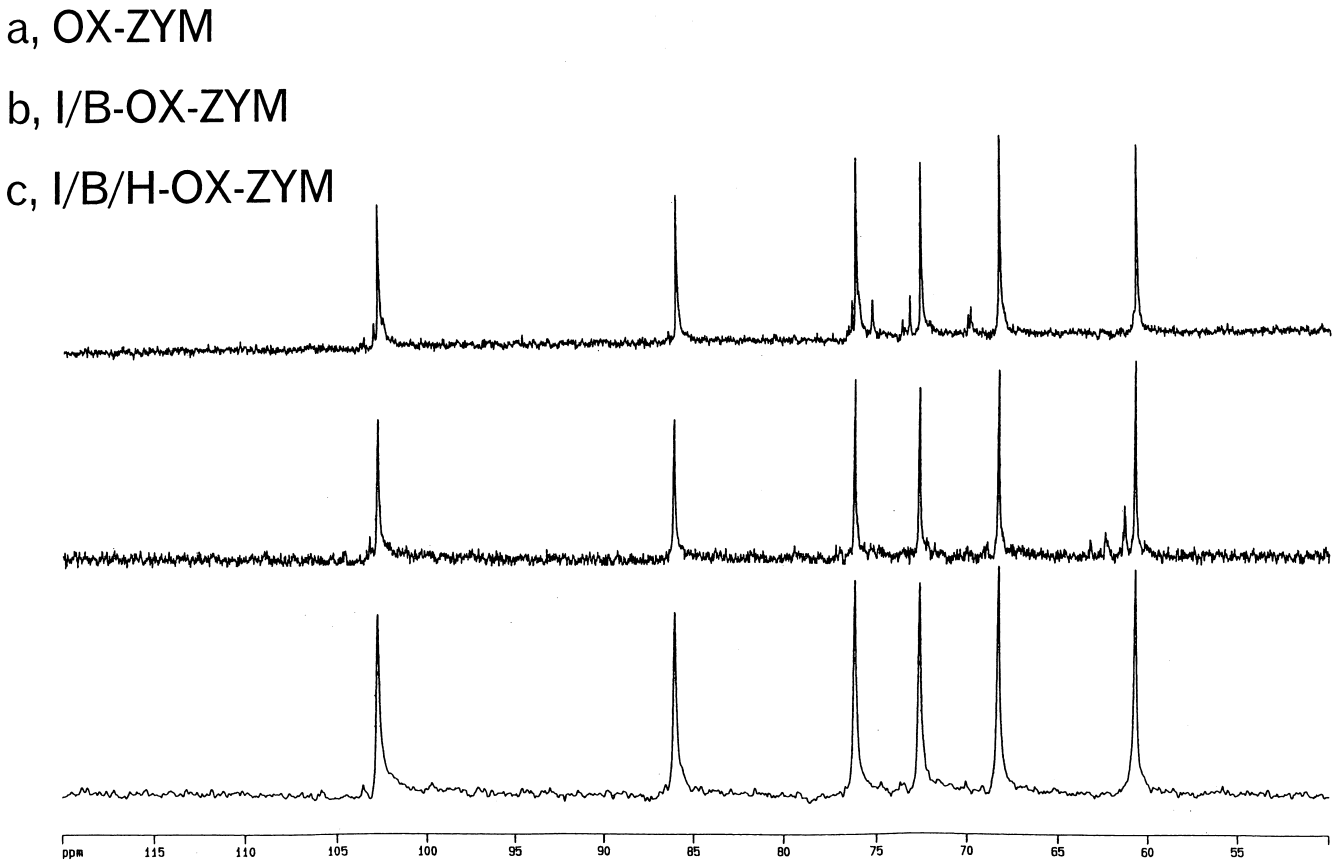

Fig. 4. ^{13}C NMR spectra of OX-ZYM and derivatives. Carbon 13 NMR spectra of OX-ZYM, I/B-OX-ZYM, and I/B/H-OX-ZYM in DMSO-d_6 (30 mg/ml) at 70°C.

Table 2
Some properties of zymosan derivatives

Fraction name	Ratio of β1,6/β1,3 + β1,6[a]	Acidic part in zymolyase resistant fraction (%)[b]	Acidic part in total (%)[c]
ZYM	0.76	36.6	27.8
ZYC	0.36	4.2	1.5
OX-ZYM (25/200)[d]	0.25	15.5	3.9
OX-ZYM (50/200)[d]	0.24	12.6	3.0
OX-ZYM (100/200)[d]	0.22	17.4	3.8
B-OX-ZYM[e]	0.26	13.0	3.4
I/B-OX-ZYM[e]	0.11	17.1	1.9

[a] Calculated from total carbohydrate content in fraction A/total.
[b] Calculated from adsorbed carbohydrate content to DEAE-Sephadex in fraction A.
[c] (Ratio of β1,6/β1,3 + β1,6) × (Acidic part in zymolyase resistant fraction (%)).
[d] 25(50, 100)/200, OX-ZYM was prepared by 25(50, 100) ml NaClO/200 ml 0.1 N NaOH.
[e] Prepared from OX-ZYM (100/200).

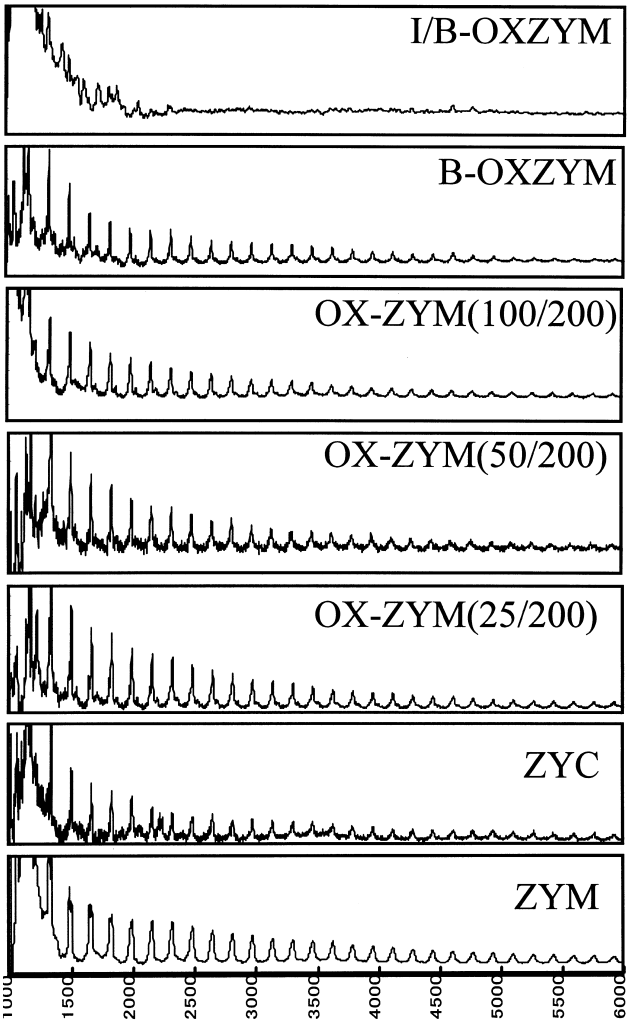

Fig. 5. MALDI-TOF-MS spectra of fraction A of OX-ZYM and derivatives. MALDI-TOF-MS spectra of fraction A of zymolyase digested OX-ZYM and derivatives. DHB was used as matrix. Experimental detail was shown in materials and methods.

DMSO-d_6 (Fig. 4). OX-ZYM shows strong signals attributable to 1,3-β-glucan chain, and weak signals attributable to 1,6-β-glucan chain (Ohno et al., 1984; Ohno et al., 1999). I/B-OX-ZYM and I/B/H-OX-ZYM lost latter signals. Similarly, loss of 1,6-β-linkage by periodate oxidation was also confirmed in this spectrum.

Table 2 summarizes the properties of OX-ZYM and derivatives. Ratio of 1,6-β-glucan moiety was calculated from the percentage of fraction A. The ratio was around 25% in OX-ZYM and slightly reduced when using higher concentration of NaClO. The ratio of ZYC was 35%, suggesting presence of higher 1,6-β-linkage concentration. As expected, ratio of fraction A was significantly reduced in I/B-OX-ZYM. In the case of ZYM, the ratio was significantly high, but could be due to the presence of other carbohydrate constituents, such as mannan and nucleic acid, both of which showed positive reaction by the phenol–sulfuric acid method. The middle column in Table 2 shows the ratio of anionic fraction. Fraction A was applied to DEAE-Sephadex chromatography and the bound fraction calculated. Ratio of anionic fraction in each derivative was increased by stronger oxidation. The acidic part was estimated to be only $2 \sim 4\%$ in total carbohydrate. In addition, actual percentage of anionic group in total carbohydrate should be significantly lower than those value, or in other word, the value does not mean significant increase of anionic groups during oxidation. Because the polysaccharide fragment containing only one anionic group could also bind to DEAE-Sephadex. It is also supported by NMR analysis that no detectable signals corresponding to the carboxyl group were present in carbon-13 NMR (data not shown). ZYM also contained significantly high percentage of acidic fraction, it is probably due to other carbohydrate constituents. The ratio of acidic fraction was reduced in B-OX-ZYM (17.4–13%). Borohydride reducible residue would be corresponding to aldehyde group. The structure of the anionic moiety incorporated during oxidation was not examined in detail, but could be a mixture of aldehyde and carboxylate.

Table 3
Antitumor activity of OX-ZYM against solid form of Sarcoma 180 (i.v.) (sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously into ICR male mice(day 0). Each sample was administered 250 μg/mouse as saline solution by intravenous injection on days 7, 9, and 11 (in experiment 1). Fraction A was administered 1000 μg/mouse as saline solution by intraperitoneal injection on days 7,9,11,13, and 15 (in experiment 2)

Sample	Mean ± SD (g)[a]	% Inhibition	CR/n	*t*-test[b]
Experiment 1				
ZYM	1.6 ± 2.6	79.8	5/10	$p < 0.001$
OX-ZYM (25/200)	1.4 ± 4.6	81.6	6/11	$p < 0.001$
OX-ZYM (100/200)	0.2 ± 0.4	97.6	6/10	$p < 0.001$
I/B-OX-ZYM	0.4 ± 0.9	94.7	4/10	$p < 0.001$
I/B/H-OX-ZYM	1.8 ± 2.2	76.6	2/10	$p < 0.001$
Control	7.8 ± 3.6		0/11	
Experiment 2				
Fraction A	8.5 ± 5.2	8.7	0/10	n.s.
Control	9.3 ± 4.9		0/11	

[a] Each tumor weight was determined at day 35 after tumor inoculation.
[b] The significant differences from control was evaluated according to Student's *t*-test. CR, complete regression; n.s., not significant.

Fig. 5 shows the molecular weight distribution of fraction A, assessed by matrix assisted laser desorption mass spectrometry (MALDI-TOF-MS). Fig. 5 shows the spectra of molecular weight range of 1000–6000. The molecular ions were detected in all the range values, and each ion had 162 mass difference. Calculated from the spectrum, degree of polymerization (DP) of 1,6-β-glucan moiety was suggested to be about 10–50, and the ladder was similar in all the fractions tested, except for periodate oxidized fraction A. This fraction did not give any specific mass ions. In addition, the ladder of OX-ZYM and ZYC were similar, suggesting the molecular weight heterogeneity would not be incorporated during NaClO oxidation.

Therefore, from the above data, OX-ZYM was suggested to be mainly composed of 1,3-β- and 1,6-β-glucan moieties, and these moieties were chemically connected to each other, since the zymolyase digestion of OX-ZYM-particle resulted in highly water soluble β-glucan fragments (Fraction A). Furthermore, ratio of 1,3-β-linkage was higher than that of 1,6-β-linkage.

3.3. Biological activity of OX-ZYM and derivatives

The main purpose of this study was to demonstrate the contribution of minor components on the biological activity of ZYM, thus we compared several activities shown by ZYM. Antitumor activity of OX-ZYM and derivatives were shown in Table 3. Sarcoma 180 was subcutaneously transplanted to ICR mice. One week later, 250 μg of OX-ZYM and derivatives were intravenously administered to these mice, and every other day for 3 times. Ten or 11 mice for each group was used. Strong antitumor activity was shown ($p < 0.001$) by all the derivatives, except for fraction A, as assessed by mean tumor weight as well as number of completely regressed mice. It is now concrete that the antitumor activity of ZYM was mediated by β-glucan moiety. Comparing the activity of OX-ZYM and derivatives, I/B-OX-ZYM showed comparable, while I/B/H-OX-ZYM lower activity. In addition, fraction A, low-molecular weight 1,6-β-glucan segment, did not show any antitumor activity. Molecular weight of fraction A was significantly smaller than other particulate derivatives,

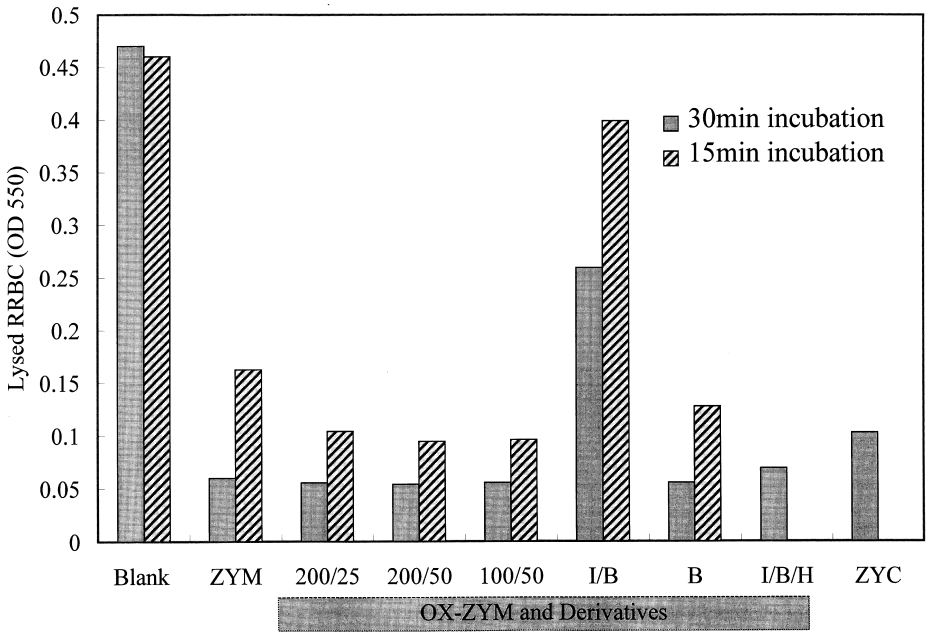

Fig. 6. Activation of alternative pathway of complement by OX-ZYM and derivatives. Aliquots of human serum were mixed and incubated with OX-ZYM and derivatives. The resulting sera were collected by centrifugation and the remaining activity measured as RRBC target, as described in materials and methods.

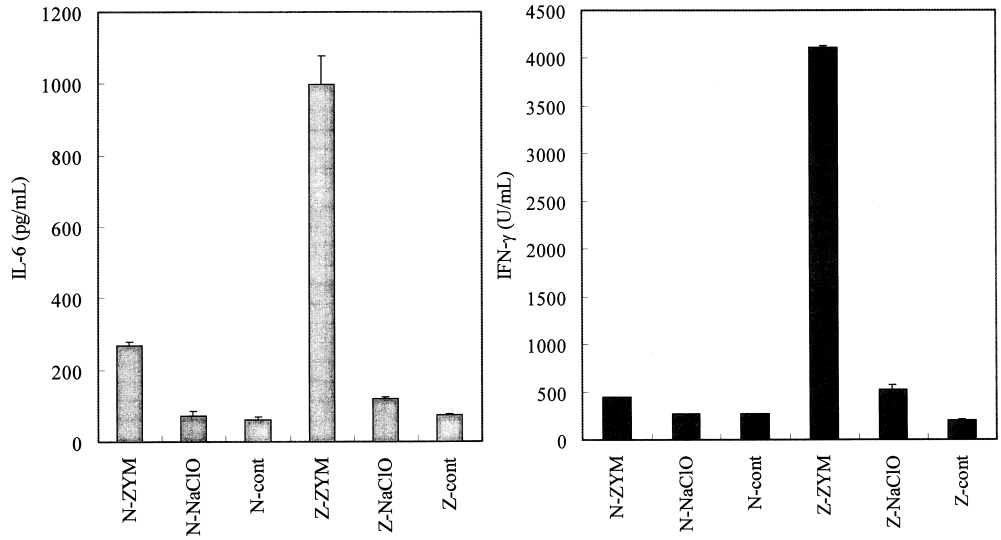

Fig. 7. Cytokine syntheses of ZYM-immune murine spleen cells by ZYM or OX-ZYM in vitro. Spleen cell suspension of ICR mice immunized with ZYM were prepared and stimulated with ZYM and OX-ZYM. After 48 h incubation in humidified CO_2 incubator, culture supernatant was collected and interleukin 6 and interferon-γ concentrations measured by ELISA. Experimental details were shown in materials and methods: N-ZYM, normal mouse spleen, stimulated with ZYM in vitro; N-NaClO, normal mouse spleen, stimulated with OX-ZYM in vitro; N-cont, normal mouse spleen, in vitro cultured without stimulation; Z-ZYM, ZYM immune mouse spleen, stimulated with ZYM in vitro; Z-NaClO, ZYM immune mouse spleen, stimulated with OX-ZYM in vitro; Z-cont, ZYM immune mouse spleen, in vitro cultured without stimulation.

thus multimeric and aggregated form of the fraction A moiety could be an important factor. Furthermore, I/B-OX-ZYM lost 1,6-β-glucan chain but contained hydrophilic polyol moiety, thus structure or property of the surface of particle could be important, in addition to the network of fibrous 1,3-β-glucan moiety.

Activation of Alternative Pathway of Complement, APC, was also tested. Fig. 6 indicates hemolysis of Rabbit Red Blood Cells. Activator of APC reduced the hemolysis. Comparing derivatives, I/B-OX-ZYM shows significantly weaker activity, and interestingly, partial hydrolysis again showed re-appeared strong activity. These facts strongly suggested the negative contribution of partially degraded polyol residues to APC.

Fig. 7 shows antigen-specific secondary response of cytokine synthesis on splenic cell culture of ZYM-immune mice. Normal mice were also used as control. The cells, probably antigen-specific T-cells, produced significant amount of interferon-γ and interleukin 6 in response to ZYM, while they did not respond to OX-ZYM at all. It is strongly suggested that the ZYM specific T-cell response could be induced by minor components.

4. Discussion

ZYM is a particulate β-glucan fraction prepared from yeast, *Saccharomyces cerevisiae*, which strongly activates the APC in the presence of a serum component, properdin. ZYM is reported to be prepared by repeated hot water extraction and tryptic degradation (DiCarlo & Fiore, 1958). The protein part of the yeast cell-wall, manno-protein would be sensitive to the tryptic digestion, however, ZYM is not a pure β-glucan preparation and still contains proteins, peptides, nucleic acids and the mannan, in addition to the cell wall β-glucan. ZYM is a representative agonist of the functional β-glucan receptor, however, there is no confirmation that β-glucan is responsible for all of the activity shown by ZYM. The latter part of the constituents limit the precise studies on the relationship between structure and biological activity of β-glucan.

Because of the fact that β-glucan is able to remain in murine organs for over six months when administered intravenously (Miura et al., 1995b, 1996b, 1998), and that one of the most powerful tools of immune system to degrade foreign materials is known as the oxidative degradation mediated by NaClO, we have applied NaClO treatment for ZYM and succeeded to prepare nitrogen-free β-glucan preparation, and confirmed that some of the biological activity induced by ZYM was mediated by minor constituents, such as protein, nucleic acid or mannan.

Structure of OX-ZYM was suggested to be 1,3-β-glucan joined to small molecular weight 1,6-β-glucan chain with both of the β-glucan chains containing few branching points. Compared with the structure of a commercially available β-glucan particle, ZYC, ratio of 1,6-β-linkage was reduced, probably due to easier solubilization of 1,6-β-glucan part during oxidation. This result was further supported by the data that solubilized fraction of ZYM during NaClO oxidation was mainly composed of 1,6-β-glucan moiety, assessed by ^{1}H NMR spectroscopy (data not shown). In addition, enzymic degradation and ion-exchange chromatography of OX-ZYM suggested the incorporation of small amount of anionic moieties. Indirect

evidence by borohydride reduction suggested that the anionic residue was mainly composed of carboxyl group and about 30% aldehyde. However, the content of these residues were low, thus could not be detected by spectrometry and examined in detail.

One of the interesting evidence we obtained in this study, was the normal susceptibility of 1,6-β-glucan chain to periodate oxidation, and we obtained particulate form of 1,3-β-glucan (I/B/H-OX-ZYM). In contrast, as we have already reported, periodate oxidation of ZYM could not completely remove 1,6-β-glucan chain (Ohno et al., 1998). These facts strongly suggested that some of the constituents, at least in part, produced physical barrier in the cell-wall to the external chemicals.

Antitumor activity and APC was found to be due to β-glucan part of ZYM. We have already reported that these activities were also shown by soluble β-glucans (Iino et al., 1985; Saito, Nishijima, Ohno, Nagi, Yadomae & Miyazaki, 1992; Suzuki, Ohno, Saito & Yadomae, 1992; Yadomae & Ohno, 1996). We have also shown that curdlan, 1,3-β-glucan obtained from *Alcaligenes faecalis* var *Myxogenes* 10C3 did not show antitumor activity, but after the NaClO oxidation, it became active (Nono, Ohno, Masuda, Oikawa & Yadomae, 1991). I/B/H-OX-ZYM prepared in this study was successively changed to be linear, but showed strong antitumor activity. Considering the fact that it contained small amounts of anionic groups, the antitumor mechanism of both glucans could possibly be similar.

We also showed the activation of APC by OX-ZYM and derivatives, except for I/B-OX-ZYM. Mechanism of APC activation would be strongly associated with the stabilization of complement factor C3b on the surface of OX-ZYM to produce autocatalytic C3 convertase (C3bBb) (Hong, Kinoshita, Pramoonjago, Kim & Seya, 1991; Pangburn, 1989; Sahu, Isaacs, Soulika & Lambris, 1998). It is quite interesting that incorporation of the partially oxidized polyol group nearby, strongly destabilized the enzyme.

Polysaccharide is generally not a useful antigen compared with protein and peptides, however, specific antibodies could be produced in mice and/or rabbit by immunization with appropriate adjuvant. We have prepared anti-β-glucan antibody against 6-branched 1,3-β-glucan, grifolan (Adachi, Ohno & Yadomae, 1994; Adachi, Miura, Ohno, Tamura, Tanaka & Yadomae, 1999). We have also found that OX-ZYM specific antibody was produced by repeated immunization with ZYM (manuscript in preparation), and suggested that ZYM could induce specific immune response in mice. Considering these background information, we tested the antigen specific T-cell response of ZYM immune mice against OX-ZYM. We detected ZYM specific cytokine synthesis of spleen cells but was not specific for OX-ZYM. T-cell response is generally induced after MHC–peptide–TCR interaction, thus polysaccharide is generally conjugated with protein antigen for enhancement of the antigenicity (Ambrosino, Bolon, Collard, Van Etten, Kanchana & Finberg, 1992; Ishioka et al., 1992; Seppala & Makela, 1989). The data shown in the present study suggested that ZYM specific T-cell response required protein component.

In conclusion, we clarified β-glucan dependent (antitumor and APC) and independent (T-cell response) activities of ZYM by using OX-ZYM and derivatives. OX-ZYM and derivatives could be useful tools for the analysis of structure and activity relationship of β-glucan.

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