ELSEVIER

FEMS Immunology and Medical Microbiology 24 (1999) 411–420

FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY

Chemical and immunochemical characterization of limulus factor G-activating substance of *Candida* spp.

Michiharu Uchiyama [a], Naohito Ohno [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*

Received 24 March 1999; accepted 27 March 1999

Abstract

The limulus test is a well-established method for the diagnosis of both Gram (−) sepsis and invasive fungal infection. To diagnose deep-seated fungal infections, a (1→3)-β-D-glucan-specific chromogenic kit (Fungitec G test MK) has been developed and applied clinically. It is suggested that the limulus reactive substance was released from the fungi to the blood, however, its chemical properties were not precisely examined in detail because of the limited quantity available. In this study, we used chemically defined liquid medium to culture *Candida* spp. and collected the water soluble fraction, CAWS. The yield of CAWS was circa 100 mg/l, independent of the strain of *Candida*. CAWS reacted with limulus factor G (Fungitec G test MK) at concentrations as low as 100 ng/ml. Limulus factor G reactivity of CAWS was sensitive to (1→3)-β-glucanase, zymolyase and was, at least in part, bound to ConA-agarose. The ConA-bound fraction also reacted with anti-β-glucan antibody. CAWS is mainly composed of mannan and (1→6)-β-glucan, in addition to protein, assessed by ^{1}H-NMR spectroscopy. CAWS also reacted with typing sera of *Candida* spp., specific for cell wall mannan. Chemical, immunochemical and biochemical analyses of CAWS strongly suggested that the limulus factor G-activating substance was a mannan-β-glucan complex, present within the architecture of the yeast cell wall.

Keywords: Fungal infection; Diagnosis; Limulus test; β-Glucan; *Candida*; Cell wall

1. Introduction

Candida spp. are medically important because of its ability to induce a disseminated candidiasis and candidemia in hospitalized immuno-compromized patients [1]. The cell wall of *Candida* is mainly composed of two polysaccharides, mannan and β-glucan [2–7]. At least a part of β-glucan is basically insoluble in H_2O or NaOH. The appearance of β-glucan in the blood is specifically associated with deep-seated fungal infections [8–10]. The limulus test is a well-established method for the diagnosis of both Gram (−) sepsis and invasive fungal infection. To diagnose deep-seated fungal infections, a (1→3)-β-D-glucan-specific chromogenic kit (Fungitec G test MK) has been developed and applied clinically [11–13]. The

* Corresponding author. Tel. +81 (426) 76 5561; Fax: +81 (426) 76 5570; E-mail: ohnonao@ps.toyaku.ac.jp

0928-8244/99/$20.00
PII: S0928-8244(99)00058-9

soluble β-glucan in sera must be a metabolite of *Candida* [14,15]. By batch culture, *Candida* produced β-glucan in the culture supernatant, but the yield was low compared with the content of the cell wall β-glucan which is essentially water insoluble [16]. It is probable that the latter β-glucan is solubilized after partial degradation resulting from interaction with the host defense system [15]. In this context, we have analyzed the fate of *Candida* β-glucan and other constituents by using metabolic ^{3}H-labelling and chemical oxidative degradation using HOCl [17]. We have shown that the majority of the cell wall β-glucan was deposited in organs over quite a long period of time with gradual degradation. Sodium hypochlorite is a good tool to solubilize cell wall β-glucan, but requires drastic chemical treatment [18,19].

To analyze the architecture of limulus reactive β-glucan in blood during *Candida* infection, we used chemically defined medium to grow *Candida* and prepared a water soluble fraction, CAWS, for analysis by chemical and immunochemical methods.

2. Materials and methods

2.1. Materials

All strains of *Candida albicans, Candida parapsilosis* and *Saccharomyces cerevisiae* were purchased from the Institute for Fermentation (Osaka, Japan), maintained on Sabouraud agar (Difco, USA) at 25°C and transferred once every 3 months. Fungitec G test MK and zymolyase were from Seikagaku (Tokyo, Japan) and distilled water (DIW) was from Otsuka (Tokyo, Japan). Zymocel™ was from Alpha-Beta Technology (MA, USA). The visking tube (MW cut-off: 1000) was from Spectrum Medical Industries.

2.2. Media

C-limiting medium, originally described by Shepherd and Sullivan [20], was used to grow all strains of yeast otherwise stated. C-limiting medium contained (per liter) 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 25μg, final pH 5.2. 5 l of media was placed in a glass jar of a microferm fermentor (New Brunswick) and cultured at 27°C with 5 l min^{-1} of aeration and with 400 rpm of stirring.

2.3. Carbohydrate analyses

The carbohydrate content was determined by the phenol-sulfuric acid method. Component sugars were determined by capillary gas-liquid chromatography (Ohkura Riken, Tokyo, Japan) of alditol acetate derivatives after complete hydrolysis by 2 M trifluoroacetic acid. A capillary column of fused silica (J and W Scientific, CA, USA: 30 m × 0.262 mm, liquid phase: DB-225, 0.25 μM) was used at 220°C. The molar ratio of glucose and mannose (G/M) was calculated from the peak area of each component (glucose as 1).

2.4. Measurement of (1→3)-β-D-glucan by the Fungitec G test MK

The activation of factor G (limulus reactivity) by (1→3)-β-D-glucans was measured by a chromogenic method using a (1→3)-β-D-glucan-specific reagent (Fungitec G test MK, Seikagaku, Tokyo, Japan), which eliminates factor C [11–13]. Each (1→3)-β-D-glucan was dissolved in 0.5 N NaOH (1 mg ml^{-1}) and diluted with 0.01 N NaOH. Usually, dilutions were made with 0.01 N NaOH and a sample solution was used directly for the limulus reaction without neutralization. Diluted NaOH was confirmed to be usable for the limulus reaction because of the high buffer action of the reagent. Reactions were performed in a flat-bottomed 96 well Toxipet plate 96F (Seikagaku) 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 SK603, Seikagaku). 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.

2.5. Zymolyase digestion of CAWS

CAWS (20 mg) suspended in 10 ml of acetate buffer (50 mM, pH 6.0) was mixed with 1 mg of zymolyase 100T (Seikagaku). After overnight incubation at 45°C, the reaction mixture was boiled for 3 min to inactivate the enzyme. The resulting solution was diluted and applied to Fungitec G test MK.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Immune plates (Nunc 442404, F96 Maxisorp) were used for all ELISA experiments in this study. Phosphate-buffered saline containing 0.05% Tween 20 (PBST) was used to wash the plates. Carbonate buffer (pH 9.6, 0.1 M) was used to bind antigen or antibody. Plates were blocked by 0.5% bovine serum albumin containing PBST.

2.7. Preparation of anti-β-glucan antibody

Zymocel (200 μg per mouse) was intravenously administered to ICR mice (5 weeks, male, Japan SLC) once a week for 6 weeks. Thereafter, blood was collected and sera prepared. Sera were frozen at −25°C.

2.8. NMR analysis

Solubilized fractions and authentic materials were dissolved in D_2O or DMSO-d_6 and the ^1H- and ^{13}C-NMR spectrum measured at 70°C. Bruker DPX400 instruments equipped with 'XWIN-NMR' software were used.

2.9. Concanavalin A (Con A) agarose chromatography

CAWS (30 mg) was applied to Con A agarose (10 ml: Wako Pure Chemical, Tokyo, Japan) suspended in 50 mM Tris-HCl buffer, pH 7.4 containing 0.15 M NaCl. After collecting the passed through fraction, the column was washed with 0.2 M α-methylmannoside, dissolved in the same buffer. The Con

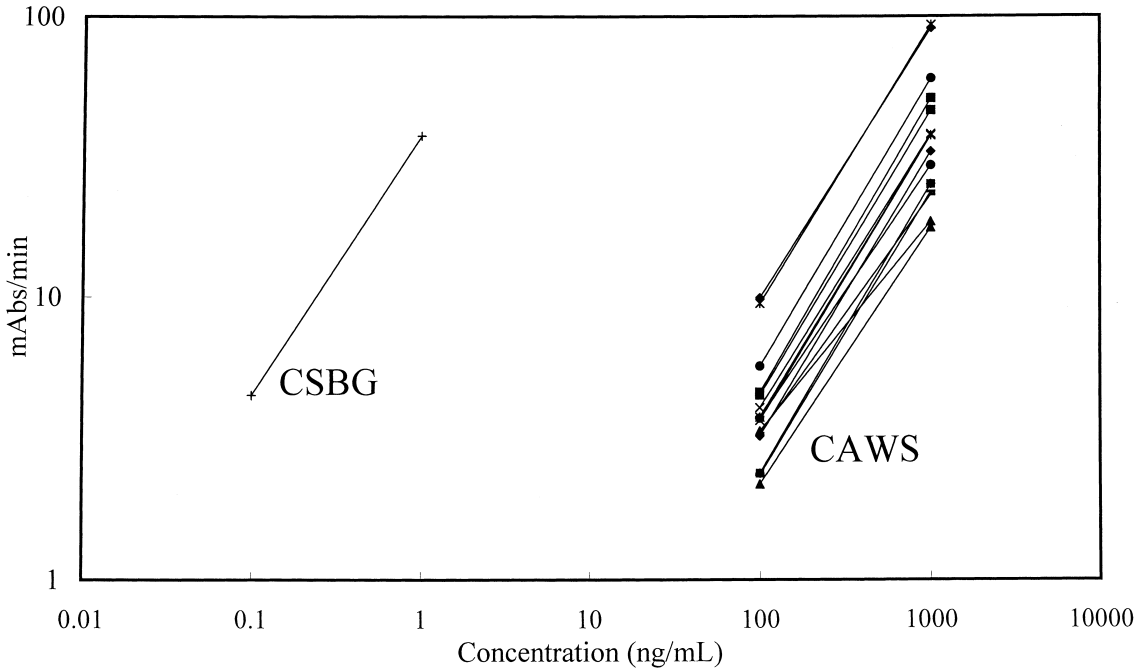

Fig. 1. Limulus factor G reactivity of CAWS by Fungitec G test MK. CAWS preparations listed in Table 1 were dissolved in 0.5 N NaOH and 10-fold dilutions were prepared by distilled water. Fungitec G test MK reactivities of these solutions were determined as described in Section 2. CSBG was used as standard material.

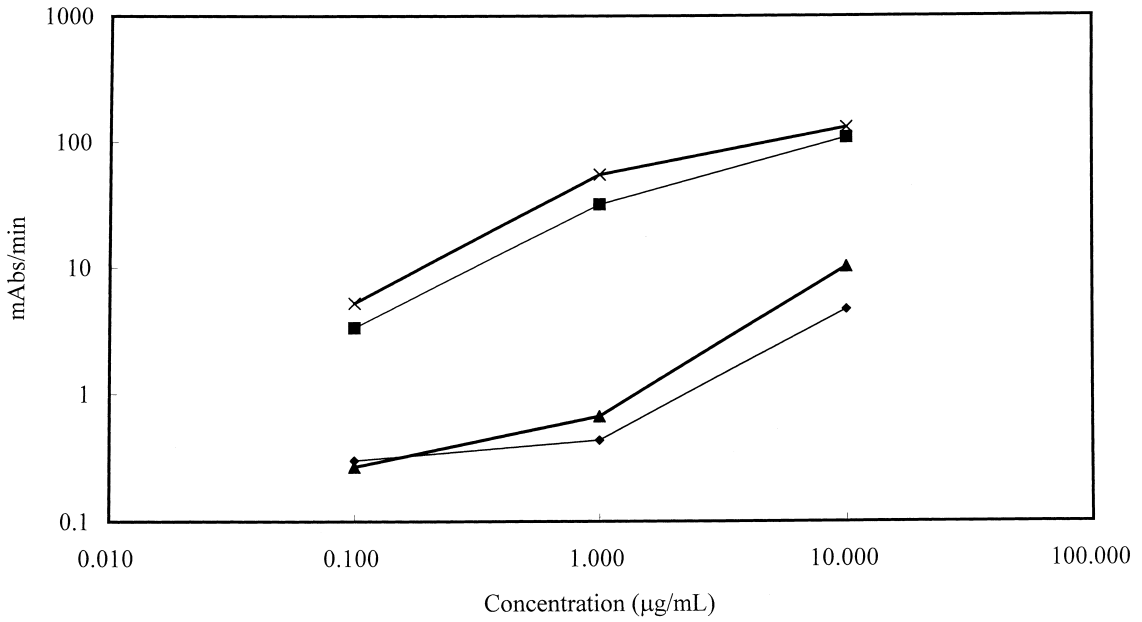

Fig. 2. Sensitivity of CAWS to zymolyase digestion assessed by Fungitec G test MK. CAWS from *C. albicans* 1385 and *C. parapsilosis* 0640 were dissolved in acetate buffer and digested with zymolyase 100T as described in Section 2. The resulting solutions were treated with 0.5 N NaOH, diluted with distilled water and used for Fungitec G test MK as described in the legend of Fig. 1. ×, *C. albicans* 1385; ■, *C. parapsilosis* 0640; ▲, *C. albicans* 1385 after zymolyase digestion; ◆, *C. parapsilosis* 0640 after zymolyase digestion.

A-bound fraction was dialyzed extensively by distilled water.

3. Results

3.1. Preparation of the limulus factor G reactive water soluble fraction, CAWS

The liquid culture medium for *Candida*, used in this paper, was originally described by Shepherd and Sullivan [20]. We applied this medium to grow various strains of *Candida*. All of the strains used in this study grew well in this medium. To collect the water soluble β-glucan fraction, equal volumes of ethanol were added to the whole culture and precipitate collected which included cells and secreted macromolecules. The precipitate was then suspended in an aliquot of distilled water and the solubilized part was collected as CAWS. Strain, culture condition, yield and physicochemical properties are shown in Table 1. All of the strains produced a similar amount of CAWS (∼100 mg l^{-1}). Mannose was the most abundant sugar component in CAWS.

Fig. 1 shows the results of the limulus G test (Fungitec G test MK) of CAWS. All of the samples show a similar reactivity and the minimum concentration to show a positive reaction was approximately 100 ng ml^{-1}. In contrast, the minimum concentration of a purified (1→3)-β-D-glucan preparation of the *Candida* cell wall, CSBG, was 100 pg ml^{-1}. To confirm no cross-reaction, CAWSs, from Ca1385 and Cp0640, were digested by zymolyase 100T, 1,3-β-glucanase and the reactivity was compared. As shown in Fig. 2, zymolyase-digested CAWSs show a significantly lower reactivity suggesting that the limulus factor G reactive substance(s) present in CAWS certainly include a (1→3)-β-D-glucan moiety.

3.2. Immunochemical characterization of CAWS

Sugar component analysis of CAWSs shows mannose as the main neutral sugar (Table 1). It is well-documented that the cell wall mannan is the major immunochemical determinant of *Candida* species.

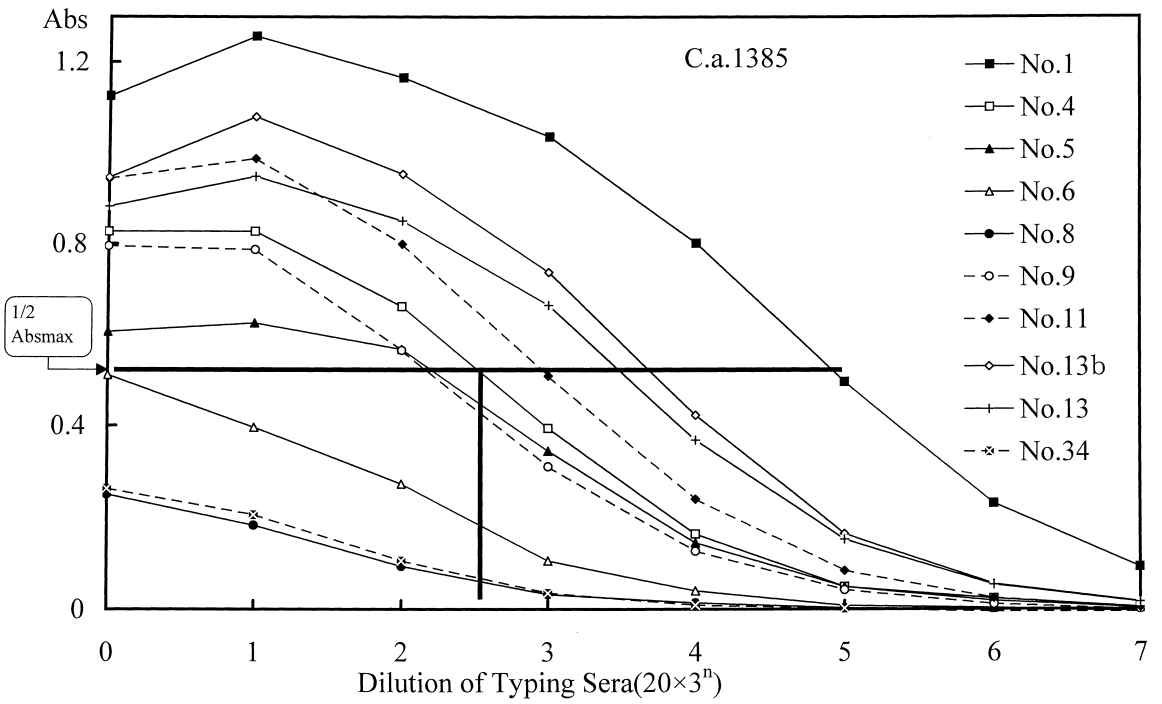

Fig. 3. Representative ELISA reaction of CAWS to anti-*Candida* mannan sera. CAWSs of *C. albicans* 1385 were adsorbed onto an ELISA plate. Anti-mannan typing sera were diluted and added to each well. After extensive washing, bound antibody was determined by anti-rabbit IgG-POX. Experimental details are shown in Section 2.

Immunochemical reactivity of CAWSs to the commercially available *Candida* typing sera (rabbit sera) was examined by ELISA. Fig. 3 shows the representative results of ELISA. In this experiment, CAWS from *C. albicans* IFO1385 was used as the plate-bound antigen and serially diluted sera were added to the well. The plate-bound antibody was detected by a peroxidase-labelled anti-rabbit IgG, followed by TMB reagent for color development. Many of the typing sera strongly reacted with CAWS. Dilution

Table 1
Strain, culture condition, yield and some properties of CAWS

Strain	Culture		Spinning	Yield (g per 5 l)	Carbohydrate%	Protein%	Man/Glc
	Temperature (°C)	Days					
C. albicans 1385	27	2	Yes	0.38 ± 0.07	78 ± 6.6	15 ± 6.3	6.3 ± 1.3
	37	2	Yes	0.32 ± 0.18	80 ± 34	6.1 ± 2.5	11 ± 12
	27	4	Yes	0.42	60.2	15.1	4.1
	27	2	No	0.45	70.5	3.5	5.6
C. albicans 0583	27	2	Yes	0.21	53.4	6.5	3.5
C. albicans 0579			Yes	0.63	76.3	11.3	9.8
C. albicans 1594			No	0.69	59.7	5.4	10.9
C. albicans 1061			Yes	1.36	91.8	15.8	17.1
C. parapsilosis 1068	27	2	Yes	0.46	56.3	7.5	13
C. parapsilosis 0640			Yes	0.21	48.9	9.7	1.1
C. parapsilosis 0708			No	0.26	85.4	4.9	7.3
C. parapsilosis 1396			No	0.1	27.3	5.7	3.9
S. cerevisiae 1136	27	2	Yes	0.74	56.4	33	19.8

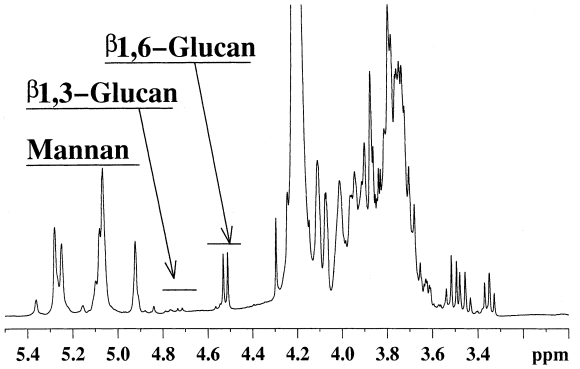

Fig. 4. ^{1}H-NMR spectrum of CAWS. CAWS of *C. albicans* 1385 was dissolved in D_2O and measured by ^{1}H-NMR as described in Section 2.

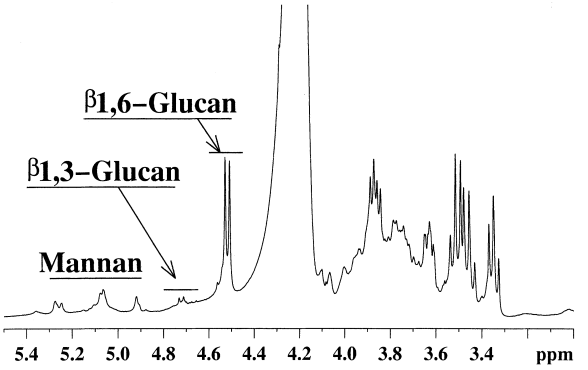

Fig. 5. ^{1}H-NMR spectrum of the zymolyase-digested cell wall. The high molecular weight fraction of zymolyase-digested *C. albicans* 1385 was dissolved in D_2O and measured by ^{1}H-NMR as described in Section 2.

of sera showing 50% of the maximum absorbance was calculated and summarized in Table 2. Similar experiments were done using CAWSs from other strains. All of the CAWSs showed a significant reactivity to typing sera, but the pattern of the reaction was different in each, probably due to the structure of the mannan antigen. Similarity of the structure of the mannan moiety of CAWS and the cell wall mannan was also supported by ^{1}H-NMR spectroscopy as described below.

3.3. NMR spectra of CAWS

The structure of CAWS was analyzed by ^{1}H-NMR spectroscopy. ^{1}H-NMR spectra were measured in D_2O at 70°C and were compared with authentic materials (Fig. 4). As shown above, CAWS was suggested to contain mannan and (1→3)-β-D-glucan moieties. The presence of mannan was supported by the strong signals shown around 5 ppm (4.8–5.4 ppm). On the contrary, signals attributable to the (1→3)-β-D-glucan moiety (around 4.7 ppm) were only slightly detected. The low intensity of the signal supported the low reactivity of CAWS to Fungitec G test MK compared with standard glucan, CSBG. In addition, the spectrum shows strong signals around 4.5 ppm, which could be attributable to the (1→6)-β-D-glucan moiety [21]. These facts strongly suggested that CAWS contains a (1→6)-β-D-glucan moiety in addition to mannan and (1→3)-β-D-glucan moieties and mannan and (1→6)-β-D-glucan moieties are the major polysaccharide chains of CAWS.

In order to confirm the architecture of CAWS, the ^{1}H-NMR spectrum of the solubilized cell wall was

Table 2
Summary of the immunochemical reactivity of CAWS monitored by 50% reactivity

		No. 1	No. 4	No. 5	No. 6	No. 8	No. 9	No. 11	No. 13b	No. 13
C. albicans	0597	4.5	2	2.4			0.3	3.3	3.9	3.7
	0583	4.4		3.7	2.7			2.2	3.8	3.7
	1061	5.4	4.4	3	3.2		0.3	1	2.1	1.8
	1068	5.8					3.1	3.9	4.9	4.8
	1385	4.5	2				1.6	2.5	3.3	3
	1594	5.4	3.5	4.2	1.9			2.4	3.2	2.5
C. parapsilosis	0640	4.1					2.2	3.3	4.2	4
	0708	4.9						1.9	3.9	3.8
	1396	4.6						2.5	3.8	3.3
S. cerevisiae	1136	3.8				0.8			1.7	

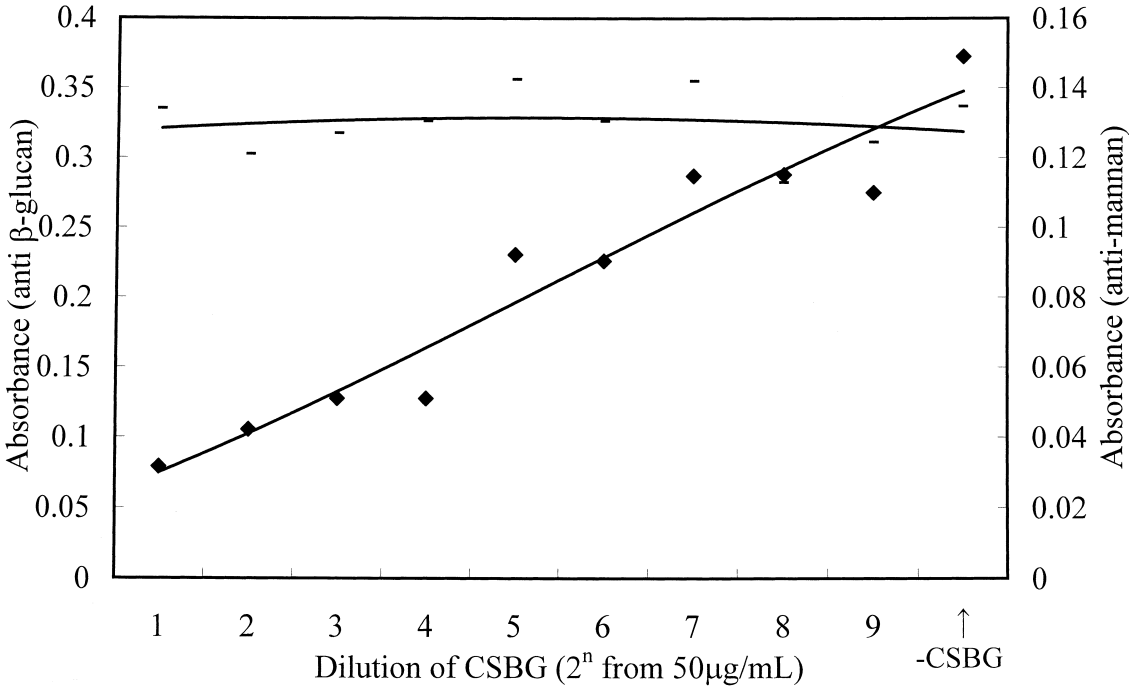

Fig. 6. Reactivity of the Con A+ fraction to anti-β-glucan and anti-mannan antibodies. The Con A-bound fraction of CAWS (*C. albicans* 1385) was adsorbed onto an ELISA plate. Anti-β-glucan (♦) or anti-mannan (–) (type 1) antisera were added to each well in the presence or absence of a standard β-glucan preparation CSBG. Antibody bound to the ELISA plate was determined by anti-mouse or anti-rabbit IgG-POX as described in Section 2. Dilution of CSBG was plotted on the X-axis.

compared. Fig. 5 shows a ^{1}H-NMR spectrum of the zymolyase-solubilized high molecular weight fraction of the acetone-dried whole cells of Ca1385. This spectrum was similar to the spectrum of CAWS, suggesting that the architecture of CAWS is similar to the cell wall.

3.4. Evidences for Factor G reactive substances as mannan-glucan complex

During the preliminary investigation, it was found that CAWS was a mixture of heterogeneous substances. To demonstrate whether Fungitec G test MK reactive substance(s) covalently bound to the mannan moiety, several experiments were performed as follows.

Fig. 6 shows the reactivity of the ConA-bound, mannan positive fraction of CAWS to anti-β-glucan antibody. The anti-β-glucan antibody was prepared by immunizing mice with a (1→3)-β-D- and (1→6)-β-D-glucan preparation, zymocel. Anti-mannan antibody used in this experiment was typing sera number 1. A soluble β-glucan preparation, CSBG prepared by the NaClO-DMSO method from *Candida* sp., was used as a competitor [22]. Immunochemical reactivity was assessed by using antigen, the ConA-bound fraction, bound to an ELISA plate. As shown in Fig. 6, both anti-β-glucan and anti-mannan antibody bound to the antigen-coated plate, strongly suggesting that the mannan fraction adsorbed onto Con A agarose contained a β-glucan moiety. Reduction of the reactivity of the anti-β-glucan antibody and not the anti-mannan antibody, by the competitive ELISA assay using CSBG, also supported the presence of a β-glucan moiety in CAWS.

Fig. 7 shows the reactivity of CAWS and the Con A passed fraction of CAWS to Fungitec G test MK. Reactivity to Fungitec G test MK was significantly reduced by Con A agarose chromatography, suggesting that the significant proportion of the reactive substance was retained on the Con A column. These facts strongly suggested that the water soluble, Fun-

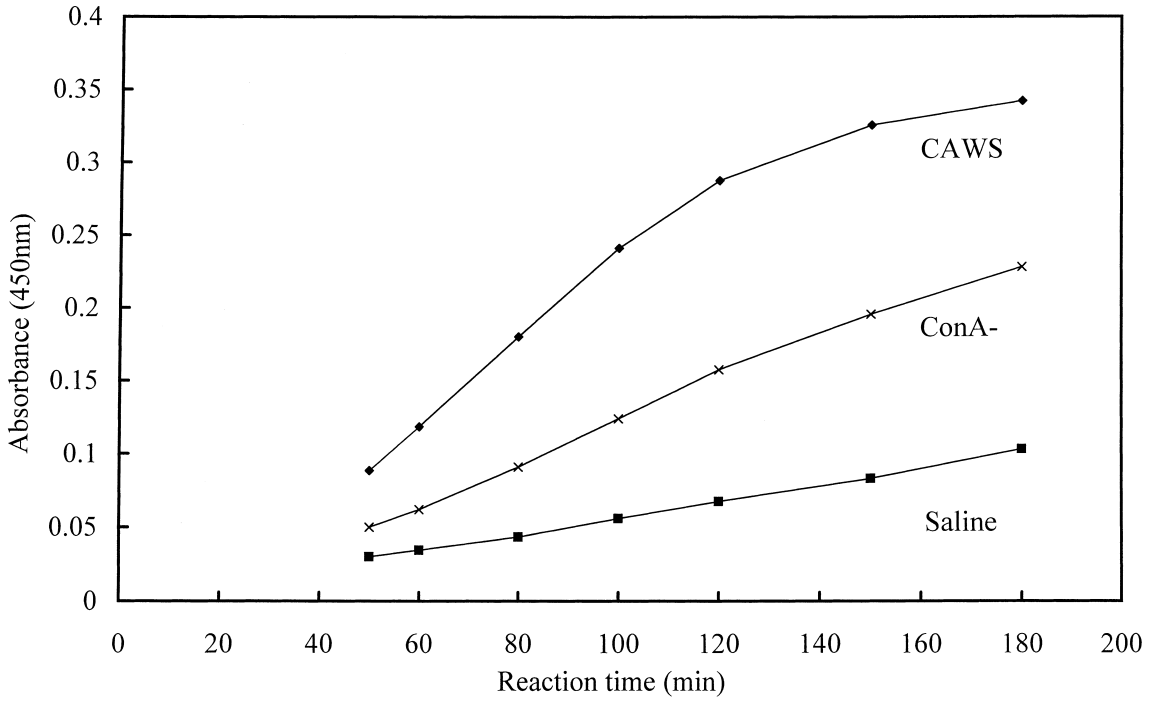

Fig. 7. Reactivity of the Con A fraction of CAWS (*C. albicans* 1385) by Fungitec G test MK. CAWS (2 mg ml^{-1} *C. albicans* 1385) was mixed with Con A agarose and incubated at 37°C for 1 h. After centrifugation, the supernatant fraction was used as Con A fraction. Each fraction was treated with 0.5 N NaOH, diluted and measured by Fungitec G test MK as described in Section 2.

gitec G test MK reactive material was, at least a part, a mannan-β-glucan complex.

4. Discussion

It is suggested that the limulus factor G reactive substance, believed to be (1→3)-β-D-glucan, is released from the fungi into the blood in patients with deep-seated mycosis. In order to diagnose fungal infections, a (1→3)-β-D-glucan-specific chromogenic kit (Fungitec G test MK) has been developed and applied clinically. However, the chemical property of the limulus factor G reactive substance was not precisely examined in detail because of the limited quantity available. In this study, we used chemically defined medium to culture *Candida* spp. and collected an extracellular, water soluble fraction, CAWS. From the chemical, immunochemical and biochemical analyses of CAWS, it is strongly suggested that the water soluble, limulus factor G-activating substance was the mannan-β-glucan complex, at least in part. However, CAWS contained only a small amount of the (1→3)-β-D-glucan moiety, the minimum concentration to react with Fungitec G test MK was 100 ng ml^{-1}, approximately 1000 times lower than the purified β-glucan, CSBG.

Recently, Klis et al. examined the architecture of the cell wall of yeast and showed that (1→3)-β-D-glucan, (1→6)-β-D-glucan and mannoprotein are covalently linked together. The data shown in this paper are consistent with their observation [21,23].

The architecture of CAWS is also interesting from the point of view of the metabolism of the fungal components. We have shown that the half clearance time of branched (1→3)-β-D-glucan from the blood was several hours and was significantly modulated by the conformational change [24]. In contrast, clearance of mannan is usually several minutes, because of the presence of a mannose receptor on various types of cells [25]. Thus the mannan-glucan complex might behave as mannan in blood.

β-Glucan is well-established as a biological response modifier, showing various beneficial activities

to the host defense system, such as anti-tumor activity, anti-microbial activity and adjuvant activity. On the other hand, mannan also shows various biological activities. It would be worth to investigate the immunomodulating activity of the mannan-β-glucan complex.

We have shown that CAWS contained the mannan-β-glucan complex. However, at this moment, we cannot rule out the presence of simple mannoprotein and β-glucan in CAWS. Probably, CAWS contains a certain part of simple mannoprotein, because it is generally understood that yeast can produce glycosylated enzymes in the medium. Further characterization of CAWS is ongoing.

References

[1] Branco, L., Pitta, M.L., Bernardes, L., Galrinho, A., Agapito, A.F., Ramos, J.M., Quininha, J., Figueiredo, L., Bento, R. and Mata, A. (1997) A review of infectious endocarditis due to *Candida*. Rev. Port. Cardiol. 16, 967–974.

[2] Osumi, M. (1998) The ultrastructure of yeast cell wall structure and formation. Micron 29, 207–233.

[3] Bishop, C.T., Blank, F. and Gardner, P.E. (1960) The cell wall polysaccharides of *Candida albicans*: glucan, mannan and chitin. Can. J. Chem. 38, 869–881.

[4] Shepherd, M.G. (1991) The structure and function of *Candida albicans* cell wall. Jpn. J. Med. Mycol. 32, 63–73.

[5] Kapteyn, J.C., Montijn, R.C., Dijkgraaf, G.J., Van den Ende, H. and Klis, F.M. (1995) Covalent association of beta-1,3-glucan with beta-1,6-glucosylated mannoproteins in cell walls of *Candida albicans*. J. Bacteriol. 177, 3788–3792.

[6] Jiang, B., Sheraton, J., Ram, A.F., Dijkgraaf, G.J., Klis, F.M. and Bussey, H. (1996) CWH41 encodes a novel endoplasmic reticulum membrane N-glycoprotein involved in beta 1,6-glucan assembly. J. Bacteriol. 178, 1162–1171.

[7] Montijn, R.C., Rinsum, J., Schagen, F.A. and Klis, F.M. (1994) Glucomannoproteins in the cell wall of *Saccharomyces cerevisiae* contain a novel type of carbohydrate side chain. J. Biol. Chem. 269, 19338–19342.

[8] Obayashi, T., Yoshida, M., Tamura, H., Aketagawa, J., Tanaka, S. and Kawai, T. (1992) Determination of plasma (1→3)-β-D-glucan: a new diagnostic aid to deep mycosis. J. Med. Vet. Mycol. 30, 275–280.

[9] Obayashi, T., Yoshida, M., Mori, T., Goto, H., Yasuoka, A., Iwasaki, H., Teshima, H., Kohno, S., Horiuchi, A., Ito, A., Yamaguchi, H., Shimada, K. and Kawai, T. (1995) Plasma (1→3)-β-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. Lancet 345, 17–20.

[10] Ikegami, K., Ikemura, K., Shimazu, T., Shibuya, M., Sugimoto, H., Yoshioka, T. and Sugimoto, T. (1988) Early diagnosis of invasive candidiasis and rapid evaluation of antifungal therapy by combined use of conventional chromogenic limulus test and a newly developed endotoxin specific assay. J. Trauma 28, 1118–1126.

[11] Aketagawa, J., Tanaka, S., Tamura, H., Shibata, Y. and Saito, H. (1993) Activation of limulus coagulation factor G by several (1→3)-β-D-glucans: Comparison of the potency of glucans with identical degree of polymerization but different conformations. J. Biochem. 113, 683–686.

[12] Tanaka, S., Aketagawa, J., Takahashi, S., Shibata, Y., Tsumuraya, Y. and Hashimoto, Y. (1993) Inhibition of high-molecular weight (1→3)-β-D-glucan-dependent activation of a limulus coagulation factor G by laminaran oligosaccharides and curdlan degradation products. Carbohydr. Res. 244, 115–127.

[13] Tanaka, S., Aketagawa, J., Takahashi, S., Shibata, Y., Tsumuraya, Y. and Hashimoto, Y. (1991) Activation of a limulus coagulation factor G by (1→3)-β-D-glucans. Carbohydr. Res. 218, 167–174.

[14] Miyazaki, T., Kohno, S., Mitsutake, K., Maesaki, S., Tanaka, K., Ishikawa, N. and Hara, K. (1995) Plasma (1→3)-beta-D-glucan and fungal antigenemia in patients with candidemia, aspergillosis, and cryptococcosis. J. Clin. Microbiol. 33, 3115–3118.

[15] Nagi, N., Ohno, N., Tanaka, S., Aketagawa, J., Shibata, Y. and Yadomae, T. (1992) Solubilization o f limulus test reactive material(s) from *Candida* cells by murine phagocytes. Chem. Pharm. Bull. 40, 1532–1536.

[16] Miyazaki, T., Kohno, S., Mitsutake, K., Maesaki, S., Tanaka, K. and Hara, K. (1995) (1→3)-beta-D-glucan in culture fluid of fungi activates factor G, a limulus coagulation factor. J. Clin. Lab. Anal. 9, 334–339.

[17] Miura, N.N., Miura, T., Ohno, N., Adachi, Y., Watanabe, M., Tamura, H., Tanaka, S. and Yadomae, T. (1998) Gradual solubitization of *Candida* cell wall β-glucan by oxidative degradation in mice. FEMS Immunol. Med. Microbiol. 21, 123–129.

[18] Ohno, N., Miura, T., Miura, N.N., Uchiyama, M., Adachi, Y., Yadomae, T., (1999) Reduction of immuno/inflammatory activities of zymosan by hypochlorite oxidation, Pharm. Pharmacol. Lett. (in press).

[19] Miura, N.N., Ohno, N., Adachi, Y. and Yadomae, T. (1996) Characterization of sodium hypochlorite degradation of β-glucan in relation to its metabolism in vivo. Chem. Pharm. Bull. 44, 2137–2141.

[20] Shepherd, M.G. and Sullivan, P.A. (1976) The production and growth characteristics of yeast and mycelial forms of *Candida albicans* in continuous culture. J. Gen. Microbiol. 93, 361–370.

[21] Kollár, R., Reinhold, B., Petráková, E., Yehi, H.J.C., Ashwell, G., Drgonová, J., Kapteyn, J.C., Klis, F.M. and Cabib, E. (1997) Architecture of the yeast cell wall. J. Biol. Chem. 272, 17762–17775.

[22] Ohno, N., Uchiyama, M., Tsuzuki, A., Tokunaka, K., Miura, N.N., Adachi, Y., Aizawa, M.W., Tamura, H., Tanaka, S. and Yadomae, T. (1999) Solubilization of yeast cell-wall β-(1→3)-D-glucan by sodium hypochlorite oxidation and dimethyl sulfoxide extraction. Carbohydr. Res 316, 161–172.

[23] Lipke, P.N. and Ovalle, R. (1998) Cell wall architecture in yeast. J. Bacteriol. 180, 3735–3740.
[24] Miura, N.N., Ohno, N., Adachi, Y., Aketagawa, J., Tamura, H., Tanaka, S. and Yadomae, T. (1995) Comparison of clearance rate from blood stream of triple- and single-helical schizophyllan in mice. Biol. Pharm. Bull. 18, 185–189.
[25] Bijsterbosch, M.K., Donker, W., van de Bilt, H., van Weely, S., van Berkel, T.J. and Aerts, J.M. (1996) Quantitative analysis of the targeting of mannose-terminal glucocerebrosidase. Eur. J. Biochem. 237, 344–349.