Characterization of a haemolytic factor from *Candida albicans*

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The culture supernatant of *Candida albicans* promoted the disruption of human red blood cells (RBCs). The haemolytic activity was detected in a sugar-rich fraction (about 200 kDa) from Sephacryl S-100 chromatography. As the haemolytic activity was adsorbed by concanavalin A-Sepharose, the haemolytic factor may be a mannoprotein. The activity was inactivated by periodate oxidation, indicating that the sugar moiety of the mannoprotein played an important role in the haemolysis. The structure of the sugar moiety of the mannoprotein was identified as a cell-wall mannan by ¹H-NMR analysis, and purified *C. albicans* mannan promoted the disruption of RBCs. The binding of mannan to RBCs was demonstrated by flow cytometric analysis and was inhibited by the addition of band 3 protein inhibitor, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS). The haemolysis caused by mannan was inhibited by DIDS, SITS (4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid) and bis(sulfosuccinimidyl) suberate, but not by pyridoxal 5-phosphate. These results indicated that a mannoprotein released from *C. albicans* bound to the band 3 protein on RBCs, thereby promoting their disruption.

Keywords: *Candida albicans*, mannan, band 3 protein, haemolysis

INTRODUCTION

The opportunistic pathogen *Candida albicans* is a serious agent of infection in the immunocompromised host. *C. albicans* is a dimorphic fungus which can alternate between yeast and hyphal phases, depending on the growth conditions (Blasi *et al.*, 1994). The cell wall of *C. albicans* contains a mannan which has a linear 1,6- α -linked mannose backbone with side chains of 1,2- α -, 1,2- β - and 1,3- α -linked mannose units (Shibata *et al.*, 1995). The antigenicity of *Candida* species depends on the structure of their mannans, and differences of antigenicity in mannans are used in the classification of *Candida* species (Ataoglu *et al.*, 1993; Kobayashi *et al.*, 1992). *C. albicans* possesses a number of factors that could be involved in the invasive process. Adhesins,

dimorphism and the secretion of specific hydrolytic enzymes have been suggested as possible virulence factors (Cameron & Douglas, 1996; Colina *et al.*, 1996; Ibrahim *et al.*, 1995).

Adherence to endothelial cells by *C. albicans* is mediated by a glycoprotein which is known to bind fibronectin; adherence is blocked by a peptide containing an Arg-Gly-Asp sequence (Pendrak & Klotz, 1995; Sawyer *et al.*, 1992). When *C. albicans* is phagocytosed by endothelial cells, it converts to the hyphal form and damages the endothelial cells (Zink *et al.*, 1996). Hydrolytic enzymes such as proteases and phospholipases are known to promote invasiveness in disseminated candidiasis (Fallon *et al.*, 1997). Numerous pathogenic micro-organisms grow in the host by using haemin or haemoglobin as a source of iron (Henderson & Payne, 1994; Law & Kelly, 1995; Otto *et al.*, 1992). We previously reported that *C. albicans* secretes a haemolytic factor that causes the release of haemoglobin, which is then used as an iron source by the organisms (Watanabe *et al.*, 1997), but the haemolytic

Abbreviations: BS³, bis(sulfosuccinimidyl) suberate; ConA, concanavalin A; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; FAb 1, anti-serum against factor 1 of the genus *Candida*; PBS(–), Ca²⁺- and Mg²⁺-free PBS; PLP, pyridoxal 5-phosphate; RBC, red blood cell; SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid.

Fig. 1. Haemolytic activity in *C. albicans* culture supernatant. *C. albicans* cells were cultured in RPMI 1640 medium (1×10^4 cells ml^{-1}) at 37°C in 5% CO_2 . The culture supernatant was collected daily and mixed with RBCs (1×10^8 cells ml^{-1}) at a 1:1 ratio, and incubated at 37°C for 12 h. After incubation, the A_{405} of the supernatant was measured to estimate the degree of disruption of the RBC (●). The percentage haemolysis was determined by comparing the A_{405} resulting from incubation with the test sample with that resulting from 100% lysis with water. The growth of *C. albicans* was measured by the Alamar blue method and the number of cells was measured as the OD_{540} minus OD_{620} (■).

factor was not identified. In this study, we have characterized the haemolytic factor in the culture supernatant of *C. albicans*.

METHODS

Reagents. Concanavalin A (Con A)-Sephacryl (Pharmacia), Alamar blue (Alamar Biosciences), DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; Sigma), SITS (4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid; Sigma), BS³ [bis(sulfosuccinimidyl) suberate; Pierce], and PLP (pyridoxal 5-phosphate; Nacalai Tesque) were used in the experiments. Antiserum against factor 1 of the genus *Candida* (FAB 1) was supplied with Candida Check kits (Iatron). Con A was purchased from Sigma, and Pepstatin A, an aspartyl protease inhibitor, was obtained from Peptide Institute.

Preparation of red blood cells (RBCs). Human RBCs (A type), supplied by the Red Cross Blood Center of Miyagi Prefecture, Japan, were suspended in Ca^{2+} - and Mg^{2+} -free PBS [PBS(–)] and centrifuged at $700g$ for 5 min. The supernatant and buffy coat were removed, and the packed RBCs were further washed with PBS(–) by centrifugation. RBCs were then suspended in RPMI 1640 medium and used for the haemolytic activity assay.

Preparation of *C. albicans* culture supernatants. *C. albicans* NIH A-207 cells were maintained in Sabouraud's liquid medium with shaking at 27°C . The cells were transferred into RPMI 1640 medium (1×10^4 cells ml^{-1}) and incubated at 37°C for 4 d. The culture supernatant was collected, dialysed and freeze-dried, and then used as the concentrated *C. albicans* culture supernatant. The growth of *C. albicans* was measured by using the Alamar blue method (Pfaller *et al.*, 1994).

Haemolytic activity assay. Haemolytic activity was measured according to the method of Manns *et al.* (1994). Briefly, a sample solution was mixed with RBC (1×10^8 cells ml^{-1}) at a 1:1 ratio and incubated at 37°C for 12 h. After incubation,

the A_{405} in the supernatant was measured to estimate the degree of RBC disruption. The percentage haemolysis was determined by comparing the A_{405} of the test group with that of 100% lysis obtained with water. To confirm the relationship of *C. albicans* secreted aspartyl protease with haemolysis, pepstatin A (0.1 mg ml^{-1}) was added to the mixture of the *C. albicans* culture supernatant and RBCs.

Purification of the haemolytic factor. The concentrated *C. albicans* culture supernatant was gel filtered through a HiPrep 16/60 Sephacryl S-100 column (Pharmacia) at 1 ml min^{-1} in PBS(–), and fractions of 4 ml were collected. Total protein content was measured using a BCA protein assay kit (Pierce) with BSA as the standard. Total carbohydrate content was determined by the phenol/ H_2SO_4 method (Shibata *et al.*, 1989) with D-mannose as the standard. To detect the fraction containing mannan, an aliquot from each fraction was added to a well of a 96-well plastic plate coated with Con A, and the amount of mannan bound to the plate was measured using an ELISA, as described previously (Mikami *et al.*, 1986). FAB 1 and horseradish peroxidase-conjugated anti-rabbit IgG (Seikagaku) were used for the detection of mannan.

Adsorption of the haemolytic factor by Con A-Sepharose. The haemolytic fractions eluted from the HiPrep 16/60 Sephacryl S-100 column (around 200 kDa) were adsorbed to Con A-Sepharose by a batch affinity method at 37°C for 1 h. After adsorption, the haemolytic activity and the sugar content were measured as described above.

Periodate oxidation of the haemolytic factor. The haemolytic factor bound to Con A-Sepharose was eluted with $0.2 \text{ M } \alpha$ -methyl mannoside. The eluate was dialysed, freeze-dried and then treated with periodate oxidate, as described previously (Suzuki *et al.*, 1977). After treatment, the sample was passed through a PD-10 (Pharmacia) column to remove the buffer salts.

^1H -NMR spectra of mannoproteins in the *C. albicans* culture supernatant. The haemolytic fraction purified using the HiPrep 16/60 Sephacryl S-100 column was dialysed and freeze-dried. The ^1H -NMR spectrum of this sample was recorded using a JEOL JNM-GSX 400 spectrometer operating at 400 MHz and a probe temperature of 45°C . The sample was dissolved in D_2O , and acetone was used as the internal standard (2.217 p.p.m.).

Mannan extraction from the *C. albicans* cell wall. Mannan was prepared as described by Tojo *et al.* (1991) using Fehling solution. An aqueous solution of each bulk mannan preparation was applied to a column of DEAE-Sephadex A-50 (Pharmacia), and the 0.05 M NaCl-eluted fraction was dialysed and then freeze-dried.

Flow cytometric analysis. RBCs (1×10^6 cells) and mannan ($10 \mu\text{g}$) were incubated in an ice bath for 30 min, and the cells were then washed by centrifugation. The washed cells were treated with FAB 1 ($10 \mu\text{l}$), and then mixed with FITC-labelled anti-rabbit IgG antibody (Seikagaku). The fluorescence intensity of individual cells was measured using a FACScan analyser (Becton Dickinson). To confirm whether band 3 protein was associated with the mannan-binding to RBCs, RBCs were pretreated with a band 3 protein inhibitor, DIDS (0.1 mg ml^{-1}), in an ice bath for 30 min.

Extraction and purification of band 3 protein. This was done by a modification of the method of England *et al.* (1980).

Effects of band 3 protein inhibitors on haemolytic activity of mannan. To determine the mannan binding site on band 3 protein, RBCs were pretreated with four protein inhibitors:

Fig. 2. Purification of haemolytic factor from *C. albicans* culture supernatant. The concentrated *C. albicans* culture supernatant was gel filtered with a HiPrep 16/60 Sephacryl S-100 column and fractions of 4 ml were collected. (a) Total protein content (thin line) and total sugar content (thick line) were measured. (b) Each fraction was mixed with RBCs (1×10^8 cells ml^{-1}) at a 1:1 ratio, and the degree of haemolysis was measured (bars). To detect the fraction containing mannan, each fraction was added to a well of a 96-well plastic plate coated with Con A, and the amount of mannan bound to the plate was measured by ELISA. Reactivity with FAB 1 is shown as the A_{490} (solid line). V_0 , void volume.

DIDS, SITS, BS³ and PLP, for 30 min. Mannan (0.5 mg ml^{-1}) was added to these treated cells, and the degree of haemolysis was measured.

Statistical analysis. Values are shown as means $\pm$ SE, and statistical analyses of these data were performed using the Student's *t*-test.

RESULTS

Haemolytic activity in the *C. albicans* culture supernatant

To confirm the secretion of a haemolytic factor by *C. albicans*, the culture supernatant was mixed with human RBCs, and the degree of haemolysis was examined. The haemolytic factor was secreted after 2 d incubation, and the activity was 27.8% haemolysis per 3×10^7 *Candida* cells (Fig. 1). Since the haemolytic activity was not inhibited by heat treatment (100°C for 10 min) or by the addition of pepstatin A (an aspartyl protease inhibitor), the active component is probably not a protein such as a secreted aspartyl proteinase (data not shown).

Purification of a haemolytic factor from the *C. albicans* culture supernatant

The concentrated culture supernatant of *C. albicans* (10 mg dry wt) was gel filtered on a HiPrep 16/60 Sephacryl S-100 column. Haemolytic activity (39% haemolysis per 50 μg sugar) was detected in the sugar-rich fraction of 200 kDa; this fraction also reacted with FAB 1 (Fig. 2). The haemolytic fractions were pooled and fractionated on Con A-Sepharose. The haemolytic activity was associated with the material adsorbed by Con A-Sepharose and was not detected in the unbound fraction. These results indicated that a mannoprotein secreted from *C. albicans* promoted the disruption of RBCs. The haemolytic activity of the mannoprotein eluted from Con A-Sepharose (42.7% haemolysis per

50 μg sugar) was completely abolished by periodate oxidation, suggesting that the sugar moiety of the mannoprotein was important in inducing the haemolysis.

¹H-NMR spectrum of the mannoprotein

The polysaccharide in the haemolytic fraction eluted from the HiPrep 16/60 Sephacryl S-100 column was structurally analysed by ¹H-NMR spectroscopy. The anomeric proton chemical shifts of oligosaccharides were assigned by adapting the findings of Shibata *et al.* (1993, 1995). The polysaccharide showed signals at 4.84 and 4.85 p.p.m. (corresponding to the 1,2- β -linked mannopyranose unit), and also signals corresponding to 1,3- α - or 1,2- α -linked mannose units (5.38, 5.27, 5.14, 5.03 and 4.91 p.p.m.). These results indicated that the haemolytic polysaccharide was a mannan derived from the cell wall of *C. albicans*.

Haemolytic activity of mannan extracted from *C. albicans*

To confirm the role of mannan in haemolysis, we purified a *C. albicans* mannan (95% carbohydrate, 3% protein). Mannose and Dextran T-40 (Pharmacia) were used as negative controls to demonstrate that the haemolysis was not due to osmolysis or non-specific activities of polysaccharides. It was found that the extent of RBC haemolysis was dependent upon the amount of mannan (Fig. 3). The addition of mannose or dextran, however, did not induce haemolysis.

Mannan binding to the surface of RBCs

We conducted flow cytometric analysis to demonstrate the existence of a mannan-binding site on RBCs. As shown in Fig. 4, the fluorescence intensity of mannan-

Fig. 3. Haemolytic activity of mannan extracted from *C. albicans*. The cell-wall mannan (■) was dissolved in RPMI 1640, and mixed with RBCs (1×10^8 cells ml^{-1}) at a 1:1 ratio. After 12 h incubation, the A_{405} in the supernatant was measured to estimate the degree of disruption of the RBCs. The percentage haemolysis was determined by comparing the A_{405} resulting from incubation with the test sample with that resulting from 100% lysis with water. Mannose (□) and dextran (○) were used as negative controls.

Fig. 4. Measurement of mannan binding to RBCs by flow cytometry. RBCs (1×10^6 cells) and mannan (10 µg) were incubated in an ice bath for 30 min, and the cells were washed by centrifugation. The washed cells were treated with FAb 1 (10 µl), and then incubated with FITC-labelled anti-rabbit IgG antibody. The fluorescence intensity of each cell was measured using a FACScan analyser. None, RBCs only; Mannan, RBCs treated with mannan; Mannan+DIDS, RBCs pre-treated with DIDS in an ice bath for 30 min prior to the addition of mannan.

treated RBCs was significantly enhanced compared with that of RBCs not treated with mannan. The binding of mannan to RBCs was strongly inhibited by the treatment of cells with 0.1 mg ml^{-1} DIDS, an anion-transport inhibitor which specifically binds to a transmembrane protein, in this case, band 3 protein. This suggested that the mannan binds to the band 3 protein of RBCs.

Effect of anion transport inhibitors on haemolytic activity of mannan

The effect of DIDS on the haemolytic activity of mannan was measured to confirm the relationship between the binding of mannan to band 3 protein and haemolysis.

Table 1. Effects of anion transport inhibitors on the haemolytic activity of mannan

Inhibitor	Concn (mM)	Inhibition (%)*
DIDS	0.10	86.0 ± 2.2
	0.01	37.4 ± 5.3
	0.001	5.2 ± 3.0
SITS	0.50	91.8 ± 1.0
	0.10	37.8 ± 1.0
	0.01	20.0 ± 2.0
BS ³	4.00	78.5 ± 0.6
	2.00	61.1 ± 0.4
	1.00	27.3 ± 0.9
PLP†	0.25	0.4 ± 0.1
	0.10	0.2 ± 0.1

* Inhibition (%) = [percentage haemolysis by mannan – percentage haemolysis by (mannan + inhibitor)] $\times$ 100, divided by percentage haemolysis by mannan.

† Inhibition activity of PLP at 1.0 mM was not examined, because of haemolysis by PLP.

The haemolytic activity of mannan was inhibited by the addition of DIDS (Table 1). A family of stilbene disulfonate derivatives, DIDS, SITS and BS³, inhibits anion transport by binding covalently or noncovalently to the Lys-539 or Lys-542 of band 3 protein (Yamaguchi *et al.*, 1995). PLP reacts with Lys-851 in band 3 protein, and is an inhibitor of band 3-mediated anion exchange (Okubo *et al.*, 1994). The stilbene disulfonate derivatives inhibited the haemolysis, but PLP did not (Table 1). These results suggest that the binding of the mannan to RBCs was mediated by Lys-539 or Lys-542 in band 3 protein.

DISCUSSION

Some pathogenic micro-organisms secrete haemolytic factors in order to obtain haemoglobin or haemin as a source of iron (Belanger *et al.*, 1995; Lewis & Dyer, 1995). We previously reported that haemoglobin utilization by *C. albicans* was related to a morphological change (Watanabe *et al.*, 1997). A complement-mediated haemolysis induced by secretion from *C. albicans* has been observed (Manns *et al.*, 1994), but the secretion of a haemolytic factor by *C. albicans* was not reported. In the present paper, we have demonstrated the secretion of a haemolytic factor by *C. albicans* and characterized this factor.

The disruption of RBCs was significantly promoted by the addition of *C. albicans* culture supernatant (Fig. 1), indicating that a haemolytic factor was released from *C. albicans*. Since *C. albicans* secretes an aspartyl protease for invasion into the host (Colina *et al.*, 1996; Zink *et al.*, 1996), such an enzyme has been suggested as the cause of the haemolysis. However, the addition of pepstatin A, an aspartyl protease inhibitor, or treatment

at 100 °C for 10 min did not affect the haemolytic activity (data not shown). Thus, we suggest that the haemolytic factor is not enzymic. The haemolytic activity was associated with a sugar-rich fraction of approximately 200 kDa. This fraction also reacted with Con A and FAb 1 (Fig. 2). These results demonstrate that the haemolytic activity was probably caused by a mannoprotein. When the sugar moiety of the mannoprotein was disrupted by periodate oxidation, the haemolytic activity was lost. This suggests that the sugar moiety of the mannoprotein is important for binding to RBCs and/or for inducing haemolysis. Structural studies indicated that the haemolytic fraction contained sugar residues typical of cell-wall mannans, and purified bulk mannan also showed haemolytic activity. Both mannan binding to RBCs and the haemolytic activity of mannan were strongly inhibited by treatment with DIDS (Fig. 4, Table 1). As DIDS is an anion-transport inhibitor which binds to band 3 protein, band 3 protein might be involved in the binding of, and haemolysis by, mannan. The binding of mannan to RBCs was inhibited by DIDS, indicating that DIDS masked the mannan-binding site of the band 3 protein. A family of stilbene disulfonate derivatives, DIDS, SITS and BS³, which inhibit anion transport by binding covalently or noncovalently to Lys-539 or Lys-542 of band 3 protein (Yamaguchi *et al.*, 1995), inhibited the haemolysis (Table 1). Thus, it was suggested that the mannan bound to band 3 protein on RBCs, and that Lys-539 or Lys-542 of band 3 protein might be an important site for the binding of mannan. The results with PLP supported this conclusion. Since band 3 protein catalyses anion exchange and seems to be associated with spectrin via ankyrin (Yamaguchi *et al.*, 1995), we suggest that the mannan destabilizes the RBC membrane through the function of band 3 protein.

As haemolytic activity was detected in the culture supernatant of *C. albicans in vitro*, some mannoprotein may be produced at local sites *in vivo*. However, large amounts of mannoprotein are not detected in patients with candidiasis. Although systemic haemolysis may not be caused by the mannoprotein, haemolysis around infected sites may enhance the growth of *C. albicans* because haemoglobin is an important factor for *Candida* growth in the host.

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