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Solubilization of yeast cell-wall β-(1→3)-D-glucan by sodium hypochlorite oxidation and dimethyl sulfoxide extraction

Naohito Ohno [a], Michiharu Uchiyama [a], Aiko Tsuzuki [a], Kazuhiro Tokunaka [a], Noriko N. Miura [a], Yoshiyuki Adachi [a], Maki W. Aizawa [b], Hiroshi Tamura [b], Shigenori Tanaka [b], Toshiro Yadomae [a,*]

[a] *Tokyo University of Pharmacy and Life Science, School of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

[b] *Seikagaku Corporation, 3-1253 Tateno, Higashiyamato, Tokyo 207-0021, Japan*

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Abstract

The limulus test is a well-established method for the diagnosis of both Gram-negative sepsis and invasive fungal infection. To diagnose fungal infections, a β-(1→3)-D-glucan-specific chromogenic kit (Fungitec G test MK) has been developed and applied clinically. We are concentrating our main efforts on developing a better standard to improve the precision of this method. To this end, we have successfully developed a protocol to obtain a soluble *Candida* spp. β-(1→3)-D-glucan (CSBG) by sodium hypochlorite (NaClO) oxidation and subsequent dimethyl sulfoxide (Me_2SO) extraction (yield of $9.6 \pm 4.1\%$) of acetone-dried whole-cell preparations. The β-glucan fraction is free from the cell-wall mannan, gives a symmetrical peak by gel filtration, and is soluble in dilute NaOH. The product is composed mainly of β-(1→3)- and β-(1→6)-D-glucosidic linkages. The specific activity of the β-glucan is comparable with pachyman when combined with the Fungitec G test as the standard glucan and reacted as low as 10^{-11} g/mL.

Keywords: β-Glucan; *Candida* spp.; Cell wall; MALDIMS; Limulus test

1. Introduction

Candida spp. is a medically important genus of fungi that induces disseminated candidiasis and candidemia in hospitalized, immunocompromised patients. The cell wall of *Candida* is mainly composed of two polysaccharides, mannan and β-glucan, and at least a part of the β-glucan, mainly the β-(1→3)-D-glucan moiety, is basically insoluble in H_2O or NaOH and is quite difficult to extract [1,2]. To analyse the architecture of the yeast cell-wall, traditional procedures, such as repeated acid and/or alkaline extraction, have been applied; however, selectivity and yield of the extracts have not been satisfactory for the precise structural determination [1,2]. Selective hydrolytic enzymes such as zymolyase are helpful to lyse and analyse the cell wall [3,4]. Several protocols have been reported to solubilize the cell wall β-(1→3)-D-glucan moiety, such as sulfate and phosphate derivatives [5,6]. However, easier procedures still need to be established.

* Corresponding author. Tel.: +81-426-765573; fax: +81-426-765570.

E-mail address: ohnonao@ps.toyaku.ac.jp (T. Yadomae)

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Our studies have shown that systemically administered soluble forms of antitumor β-glucans, such as SSG (from *Sclerotinia sclerotiorum* IFO 9395), SPG (from *Schizophyllum commune*) and GRN (from *Grifola frondosa*), gradually disappeared from the bloodstream and deposited in the body, especially in the liver and spleen, over a very long period, i.e., more than a few months, without undergoing significant structural changes [7–9]. Analysis of the fate of these β-glucans is important to the molecular understanding of the immunomodulators. We have also demonstrated that intravenously administered *Candida* spp. distributed in the liver and the spleen, and the β-(1→3)-D-glucan moiety was deposited in these organs for quite a long period of time, as assessed by the limulus G-test [10]. Degradation and excretion of other components, such as proteins, nucleic acids, lipids, and carbohydrates, proceeded within a couple of days as assessed by metabolic labeling methodologies [10]. The deposited insoluble cell-wall β-(1→3)-D-glucan has been gradually solubilized. This β-glucan deposition and slow metabolism would be due to the absence of β-glucan hydrolase in the host. The only mechanism to degrade β-glucan that can be reasonably postulated is oxidative degradation by phagocytes, which utilize species such as O_2^-, H_2O_2, and hypochlorous acid (HOCl) [11]. The cell-wall mannan, present as a constituent of mannoproteins, is also a major cell-wall polysaccharide; however, it is highly water soluble and thus would be metabolized faster than the β-glucan components. These facts strongly suggested that a β-(1→3)-D-glucan might have the property to strongly resist oxidative degradation, in comparison with other cellular components.

Hypochlorous acid is a strong oxidant derived from H_2O_2 with the aid of leukocyte-derived peroxidases. Two main peroxidases exist in phagocytes: (1) myeloperoxidase (MPO) in neutrophils and monocytes, and (2) eosinophil peroxidase (EPO). Macrophages may take advantage of the MPO released from other cells at inflammatory sites to convert H_2O_2 to HOCl [11].

The purpose of this study is to establish an easier method to solubilize the yeast cell-wall β-(1→3)-D-glucan and to apply the product for the limulus G-test. As described above, HOCl oxidation would be advantageous for selective solubilization of the cell-wall β-(1→3)-D-glucan. In this paper, we have developed a method to solubilize the cell-wall β-glucan, especially β-(1→3)-D-glucan, using NaClO and dimethyl sulfoxide (Me_2SO). This procedure allowed us to prepare yeast cell-wall β-glucan in very high yield and in a shorter period of time.

2. Materials and methods

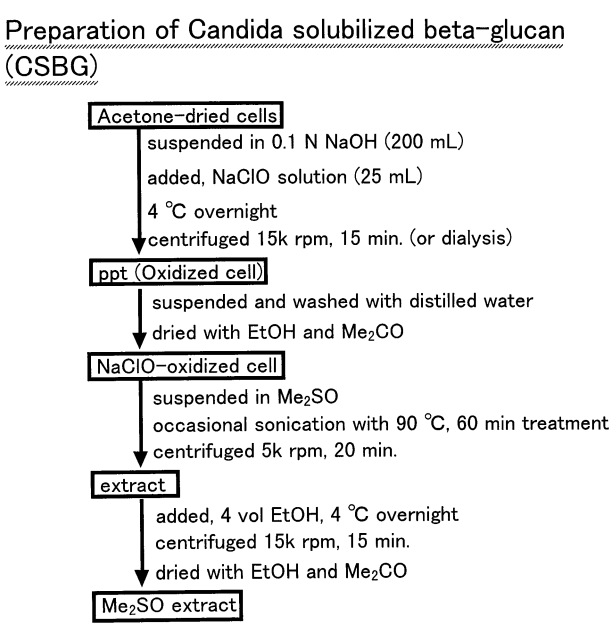
Materials.—All strains of *Candida albicans*, *Candida parapsilosis*, and *Saccharomyces cerevisiae* were purchased from the Institute for Fermentation, Osaka, maintained on Sabouraud agar (Difco, USA) at 25 °C and transferred once every 3 months. Sodium hypochlorite solution and sodium hydroxide were purchased from Wako Pure Chemical Industries, Ltd. The limulus G-test (Fungitec G test MK) and zymolyase (20T and 100T) were from Seikagaku Corp. (Tokyo), and distilled water (DIW) was from Otsuka Co., Ltd. (Tokyo). The visking tube (MW cutoff, 1000) was from Spectrum Medical Industries, Inc.

Media.—C-limiting medium originally described by Shepherd and Sullivan [12] was used to grow all strains of yeast unless otherwise stated. C-limiting medium contained the following (per L): sucrose, 10 g; $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 2 g; $CaCl_2{\cdot}2H_2O$, 0.05 g; $MgSO_4{\cdot}7H_2O$, 0.05 g; $ZnSO_4{\cdot}7H_2O$, 1 mg; $CuSO_4{\cdot}5H_2O$, 1 mg; $FeSO_4{\cdot}7H_2O$, 0.01 g; biotin, 25 μg; final pH 5.2. Five liters of media were placed in a glass jar of a microferm fermentor (New Branswick) and cultured at 27 °C with 5 L/min of aeration and with stirring at 400 rpm.

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&W Scientific, Inc., CA, 30 m × 0.262 mm, liquid

phase; DB-225, 0.25 μM) 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).

Measurement of β-(1→3)-D-glucan by the Fungitec G test MK.—Activation of factor *G* (limulus reactivity) by β-(1→3)-D-glucans was measured by a chromogenic method using a β-(1→3)-D-glucan-specific reagent (Fungitec G-test MK, Seikagaku Corp., Tokyo), which eliminates factor *C* [13,14]. Each β-(1→3)-D-glucan was dissolved in 0.3 M NaOH (1 mg/mL) and diluted with 0.01 M NaOH. Usually, enough dilution was done by 0.01 M NaOH addition so that the sample solution was used directly for the limulus reaction without neutralization. Dilute NaOH was confirmed to be usable for the limulus reaction because of the high buffer action of the reagent. Reactions were performed in flat-bottomed 96-well Toxipet plates 96F (Seikagaku Corp.) as follows. Samples (50 μL) were placed in the wells, and the Fungitec G test MK reagent (50 μL) was added to each well. The plate was incubated at 37 °C, and during incubation the absorbance at 405 nm (reference: 492 nm) was measured kinetically using a microplate reader (Wellreader SK601, Seikagaku Corp.). Disposable plastic materials for tissue culture or clinical use were employed, and all glassware was sterilized at 260 °C for 3 h. All operations were performed in triplicate under aseptic conditions.



Scheme 1.

Preparation of cell-wall β-glucan by NaClO oxidation, followed by Me_2SO extraction.—Preparation of the NaClO-oxidized yeast was followed by the procedure used in a previous paper [15] (Scheme 1). Briefly, yeast cells (2 g) were suspended in 200 mL of 0.1 M NaOH and oxidized with appropriate volume of NaClO solution for 1 day at 4 °C. After the reaction was completed, the reaction mixture was dialyzed extensively with distilled water to collect the nondialyzable and insoluble fraction, or the reaction product was directly centrifuged to collect the insoluble fraction. Insoluble fractions were dried by washing with ethanol and acetone. Each dried fraction was suspended in Me_2SO and extracted with occasional sonication and boiling. After centrifugation to remove any insoluble fraction, the solubilized part was again precipitated with ethanol and acetone. The resulting material was designated as CSBG (*Candida* spp. β-(1→3)-D-glucan).

Zymolyase digestion of CSBG.—CSBG (20 mg) suspended in 10 mL of acetate buffer (50 mM, pH 6.0) was 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.

To monitor the zymolyase digestivity of the glucan preparation, each glucan (2 mg) suspended in 200 μL of distilled water was mixed with 3 M NaOH (25 μL) and immediately neutralized by adding 3 M HCl (25 μL). Acetate buffer (50 mM, pH 6.0) (2 mL) was added, followed by 200 μg of zymolyase 20T. After overnight incubation at 37 °C, the reaction mixture was boiled for 3 min to inactivate the enzyme. The resulting reaction mixture was applied to a centricut filter having a MW cutoff at 3500 Da. Each filtrate fraction was collected, and the carbohydrate content was measured by the phenol–sulfuric acid method.

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 described previously [16].

Preparation of peripheral blood mononuclear cells.—Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors. The blood cells were centrifuged (3000 rpm at 4 °C for 20 min) to obtain a leukocyte-rich buffy coat. The cells were then suspended in 8 mL of phosphate-buffered saline (PBS) and mixed with an equal volume of HISTOPAQUE (density: 1.077; Sigma Chemical Co., USA) in a 15 mL centrifuge tube (Falcon 352196, Becton Dickinson, Lincoln Park, NJ) and centrifuged at 2500 rpm for 25 min at room temperature, as recommended in the supplier's instruction sheet. After centrifugation, the PBMC fraction layered in the middle of the suspension was washed three times with PBS. The quality of the PBMC fraction was monitored by microscopy with Diff-Quick stain. PBMC (2×10^6 mL/well) were cultured in RPMI 1640 containing 10% autologous serum in a polypropyrene tube at 37 °C in a humidified 5% CO_2 incubator.

ELISA for cytokine determination.—Interleukin 8 concentration was determined by using an indirect enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Anti human IL-8 and recombinant IL-8 were purchased from Pharmingen, San Diego, CA, USA.

NMR analysis.—Solubilized fractions and authentic materials were dissolved in D_2O or Me_2SO-d_6, and the ^1H and ^{13}C NMR spectra were determined at 55 or 70 °C. Bruker DPX400 and DRX500 instruments equipped with 'XWIN-NMR' software were used.

MALDIMS.—MALDI-TOF-MS analysis was conducted using a Perceptive Biosystems Voyager DP-RD equipped with delayed extraction and a nitrogen laser. All analyses were conducted using 2,5-dihydrobenzoic acid (Tokyo Kasei Co.) as matrix.

3. Results

Preparation of cell-wall β-glucan by NaClO oxidation, followed by Me_2SO extraction.—Acetone-dried yeast cells were suspended in 0.1 M NaOH and oxidized with NaClO solution for 1 day at 4 °C. After the reaction was completed, the reaction mixture was centrifuged to collect a particulate substance, and the residue was washed extensively with water and dried with ethanol and acetone. During establishment of the preparation procedure, dialysis of the whole reaction mixture was performed instead of centrifugation. Components and yields were comparable in either method; thus we chose the centrifugation because of its ease. The resulting particulate mass was suspended in Me_2SO and dissolved by occasional boiling and sonication. After centrifugation, the extract was precipitated with ethanol and dried with acetone. The resulting fraction was designated as CSBG. We used several strains of yeast which include *C. albicans*, *C. parapsilosis*, and *S. cerevisiae*. Culture and extracting conditions, yield, and components are summarized in Table 1. Component sugars were determined after trifluoroacetic acid hydrolysis. It is apparent that by using this procedure, almost all of the cell-wall mannan moiety was solubilized during NaClO oxidation. The content of mannan was slightly higher in the glucan fractions prepared by dialysis. The highest yield of CSBG was 89% from the NaClO-oxidized yeast. The yield was relatively high, but varied depending on the strain and/or the concentration of NaClO used. Table 1 includes all of the experimental trials, thus including errors and lower yields that occurred during handling of the materials.

Limulus reactivities to activate factor G of CSBG from various strains are compared and summarized in Fig. 1. CSBGs were dissolved in 0.3 M NaOH and diluted in order to fit the conformation to the single helix conformer, which is the conformer responsible for the limulus G-test. All of the glucan preparations show activity as low as 1×10^{-11} g/mL. The activity was comparable with a temporally standard material, pachyman, which was combined with the Fungitec G test MK. These facts strongly suggested that CSBG could be a candidate standard molecule for the limulus G-test.

Table 1
Yield and properties of CSBG

Name culture condition	NaClO (mL) [a]	Yield of NaClO (mg) [b]	Treatment method[c]	Yield of Me_2SO (mg) [d]	Yield of Me_2SO (%, overall) [e]	Molar ratio G/M [f]	% of zymolyase–sensitive part [g]
C. albicans 1385	50 (×3) [h]	153	dialysis	70	5.4	100/11.36	69
	50 (×5)	303	dialysis	21	3.2	100/4.26	81
	25	417	dialysis	52	10.8	100/3.61	
(37 °C) [i]	25	400	dialysis	60	12.0	100/4.59	64
C. albicans 1594	25	370	dialysis	78	14.4		
C. albicans 1385	50 (×5) [j]	221	centrifuge	89	9.8	100/2.31	89
	25 [k]	346	centrifuge	71	12.3	100/2.92	62
C. albicans 1594	25	530	centrifuge	16	4.2	100/4.49	58
C. albicans 0583	25	434	centrifuge	49	10.6	100/0.88	83
C. parapsilosis 0640 (100 rpm) [l]	25	530	centrifuge	39	10.3	100/2.86	62
S. cerevisiae [m]	25	233	centrifuge	32	3.7	100/0.83	75
C. parapsilosis 0640 (YPG) [n]	25	397	centrifuge	71	14.1	100/2.22	
C. parapsilosis 0708	25	454	centrifuge	69	15.7	100/1.84	
C. parapsilosis 1068	25	387	centrifuge	42	8.1	100/1.39	
			treatment				

[a] In 200 mL NaOH (final dilution in parentheses).
[b] From 2 g acetone-dried cell.
[c] After NaClO degradation.
[d] From 100 mg NaClO-treated cell.
[e] From 2 g acetone-dried cell.
[f] Glucose/mannose ratio of Me_2SO extracts by GLC: alditol acetate method.
[g] MW < 3500.
[h] In 100 mL 0.1 M NaOH.
[i] Cultured at 37 °C.
[j] Ca1385b.
[k] Ca1385a.
[l] Spinning rate.
[m] Commercially available baker's yeast.
[n] Cultured in YPG medium.

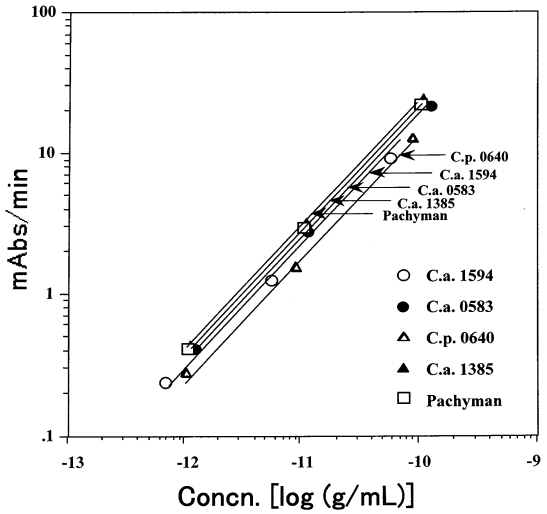


Fig. 1. Limulus activity of CSBG from various strains of *Candida*.

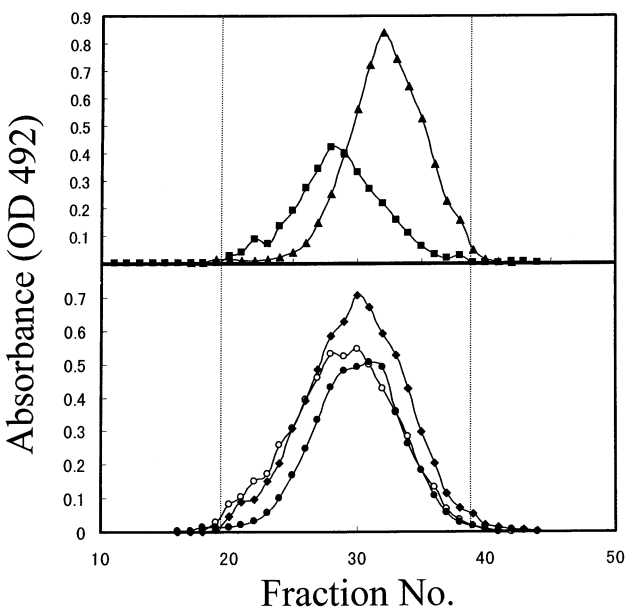


Fig. 2. Gel-filtration chromatography of CSBG from a column of Toyopearl HW65. A column (1 × 40 cm) of Toyopearl HW65 was equilibrated with 0.3 M NaOH. CSBG (2 mg) was applied and each 0.5 mL fraction was collected. An aliquot (200 μL) of each fraction was applied for phenol–sulfuric acid analysis. (upper) ■; *C. albicans* 1385a, ▲; *C. albicans* 1385b, (lower) ●; *C. albicans* 1594 □; *C. albicans* 0583 ◆; *C. parapsilosis* 0708.

Structure of solubilized cell-wall glucan.— The physicochemical properties of CSBG were determined by elemental analysis, gel filtration, NMR spectroscopy, and by a β-(1→3)-glucanase digestion. In this study, CSBG obtained from *C. albicans* IFO1385 treated with 25/200 NaClO solution (*C. albicans* 1385a) was used unless otherwise stated. Elemental analysis showed CHN as C, 40.71; H, 6.76; and N, 0.20%, respectively. The values were close to the theoretical values of pure polysaccharide. The results strongly suggest that CSBG is a β-(1→3)-D-glucan free from protein as well as chitin.

The CSBG fraction was soluble in Me_2SO and NaOH, but could scarcely be dissolved in distilled water, even after extensive sonication. However, once dissolved in NaOH, it remained soluble, even after neutralization. Fig. 2 shows the gel-filtration profile of CSBG from Toyopearl HW65 using 0.3 M NaOH. CSBGs from *C. albicans* 1385a (25 mL NaClO/200 mL NaOH), *C. albicans* 1385b (50 mL NaClO/200 mL NaOH), *C. albicans* 1594, *C. albicans* 0583, and *C. parapsilosis* 0708 were used. All of the CSBGs tested gave a main-peak between V_o and V_t of HW65, suggesting an average MW of about 10^6 Da; however, MWs were widely dispersed from high- to low-MW materials. Comparing *C. albicans* 1385a and 1385b, the average MW was apparently reduced by stronger NaClO oxidation. The results strongly suggested that chemical degradation of the main chain by the stronger/prolonged NaClO oxidation had occurred.

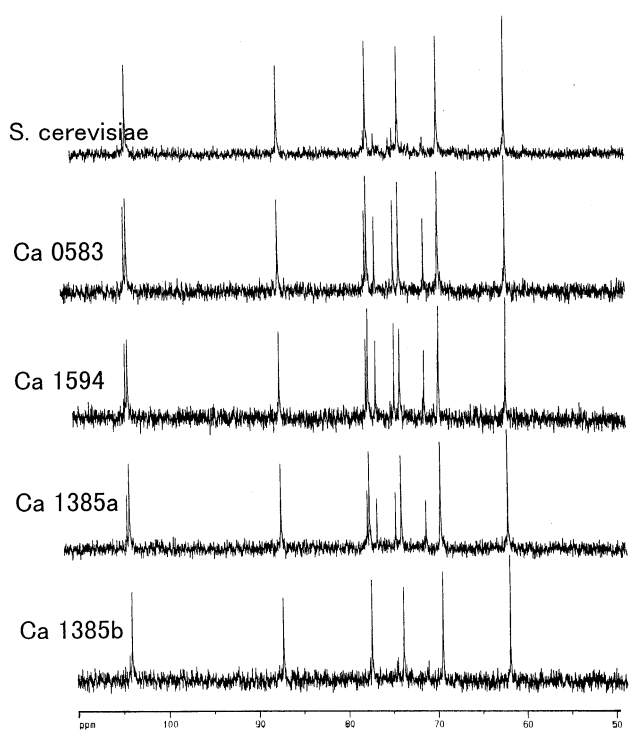


Fig. 3. ^{13}C NMR spectra of CSBGs in Me_2SO-d_6.

Table 2
Assignment of ^{13}C NMR chemical shifts[a]

	C-1	C-2	C-3	C-4	C-5	C-6	
GRN-A	102.8	72.4	86.6	68.3	76.2	60.7	
GRN-B	102.8	72.4	86.2	68.3	76.2	60.7	
GRN-C	102.8	72.4	85.8	68.3	74.5	68.3	terminal
GRN-D	102.8	73.5	76.5	70.0	76.2	60.9	nonreducing end
SSG-I	102.9	72.5	86.2	68.3	76.2	60.7	
SSG-II	102.9	72.5	86.0	68.3	74.5	68.3	terminal
SSG-III	102.9	73.5	76.4	70.0	76.2	60.9	nonreducing end
Islandican	102.9	73.2	76.3	69.8	75.3	68.3	
Curdlan	102.7	72.6	85.9	68.2	76.1	60.7	
Ca1385a	102.8	72.6	86.1	68.3	76.2	60.8	(1→3)
	103.1	73.3	76.4	69.9	75.3	68.3	(1→6)

[a] GRN and SSG are 6-branched β-(1→3)-D-glucans. A to D and I to III represent as follows: –[A–B–C(D)–]*n*, –[I–II(III)–]*n*.

Fig. 3 shows ^{13}C NMR spectra of CSBG from several strains of *C. albicans* and *S. cerevisiae* in Me_2SO-d_6 at 70 °C. *S. cerevisiae* and *C. albicans* 1385b (50 mL NaClO/200 mL NaOH) shows six strong signals attributable to β-(1→3)-D-glucan. These signals were comparable with those of a commercially available β-(1→3)-D-glucan, curdlan. *C. albicans* 0583, *C. albicans* 1594, and *C. albicans* 1385a (25/200) all show minor signals in addition to signals attributable to β-(1→3)-D-glucan. The intensities of these minor signals were different with each strain. The latter signals were also attributable to the β-(1→6)-D-glucan, islandican. In addition, the chemical shifts were not consistent with a highly β-(1→6)-branched β-(1→3)-glucan, such as GRN [17]. GRN contained mono-β-glucosyl substitution at C-6. These chemical shifts also strongly suggested the presence of a linear β-(1→6)-D-glucan part in CSBG. Assignment of the ^{13}C signals is summarized in Table 2. To determine the ratio of (1→3)- and (1→6)-linked glucan moieties, ^1H NMR spectra were measured and the anomeric signals were compared. Calculated from the integral value, the percentage of the (1→6)-linkage was the lowest in *C. albicans* 1385b and highest in *C. albicans* 1594 (Table 3). The ratio of (1→3)- and (1→6)-glucan was also further estimated by zymolyase, endo-β-(1→3)-D-glucanase, digestion (Table 1). Each digest was separated into high- and low-MW fractions by centrifugal filtration. The percentage of the low-MW fraction was determined by the phenol–sulfuric acid method. As shown in Table 1, 60–80% of CSBG was degraded to the low-MW glucans. The ratios so determined were consistent with the results of ^1H NMR spectroscopy.

The zymolyase-resistant part was further analysed after separation by Toyopearl HW40 chromatography (Fig. 4) to mono- and oligo-, and polysaccharide fractions. The void fraction of the HW40 column was further separated by HW50 and HW55 chromatography (data not shown) and analysed by ^1H NMR spectroscopy (Fig. 5) and also by matrix-assisted laser-desorption–time-of-flight mass spectrometry (MALDI-TOF-MS) (Fig. 6). Almost all of the signals in Fig. 5 were attributable to the β-(1→6)-glucosyl unit. The DP of the polysaccharide fraction was from 10 to over 50. These results strongly suggest that the zymolyase-resistant part was a β-(1→6)-D-glucan moiety having various degrees of polymerization.

Table 3
Ratios of (1→3):(1→6) linkages calculated from ^1H NMR spectra

	1,3:1,6
Ca 1385b	1:0.191
Ca 1385a	1:0.438
Ca 0583	1:0.602
Ca 1594	1:0.692
S. cerevisiae	1:0.284

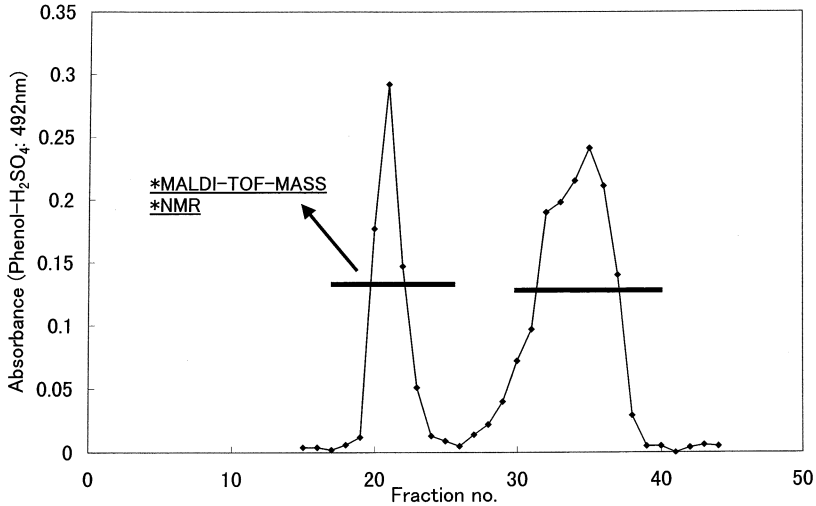


Fig. 4. Gel-filtration chromatography of the zymolyase digest of CSBG from a column of Toyopearl HW40. A column (1 × 40 cm) of Toyopearl HW40 was equilibrated with distilled water. Zymolyase-digested CSBG (20 mg) was applied and each 0.5 mL fraction was collected. An aliquot (25 μL) of each fraction was applied for phenol–sulfuric acid analysis.

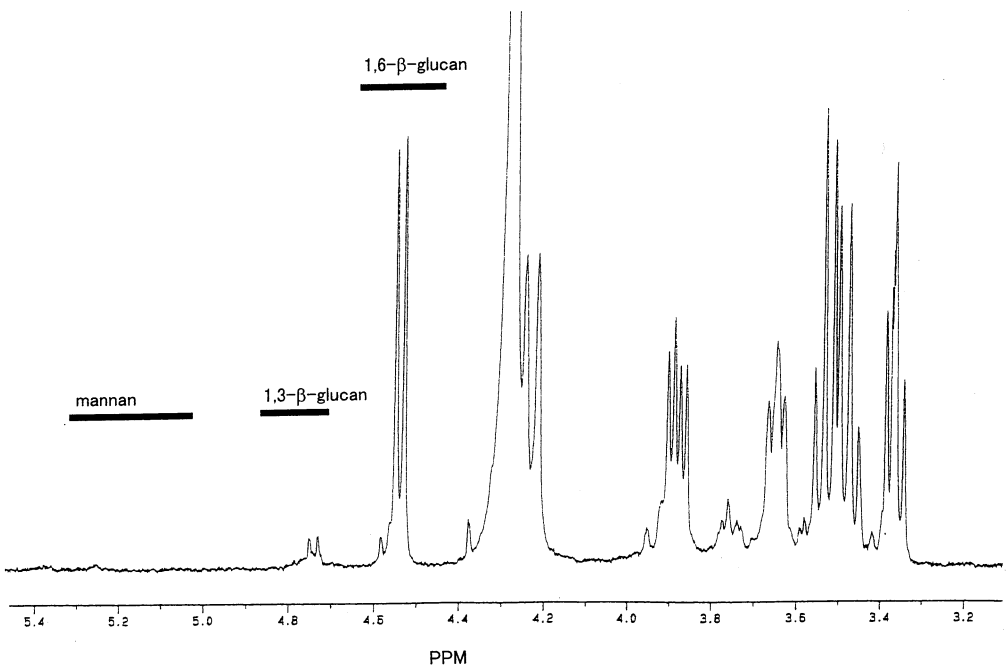


Fig. 5. ^{1}H NMR spectrum of the zymolyase-resistant part of CSBG in D_2O.

Cell-wall glucans are mainly composed of β-(1→3)-D- and β-(1→6)-D-glucans. To estimate the selectivity of the reaction, each solubilized fraction was also analysed by ^{1}H NMR spectroscopy. Fig. 7 shows a spectrum of the NaClO-solubilized fraction of *C. albicans* 1385 in D_2O at 55 °C. The signals attributable to the mannan and the β-(1→6)-D-glucan were those mainly observed, while those of the β-(1→3)-D-glucan were barely visible. These facts suggested that NaClO oxidation released the mannan and the β-(1→6)-D-glucan moieties rather selectively, and, following the Me_2SO treatment, β-(1→3)-D-glucan was extracted. The differences of the ratios of β-(1→6)-D-glucan among several CSBG preparations might be related to the conditions of NaClO oxidation and the architecture of the cell wall. The proposed structure of CSBG is shown in Fig. 8.

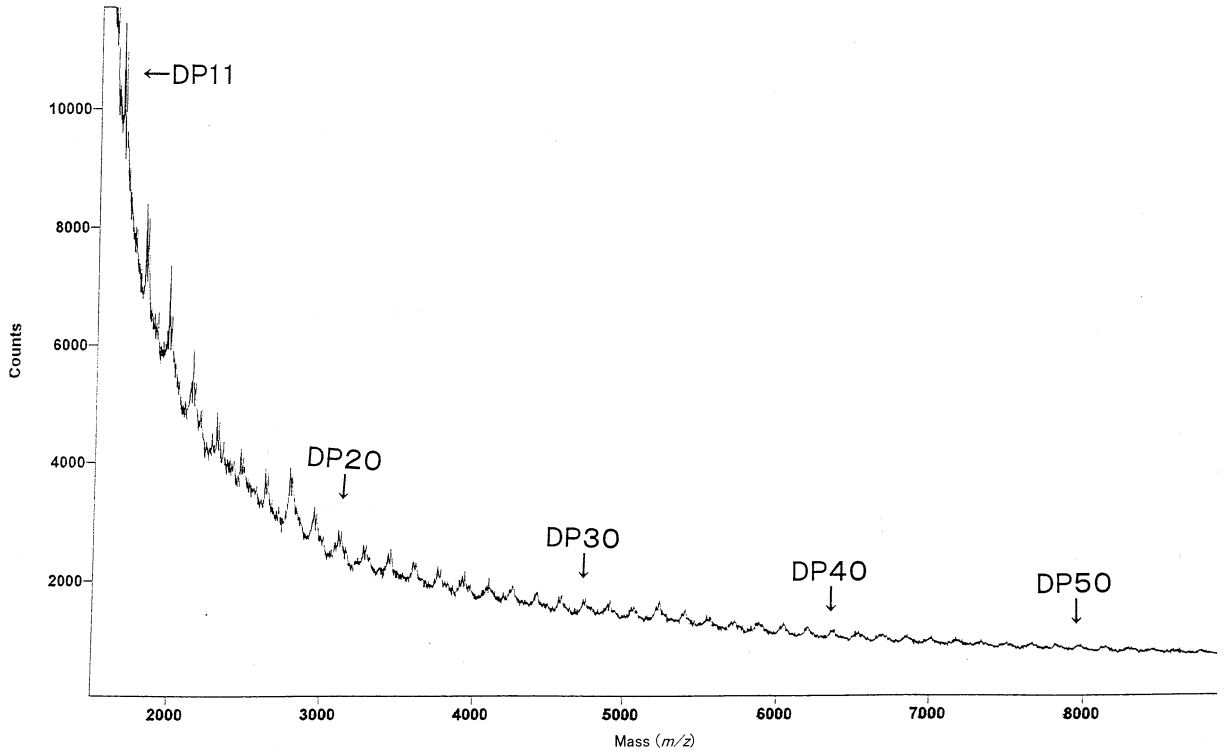


Fig. 6. MALDI-TOF-MS spectrum of the zymolyase-resistant part of CSBG. Conditions of measurement were as follows: method, LDE 1003; mode, linear; accerelating voltage, 20,000; grid voltage, 91.0%; guide wire voltage, 0.10%; delay, 125 ON; number of scans, 256; low mass gate, 1500; Savitsky–Golay order, 2; points, 2. DP indicates degree of polymerization.

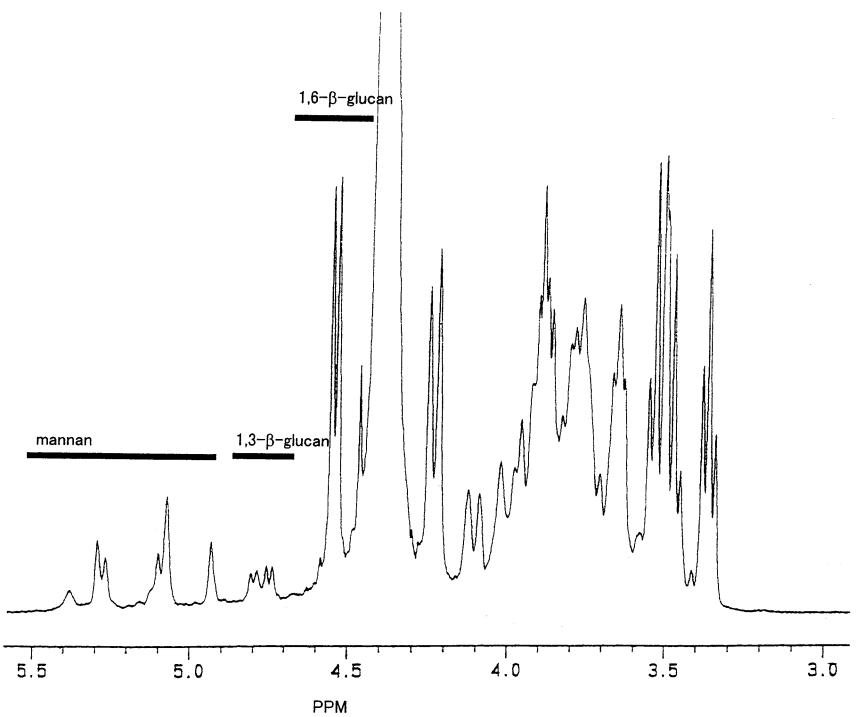


Fig. 7. ^{1}H NMR spectrum of the solubilized fraction of *C. albicans* IFO1385 by NaClO in D_2O.

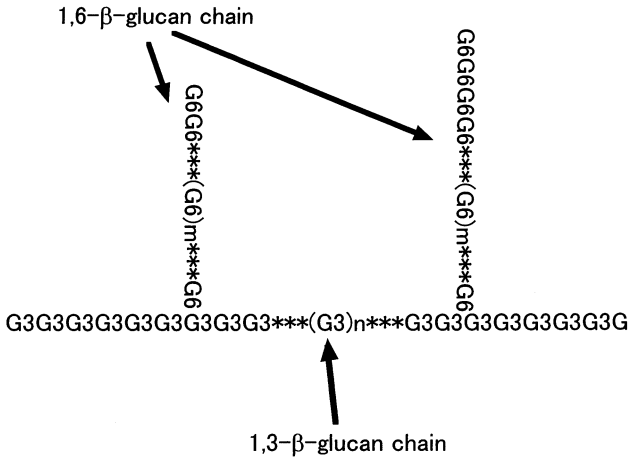


Fig. 8. Proposed structure of CSBG (number of repeats $= m, n$; variable).

Table 4
Antitumor activity of CSBG against murine Sarcoma 180 (solid)

Treatment	Dose	CR[a]	Mean ±S.D.	Inhibition (%)
Control, saline		0	5.29 ± 1.72	
SSG	250 μg × 3	8	0.76 ± 1.64	85.72[b]
SPG	100 μg × 3	2	0.93 ± 1.89	82.40[b]
CSBG (Ca1385/25)	100 μg × 3	5	1.05 ± 2.90	80.09[b]

[a] CR = complete regression, $N = 10$–11.
[b] $P < 0.001$.

Biological activities of CSBG.—β-(1 → 3)-D-Glucan usually shows immunopharmacological and immunotoxicological activities, and it is also the substance responsible for the limulus factor *G*-mediated pathway. We tested several activities such as the antitumor activity against a murine solid tumor (sarcoma 80, Table 4) and interleukin 8 production in human leukocytes (Fig. 9). CSBGs were dissolved in 0.3 M NaOH and neutralized; thus the conformations to be studied were mainly those of the single helix conformer. CSBG shows activity against murine solid tumors comparable with the clinically used antitumor glucan, SPG [18] and a highly branched β-(1 → 3)-D-glucan, SSG [19]. CSBGs from *C. albicans* 1385 and *C. parapsilosis* 0640 also stimulated the IL-8 productivity of human peripheral blood leukocytes. These facts strongly suggest that CSBG would be a candidate as an immunomodulating substance.

4. Discussion

We established a convenient, two-step, procedure to solubilize the yeast cell-wall β-(1 → 3)-D-glucan by combined use of NaClO oxidation and Me_2SO extraction. This method was applied to several strains of *Candida* and *Saccharomyces*. The structures of all the products were essentially confirmed as β-(1 → 3)-D-glucan, but covalently bound with various amounts of the β-(1 → 6)-D-glucan moiety. The proportion of the β-(1 → 6)-D-glucan moiety was lower in examples submitted to harsher NaClO oxidation. The molecular weight of the β-(1 → 6)-D-glucan moiety was widely distributed as assessed by gel-filtration chromatography and MALDI-TOF-MS analyses, suggesting that the β-(1 → 6)-D-glucan moiety was partially degraded during oxidation. Recently, Klis and co-workers extensively analysed the architecture of yeast cell-wall and proposed a linkage between β-glucans, chitin, and mannan [20–22]. The results presented in this paper well support their study.

A traditional method to prepare cell-wall β-(1 → 3)-D-glucan was repeated extractions with acid and base. This process is not convenient because of the many steps involved. Furthermore, the yield of the glucan is usually not dependable, owing to the presence of many impurities. The resulting glucan was usually again insoluble in water. Williams and co-workers have prepared sulfate and phosphate derivatives to obtain soluble β-glucan preparations [5,6]. The product prepared in this paper became water soluble once it dissolved in sodium hydroxide solution and remained dissolved, even after subsequent neutralization. Basically the method involves two steps of NaClO oxidation and Me_2SO extraction. Thus, this method has the advantage of being a rather simple means of obtaining water-soluble, yeast cell-wall β-glucan. The NaClO oxidation step is essential for this method, because β-glucan could be scarcely extracted by Me_2SO without oxidation (data

not shown). The selectivity of NaClO oxidation for preparing β-glucan has not attracted attention, but now it is apparent that the oxidation product was free from protein, nucleic acid, lipid, as well as mannan, and is thus useful to prepare yeast β-glucan, especially the water-soluble form. Controlling oxidation might be important because the molecular weight and content of β-(1→6)-D-glucan part are relatively sensitive to the strength of the oxidation. Compared with Williams' methods we did not selectively add charged (anionic) groups to the molecule. The content of anionic groups in CSBG from NaClO oxidation would be low enough because these residues could not be detected by NMR spectroscopy. We did not precisely examine the content and structure of anionic residues in the product, but a part of the zymolyase-resistant moiety of CSBG was bound to DEAE-Sephadex A25, suggesting the non-specific incorporation of some, but not many anionic groups on CSBG (data not shown).

An additional advantage of NaClO oxidation might be the disruption of the permeability barrier in the cell wall. Yeast cell walls are packed with various proteins, thus producing a barrier for transferring various materials. We have applied metaperiodate oxidation to yeast to degrade the β-(1→6)-glucan part, however, the reaction could not proceed as we thought it might, and the majority of product was the β-(1→6) part [23]. However, after NaClO oxidation, metaperiodate oxidation proceeded as per design and oxidized all of the β-(1→6)-glucan part from the cell-wall (manuscript in preparation).

Candida spp. is a medically important fungi which induces disseminated candidiasis and candidemia in hospitalized immunocompromised patients. In a series of experiments we have analysed the fate of cell-wall β-glucan deposited in organs. β-Glucans were detected in these mice for at least for 6 months by the β-glucan specific assay. During this period, the insoluble cell-wall β-glucan was gradually solubilized in these organs, probably by oxidative stress of macrophages. It was supported by the fact that a malfunctioning macrophage shows a reduced rate of degradation [24]. The major concept involved in this study is to add to the evidence that the host defense system actually uses oxidative degradation to remove the fungal cell-wall component from organs. Analysis of the NaClO oxidation products supported rather specific resistance of the cell-

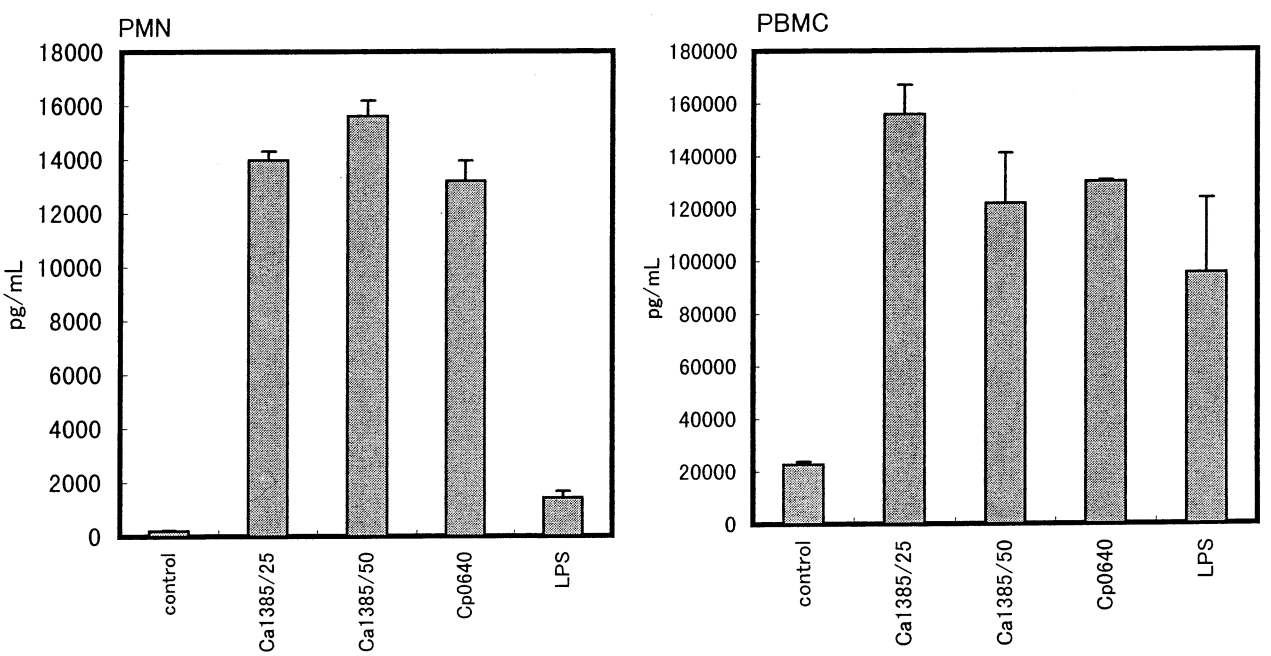


Fig. 9. IL-8 synthesis by CSBG of polymorphonuclear leukocyte (left) and peripheral blood mononuclear leukocyte (right). PMN and PBMC were stimulated for 24 h by 50 μg/mL of CSBG. Supernatant was collected and IL-8 concentration was measured by ELISA as shown in Section 2.

wall β-(1→3)-D-glucan to metabolic oxidation. Thus, the glucans prepared in this paper might be of a similar structure to the glucan deposited in organs of the patient carrying a deep-seated mycosis [25,26]. Immunotoxicological and immunopharmacological activities of the deposited glucan might be closely related to the in vivo pathophysiologic significance and are also strongly associated with symptoms and prognoses of invasive fungal infection. We have observed the immunostimulating activity of CSBG, such as the antitumor activity and the stimulation of interleukin 8 biosynthesis. Analysis of other immunostimulating activities of CSBG is now on progress. Clarification of a whole set of biological activities would be important for the molecular understanding of deep-seated mycosis.

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References

[1] R.J. Yu, C.T. Bishop, F.P. Cooper, F. Blank, H.F. Hasenclever, *Can. J. Chem.*, 45 (1967) 2264–2267.
[2] G.H. Fleet, D.J. Manners, *J. Gen. Microbiol.*, 94 (1976) 180–192.
[3] N. Shibata, H. Kobayashi, M. Tojo, S. Suzuk, *Arch. Biochem. Biophys.*, 251 (1986) 697–708.
[4] N. Shibata, K. Mizugami, S. Suzuki, *Microbiol. Immunol.*, 28 (1984) 1283–1292.
[5] A. Muller, H. Ensley, H. Pretus, R. McNamee, E. Jones, E. McLaughlin, W. Chandley, W. Browder, D. Lowman, D. Williams, *Carbohydr. Res.*, 299 (1997) 203–208.
[6] D.L. Williams, H.A. Pretus, R.B. McNamee, E.L. Jones, H.E. Ensley, I.L. Browder, *Carbohydr. Res.*, 235 (1992) 247–257.
[7] N.N. Miura, N. Ohno, J. Aketagawa, H. Tamura, S. Tanaka, T. Yadomae, *FEMS Immunol. Med. Microb.*, 13 (1996) 51–57.
[8] T. Miura, N. Ohno, M. Suda, N.N. Miura, S. Shimada, T. Yadomae, *Biol. Pharm. Bull.*, 18 (1995) 1797–1801.
[9] M. Suda, N. Ohno, Y. Adachi, T. Yadomae, *Biol. Pharm. Bull.*, 17 (1994) 131–135.
[10] N.N. Miura, T. Miura, N. Ohno, Y. Adachi, M. Watanabe, H. Tamura, S. Tanaka, and T.Yadomae, *FEMS Immunol. Med. Micro.*, 21 (1998) 123–129.
[11] B.A.M. Walker and J.C. Fantone, The inflammatory response, in: L.H. Signal, Y. Ron (Eds.), *Immunology and Inflammation*, McGraw-Hill, USA, 1994, pp. 359–386.
[12] M.G. Shepherd, P.A. Sullivan, *J. Gen. Microbiol.*, 93 (1976) 361–370.
[13] S. Tanaka, J. Aketagawa, S. Takahashi, Y. Shibata, Y. Tsumuraya, Y. Hashimoto, *Carbohydr. Res.*, 218 (1991) 167–174.
[14] T. Obayashi, M. Yoshida, H. Tamura, J. Aketagawa, S. Tanaka, T. Kawai, *J. Med. Vet. Mycol.*, 30 (1992) 275–280.
[15] N.N. Miura, N. Ohno, Y. Adachi, T. Yadomae, *Chem. Pharm. Bull.*, 44 (1996) 2137–2141.
[16] K. Iino, N. Ohno, I. Suzuki, T. Miyazaki, T. Yadomae, S. Oikawa, K. Sato, *Carbohydr. Res.*, 141 (1985) 111–119.
[17] N. Ohno, I. Suzuki, T. Yadomae, *Chem. Pharm. Bull.*, 34 (1986) 1362–1365.
[18] N. Ohno, N.N. Miura, N. Chiba, Y. Adachi, T. Yadomae, *Biol. Pharm. Bull.*, 18 (1995) 1242–1247.
[19] N. Ohno, K. Kurachi, T. Yadomae, *J. Pharmacobio-Dyn.*, 10 (1987) 478–486.
[20] J.C. Kapteyn, R.C. Montijn, G.J. Dijkgraaf, H. Van den Ende, F.M. Klis, *J. Bacteriol.*, 177 (1995) 3788–3792.
[21] R.P. Hartland, C.A. Vermeulen, F.M. Klis, J.H. Sietsma, J.G. Wessels, *Yeast*, 10 (1994) 1591–1599.
[22] R. Kolla, B.B. Reinhold, E.P. Kova, H.J.C. Yehi, G. Ashwell, J. Drgonova, J.C. Kapteyn, F.M. Klis, E. Cabib, *J. Biol. Chem.*, 272 (1997) 17762–17775.
[23] N. Ohno, T. Miura, N.N. Miura, N. Chiba, M. Uchiyama, Y. Adachi, and T. Yadomae, *Zentralbl. Bakteriol.*, 1357 (1998) in press.
[24] M. Suda, N. Ohno, T. Hashimoto, K. Koizumi, Y. Adachi, T. Yadomae, *FEMS Immunol. Med. Microb.*, 15 (1996) 93–100.
[25] M. Yoshida, T. Obayashi, A. Iwama, M. Ito, S. Tsunoda, T. Suzuki, K. Muroi, M. Ohta, S. Sakamoto, Y. Miura, *J. Med. Vet. Mycol.*, 35 (1997) 371–374.
[26] T. Obayashi, M. Yoshida, T. Mori, H. Goto, A. Yasuoka, H. Iwasaki, H. Teshima, S. Kohno, A. Horiuchi, A. Ito, H. Yamaguchi, K. Shimada, T. Kawai, *Lancet*, 345 (1995) 17–20.