1 **A novel mannose-containing sialoprotein adhesin involved in the binding of *Candida***
2 ***albicans* cells to DMBT1**
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16 **Short title:** *C. albicans* adhesin involved in the binding to DMBT1
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18

Abstract

Candida albicans colonizes the oral cavity and causes oral candidiasis and early childhood caries synergistically with cariogenic *Streptococcus mutans*. Colonization of oral tissues with *C. albicans* is an essential step in the initiation of these infectious diseases. DMBT1 (deleted in malignant brain tumors 1), also known as salivary agglutinin or gp-340, belongs to the scavenger receptor cysteine-rich (SRCR) superfamily and has important functions in innate immunity. In the oral cavity, DMBT1 causes microbial adherence to tooth enamel and oral mucosa surfaces, but the adherence of *C. albicans* to DMBT1 has not been examined. In this study, we investigated the binding of *C. albicans* to DMBT1 and isolated the fungal components responsible for the binding. *C. albicans* specifically bound to DMBT1 and strongly bound to the peptide domain SRCRP2. Binding to SRCRP2 was inhibited by *N*-acetylneuraminic acid and mannose and by lectins recognizing these sugars. The isolated component had a molecular mass of 25 kDa, contained sialic acid and mannose residues, and inhibited *C. albicans* binding to SRCRP2. The localization of the 25-kDa protein on the surface of *C. albicans* cell walls was confirmed by immunostaining and a cell ELISA using an antiserum to the protein, and Western blotting revealed the presence of the 25-kDa protein in the cell wall fraction of *C. albicans*. These results suggest that the isolated adhesin is localized on

1 the surface of *C. albicans* cell walls and that sialic acid and mannose residues in the

2 adhesin play a significant role in the binding reaction.

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4

5 **KEYWORDS**

6 adhesin, binding, *Candida albicans*, DMBT1, sialic acid, surface localization

7

1 INTRODUCTION

Candida albicans, an opportunistic pathogen, colonizes the oral cavity and is associated with many clinical manifestations (Lombardi & Ouanounou, 2020; Vila et al., 2020). *C. albicans* adheres to mucosal surfaces and causes oral candidiasis, and the conditions can range from superficial mucocutaneous overgrowths to invasive bloodstream infections. *C. albicans* also adheres to tooth surfaces and causes the development of early childhood caries synergistically with cariogenic *Streptococcus mutans* in some populations (Alkhars et al., 2022; de Carvalho et al., 2006). Colonization of oral tissues with *C. albicans* is an essential step to cause these infectious diseases. Oral mucosa and tooth enamel surfaces are covered with a film of salivary proteins, which serve as an adherence receptor for the oral microflora. Previous studies have reported the adherence of *C. albicans* cells to salivary proteins, including basic proline-rich proteins, statherin, mucin, and salivary IgA (Hoffman & Haidaris, 1993; Johansson et al., 2000; O'Sullivan et al., 1997; van der Wielen et al., 2016).

DMBT1 (deleted in malignant brain tumors 1), also known as salivary agglutinin or gp-340, is a high molecular mass glycoprotein belonging to the scavenger receptor cysteine-rich (SRCR) superfamily (Osei et al., 2018; Reichhardt et al., 2017). The DMBT1 protein is composed of conserved peptide motifs: 14 SRCR domains that are

1 separated by SRCR-interspersed domains (SIDs), 2 C1r/C1s Uegf Bmp1 domains, and a
2 zona pellucida domain (Bikker et al., 2002). DMBT1 has important functions in innate
3 immunity, inflammation, epithelial homeostasis, and tumor suppression (Reichhardt et al.,
4 2017). In the oral cavity, DMBT1 is expressed in the salivary glands and secreted
5 DMBT1 exists in two conformations, namely, in soluble and immobilized forms. In the
6 fluid phase, DMBT1 induces microbial aggregation leading to the clearance of
7 microorganisms from the oral cavity (Oho et al., 1998; Purushotham & Deivanayagam,
8 2014). In its immobilized form, DMBT1 is incorporated into the salivary film formed
9 on the oral mucosa and tooth enamel, and induces microbial adherence, leading to the
10 accumulation of microorganisms (Bikker et al., 2013; Brittan & Nobbs, 2015). Studies
11 on the interaction between DMBT1 and various pathogens including oral streptococci,
12 oral *Actinomyces,* and *Lactobacilli*, have been reported (Haukioja et al., 2008; Loimaranta
13 et al., 2005; Prakobphol et al., 2000). Regarding the involvement of DMBT1 in the
14 interaction with *C. albicans*, inhibitory effects of DMBT1 on *Candida albicans*-induced
15 complement activation and *C. albicans* binding to dendritic cell-specific ICAM-grabbing
16 nonintegrin (DC-SIGN) and Langerin have been reported (Boks et al., 2016; Reichhardt
17 et al., 2012). However, the adherence of *C. albicans* cells to DMBT1 and the possible
18 mechanism of this interaction have not been demonstrated.

1 Moreover, many surface adhesins of *C. albicans* have been identified and
2 demonstrated to be involved in biofilm formation and interactions with host tissues, cells,
3 and molecules (Chaffin, 2008; Garcia-Rubio et al., 2019). However, reports on the
4 interaction between *C. albicans* adhesins and salivary proteins are limited. In the
5 present study, the binding of *C. albicans* cells to DMBT1 was examined, and the fungal
6 components responsible for the binding were isolated. We confirmed *C. albicans*
7 binding to DMBT1 and examined the effects of sugars and lections on the binding. A
8 binding inhibitory component was isolated from *C. albicans* cell walls and the
9 localization of the isolated component on the surface of *C. albicans* cell walls was verified.
10 The results demonstrate the involvement of a novel mannose-containing sialoprotein
11 adhesin in the interaction of *C. albicans* with the binding domain of DMBT1.
12
13 **2 MATERIALS AND METHODS**
14 **2.1 Microbial strains and growth conditions**
15 The *C. albicans* type strain NBRC1385 (ATCC 18804) was obtained from the National
16 Institute of Technology and Evaluation (NITE) (Kisarazu, Japan), and was used
17 throughout this study. *S. mutans* MT8148 was obtained from our collection. *C.*
18 *albicans* NBRC1385 was cultured in Sabouraud dextrose broth (Becton Dickinson and

1 Company, Sparks, MD) under aerobic conditions at 37°C for 18 h. *S. mutans* MT8148
2 was grown under anaerobic conditions at 37°C for 18 h in brain heart infusion (Becton
3 Dickinson and Company) broth.
4
5 **2.2 Isolation of DMBT1**
6 DMBT1 was isolated from human resting saliva according to a previously described
7 method (Oho et al., 1998). Briefly, clarified whole saliva diluted in a 1:2 ratio with
8 aggregation buffer (1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl,
9 pH 7.2) was incubated with an equal volume of a cell suspension of *S. mutans* MT8148
10 at 37°C for 30 min. Cells were collected by centrifugation and washed twice with
11 aggregation buffer, and the absorbed DMBT1 was eluted with the same buffer
12 supplemented with 1 mM EDTA. The eluate was filtered (0.22-µm pore size), dialyzed
13 against aggregation buffer, and subjected to gel filtration chromatography on a Superdex
14 200 HR (GE Healthcare Bioscience, Marlborough, MA) equilibrated with aggregation
15 buffer. The eluate at the void volume was collected and used as DMBT1. Protein
16 content was determined according to the method of Lowry *et al*. (Lowry et al., 1951),
17 with bovine serum albumin (BSA) as a standard.
18

1 **2.3 DMBT1-derived peptides**

2 Eight DMBT1-derived peptides (SRCRP1-7, SID22) (Bikker et al., 2002) were
3 synthesized and purified by high-performance liquid chromatography to > 90% purity
4 (Qiagen, Tokyo, Japan; GenScript, Tokyo, Japan).

5

6 **2.4 Binding of *C. albicans* cells to DMBT1 and DMBT1-derived peptides**

7 The binding of *C. albicans* cells to DMBT1 and DMBT1-derived peptides was examined
8 using an enzyme linked immunosorbent assay (ELISA) according to a previously
9 described method (Nagata et al., 2006). *C. albicans* cells were suspended in potassium
10 phosphate-buffered saline (PBS, pH 7.0), and incubated with biotin 3-sulfo-*N*-
11 hydroxysuccinimide ester (final concentration: 0.5 mg/ml; Sigma Chemical Co., St. Louis,
12 MO) for 2 h at room temperature. Unbound biotin was removed by washing with PBS.
13 The wells of microtiter plates were coated with 100 μl of DMBT1 (5 μg/ml) or peptides
14 (20 μg/ml) diluted in PBS and incubated at 4°C overnight. The plates were washed with
15 PBS containing 0.05% Tween 20 (PBST), and residual protein-binding sites were blocked
16 by incubation with 100 μl of PBST at 37°C for 1.5 h (Holmes et al., 1995). After
17 washing, 100 μl of the suspension of biotinylated *C. albicans* cells in PBS was added to
18 the wells and incubated at 37°C for 1.5 h. After washing, the bound cells were incubated

1 with alkaline phosphatase-conjugated streptavidin (Vector Laboratories, Burlingame,
2 CA) diluted in PBST at 37°C for 1.5 h. The reaction products were visualized using *p*-
3 nitrophenylphosphate (1 mg/ml) in diethanolamine buffer (pH 9.8) containing 0.01 g/ml
4 $MgCl_2$, and the absorbance at 405 nm was measured with an iMark™ microplate reader
5 (Bio-Rad Laboratories, Richmond, CA). The number of bound cells was determined
6 using a standard curve. To examine the effect of divalent cations on the binding of *C.*
7 *albicans* cells to DMBT1 and DMBT1-derived peptides, $CaCl_2$, $MgCl_2$, or $MnCl_2$ was
8 added to the suspension of biotinylated *C. albicans* cells at a final concentration of 1 mM.
9
10 **2.5 Inhibition assay**
11 The effects of sugars on the binding of *C. albicans* cells to SRCRP2 were examined.
12 Fucose, galactose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, or *N*-
13 acetylneuraminic acid was added to a *C. albicans* cell suspension, and an ELISA was
14 performed. Since *N*-acetylneuraminic acid and mannose showed inhibitory effects on
15 *C. albicans* binding to SRCRP2, lectins recognizing these sugars were examined for their
16 inhibitory effect on fungal binding. Various amounts of *Maackia amurensis* lectin
17 (MAM; J-chemical Inc., Tokyo, Japan), which recognizes α2,3-linked sialic acid residues,
18 *Sambucus sieboldiana* lectin (SSA; J-chemical Inc.), which recognizes α2,6-linked sialic

1 acid residues, or concanavalin A (Con A; J-chemical Inc.), which recognizes α-mannose

2 residues, were added to a *C. albicans* cell suspension, and an ELISA was performed.

3

4 **2.6 Isolation of components inhibiting *C. albicans* binding**

5 The cell surface components of *C. albicans* responsible for inhibiting its binding to

6 SRCRP2 were isolated using an ÄKTA go chromatography system (Cytiva, Uppsala,

7 Sweden). Cell wall extracts were prepared according to the method described by Jeng

8 *et al.* (Jeng et al., 2005). *C. albicans* cells from a 10 l culture were collected by

9 centrifugation at 3,000 × *g* for 10 min at 4°C, washed three times with 50 mM potassium

10 phosphate buffer (pH 7.5), and resuspended in 4 ml of potassium phosphate buffer

11 containing 1 M sorbitol and a protease inhibitor cocktail (Fujifilm Wako Pure Chemical

12 Corp., Osaka, Japan). Zymolyase-100T (0.5 mg/ml, Nakarai Tesque Inc., Kyoto, Japan)

13 and 2-mercaptoethanol (0.1% [v/v]) were added, and the cell suspension was incubated

14 at 37°C for 45 min. The treated cells were centrifuged at 8,000 × *g* for 10 min at 4°C.

15 The supernatant was collected and dialyzed against Tris buffer (20 mM Tris-HCl, 0.5 M

16 NaCl, pH 7.4). The dialyzed cell wall extract was subjected to ammonium sulfate

17 precipitation at 30, 45, 60, 75, and 90% saturation. The precipitates were collected by

18 centrifugation at 12,000 × *g* for 20 min at 4°C, dissolved in PBS and dialyzed against

1 PBS. Fractions possessing binding inhibitory activity were subsequently subjected to
2 anion-exchange chromatography for further purification. The crude component after
3 ammonium sulfate precipitation was dialyzed against the starting buffer (20 mM Tris-
4 HCl, pH 8.0). The samples were passed through a 0.22-μm filter and applied to a Mono
5 Q™ 5/50 GL column (Cytiva) that had been equilibrated with the starting buffer. After
6 sample application, the column was washed with the starting buffer, and the bound
7 material was eluted with a linear gradient (0–1 M) of NaCl in the starting buffer. Each
8 fraction was analyzed for protein by monitoring the absorbance at 280 nm and by SDS-
9 PAGE followed by lectin blotting. Fractions containing mannoproteins or sialoproteins
10 were assayed for binding inhibitory activity.
11

12 2.7 Mass spectrometry analysis

13 Isolated binding inhibitory components were subjected to SDS-PAGE using 12.5%
14 polyacrylamide gels, and the gels were stained with Coomassie Brilliant Blue R-250.
15 Bands of interest were excised and submitted for peptide mass fingerprinting analysis by
16 matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-
17 TOF MS) (Cosmo Bio Co., Ltd., Tokyo, Japan) to identify the corresponding proteins.
18

1 **2.8 Immunofluorescence assay**

2 To examine the localization of binding inhibitory component on the surface of *C. albicans*
3 cells, immunostaining was performed according to a previously described method
4 (Poltermann et al., 2007). Antiserum to the isolated component that inhibits *C. albicans*
5 binding to SRCRP2 was raised in a BALB/C mouse by Cosmo Bio Co., Ltd. *C. albicans*
6 cells were incubated in PBS supplemented with 2% BSA (blocking buffer) at room
7 temperature for 1 h. After blocking, the cells were incubated with mouse antiserum
8 against the binding inhibitory component for 1.5 h at 37°C. Preimmune serum was used
9 as a control. After washing with the blocking buffer, the cells were incubated with Alexa
10 Fluor 555-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA) for
11 1 h at 37°C. Stained cells were mounted on a glass slide and observed using an Eclipse
12 50i microscope equipped with an Intenslight C-HGF1 (Nikon Instech Co., Ltd., Tokyo,
13 Japan).

14

15 **2.9 Fungal cell fractionation**

16 To examine the localization of the isolated component on the surface of *C. albicans* cells,
17 whole cells were fractionated into cell wall and cytoplasmic proteins according to
18 previously described methods (Heilmann et al., 2013; Poltermann et al., 2007), with

1 modifications. The cells were washed with PBS and then subjected to disruption in a
2 micro homogenizing system (Micro Smash™ MS-100; Tomy Seiko Co., Ltd., Tokyo,
3 Japan) with 0.5-mm glass beads in the presence of a protease inhibitor cocktail. The
4 broken cell suspension was centrifuged at 10,000 × *g* for 15 min, and the supernatant was
5 collected and designated cytoplasmic proteins. The pellet was washed three times with
6 1 M NaCl and then washed twice with Milli-Q water. The pellet was resuspended in
7 Milli-Q water and centrifuged at 1,000 × *g* for 10 min to remove contaminating intact
8 cells. After confirming the absence of intact cells by microscopic observation, the
9 supernatant was designated cell wall proteins.
10
11 **2.10 Cell ELISA**
12 A cell ELISA was performed to confirm the localization of the binding inhibitory
13 component on the surface of *C. albicans* cells. Briefly, the wells of microtiter plates
14 were coated with 100 µl of a *C. albicans* cell suspension (1 × 10^9 - 1.5 × 10^6 cells/ml) in
15 PBS at 4°C overnight. After blocking with 1% BSA in PBST, mouse antiserum against
16 the binding inhibitory component was added to the well. Preimmune serum was used
17 as a control. The antibodies bound to the component were detected with horseradish
18 peroxidase-conjugated goat anti-mouse IgG (Bethyl Laboratories Inc., Montgomery, TX).

Color development was performed using an ABTS substrate solution (Sigma Chemical Co.), and the absorbance at 405 nm was determined.

2.11 Western blotting and lectin blotting

Isolated components were subjected to SDS-PAGE and were transferred electrophoretically to nitrocellulose membranes according to the method of Burnette (Burnette, 1981). After blocking with 1% BSA in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, [pH 7.2]) plus 0.1% Triton X-100 (TBS-Triton), the membranes were treated with mouse antiserum against the binding inhibitory component. Preimmune serum was used as a control. After washing with TBS-Triton, the antibodies bound to protein immobilized on the membranes were detected with alkaline phosphate-conjugated goat anti-mouse IgG (Bethyl Laboratories, Inc.). For lectin blotting, Con A, MAM and SSA were biotinylated with biotin 3-sulfo-*N*-hydroxysuccinimide ester (Sigma Chemical Co.). The membranes were treated with a 5 μg/ml solution of biotinylated Con A, MAM or SSA in TBS-Triton, and the lectins bound to glycoproteins immobilized on the membrane were detected with alkaline phosphatase-conjugated streptavidin (Vector Laboratories).

2.12 Statistical analysis

Statistical differences between groups were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, as appropriate.

3 RESULTS

3.1 Binding of *C. albicans* cells to DMBT1 and DMBT1-derived peptides

The interaction between *C. albicans* cells and DMBT1 and DMBT1-derived peptides was examined by ELISA. *C. albicans* NBRC 1385 cells bound to DMBT1 in a cell number-dependent manner (Fig. 1A). The binding was significantly inhibited by soluble DMBT1 added to the reaction mixture in a dose-dependent manner (Fig. 1B), indicating that soluble DMBT1 is specifically involved in this binding reaction. Regarding the effect of divalent cations on the binding of *C. albicans* to DMBT1, the addition of $CaCl_2$ to the reaction mixture significantly enhanced *C. albicans* binding to DMBT1 (Table 1). The other divalent cations, magnesium and manganese, had no effect. To identify the peptide domain involved in *C. albicans* binding, DMBT1-derived peptides were examined in the presence or absence of calcium ions. Of the peptides tested, *C. albicans* cells strongly bound to SRCRP2, while the binding to the other peptides was weak (Table

2). Calcium had no effect on the binding of *C. albicans* cells to SRCRP2, while the addition of $CaCl_2$ to the reaction mixture significantly enhanced *C. albicans* binding to SRCRP5 and SRCRP6.

3.2 Inhibition of *C. albicans* binding to SRCRP2

The inhibitory effects of sugars on the binding of *C. albicans* cells to SRCRP2 were examined. *N*-acetylneuraminic acid and mannose strongly inhibited the binding to SRCRP2, whereas the other sugars showed weak inhibitory effect (Table 3). The effects of three lectins, Con A, MAM and SSA, on the binding were also examined. *C. albicans* binding to SRCRP2 was significantly inhibited by the addition of each lectin in a dose-dependent manner (Fig. 2). MAM and SSA showed stronger inhibitory effects than Con A at low concentrations, and approximately 70% inhibition was observed with 10 μg/ml SSA.

3.3 Isolation of a binding inhibitory component

Anion-exchange chromatography fractions possessing inhibitory activity against *C. albicans* binding to SRCRP2 were pooled and subjected to SDS-PAGE. Coomassie staining of the SDS gel revealed a single 25-kDa band in this fraction (Fig. 3A, lane2).

1 In lectin blotting, Con A, MAM and SSA recognized this band (Fig. 3B). Regarding the
2 binding of *C. albicans* cells to SRCRP2, the isolated 25-kDa proteins significantly
3 inhibited the binding in a dose-dependent manner (Fig. 4). Peptide mass fingerprinting
4 analysis by MALDI-TOF MS revealed that the isolated 25-kDa proteins had 56% protein
5 sequence coverage for the *C. albicans* 60S ribosomal protein L10a.
6
7 **3.4 Localization of the binding inhibitory component on the surface of *C. albicans***
8 To confirm the localization of the 25-kDa protein on the surface of *C. albicans* cells, an
9 antiserum was raised against this protein. Fluorescence microscopy revealed the
10 expression of 25-kDa protein on the surface of *C. albicans* cells (Fig. 5a). Preimmune
11 serum showed no detection (Fig. 5d). In a cell ELISA, antiserum to the 25-kDa protein
12 strongly recognized *C. albicans* cells immobilized on microtiter plate wells, in contrast
13 to preimmune serum (Fig. 6). After cell fractionation, cell wall and cytoplasmic proteins
14 were subjected to SDS-PAGE, and proteins on the gel were transferred to nitrocellulose
15 membranes. The 25-kDa protein was identified among the cell wall proteins by Western
16 blotting using the antiserum (Fig. 7B). Diffuse 97- to 123-kDa bands, a 44-kDa band,
17 and a 42-kDa band among the cell wall proteins and a 44-kDa band among the
18 cytoplasmic proteins were also detected by the antiserum. The preimmune serum

1 showed no reactivity (Fig. 7C).

2

3 **4 DISCUSSION**

4 In the present study, we demonstrated the binding of *C. albicans* cells to DMBT1. *C.*
5 *albicans* bound to immobilized DMBT1 on microtiter plates. In addition, soluble
6 DMBT1 in the reaction mixture inhibited the *C. albicans* binding to immobilized DMBT1,
7 implying that fluid-phase DMBT1 also bound to *C. albicans* cells. Reichhardt *et al*.
8 (2012) stated that no binding of fluid-phase SALSA (DMBT1) to *C. albicans* was
9 observed by flow cytometry, though the data were not shown. The cause of this
10 discrepancy may be ascribed to differences in *C. albicans* strain used or the experimental
11 condition. Among the peptides derived from DMBT1, *C. albicans* cells strongly bound
12 to SRCRP2. Several studies have reported an interaction between bacterial cells or
13 components and DMBT1-derived peptides. Bikker *et al*. (Bikker et al., 2004) reported
14 the binding of SRCRP2 to several bacterial species including *S. mutans, Streptococcus*
15 *gordonii, Escherichia coli,* and *Helicobacter pylori*, and then narrowed down the minimal
16 bacterial binding site on the SRCR domains of DMBT1 to an 11-mer peptide. End *et al*.
17 (End et al., 2009) also reported the binding of *Salmonella typhimurium* to SRCRP2.
18 Regarding bacterial components, Oho *et al*. (Oho et al., 2004) found that the surface

1 protein antigen PAc of *S. mutans*, which is involved in the interaction with DMBT1,
2 strongly bound to SRCRP2. Our results are coincided with these findings. The strong
3 binding of *C. albicans* to SRCRP2 seems to be due to the high isoelectric point (pI = 8.22)
4 of SRCRP2 (Bikker et al., 2004), which confers the high affinity for the negatively
5 charged fungal surface.
6 Calcium ions enhanced the binding of *C. albicans* to DMBT1 but had no effect on its
7 binding to SRCRP2. *S. mutans* PAc also required calcium ions for binding to DMBT1
8 but not for SRCRP2 (Oho et al., 2004). Most interactions between DMBT1 and
9 bacterial cells appear to be calcium dependent (Madsen et al., 2003; Oho et al., 1998).
10 Calcium ions have been reported to play a role in conformational changes within protein
11 molecules, resulting in the exposure of ligand-binding sites (Dainese et al., 2002;
12 Gonzalez et al., 2016). The results obtained in this study suggest that calcium ions might
13 induce a conformational change in DMBT1 to expose the binding domain for *C. albicans*.
14 In addition, SRCRP5 and SRCRP6 might be involved in the calcium-dependent binding
15 of *C. albicans* cells to DMBT1, though the degree of binding appears to be small.
16 *N*-acetylneuraminic acid and mannose showed significant inhibitory effects on the
17 binding of *C. albicans* to SRCRP2. Each lectin, MAM, SSA, and Con A, which
18 recognizes *N*-acetylneuraminic acid and mannose, also significantly inhibited the binding

1 of *C. albicans* to SRCRP2. *C. albicans* has many carbohydrate moieties on the cell wall,
2 and three basic constituents represent the major polysaccharides of the cell wall: branched
3 polymers of glucose, unbranched polymers of *N*-acetyl-D-glucosamine, and mannose
4 polymers covalently associated with proteins (Chaffin et al., 1998). The outer surface
5 of the *C. albicans* cell wall is enriched in mannoproteins, and their mannose residues are
6 involved in the interaction with host molecules (Del Rio et al., 2019; Hall & Gow, 2013).
7 In addition to these carbohydrates, *N*-acetylneuraminic acids have also been identified on
8 the cell surface of *C. albicans* (Lima-Neto et al., 2011; Soares et al., 2000). On the other
9 hand, the *C. albicans*-binding target DMBT1 possesses many kinds of carbohydrates
10 including galactose, fucose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and
11 *N*-acetylneuraminic acid (Oho et al., 1998). In our early experiments, we observed that
12 *N*-acetylneuraminic acid and mannose inhibited *C. albicans* binding to DMBT1 (data not
13 shown), and it was unclear which polysaccharides in *C. albicans* or DMBT1 were
14 involved in the interaction. Therefore, we performed an inhibition assay using SRCRP2
15 as a *C. albicans*-binding target. The results imply that sialic acid- and mannose residue-
16 containing components on the surface of *C. albicans* are involved in fungal binding to
17 SRCRP2. We then searched for sialo- or mannoproteins responsible for the interaction
18 with SRCRP2 in *C. albicans* cell wall extracts. The isolated 25-kDa protein

1 significantly inhibited the binding of *C. albicans* to SRCRP2. However, the binding
2 was not ablated with higher concentration of the 25-kDa protein, implying additional
3 DMBT1-binding adhesins may exist on the surface of *C. albicans*. Lectin blotting
4 confirmed the presence of α2,3- and α2,6-linked sialic acid residues and mannose
5 residues in the 25-kDa protein. The 25-kDa protein was identified in the cell wall
6 proteins by Western blotting. Several other protein bands were also detected by a
7 specific antiserum, indicating that these proteins have an epitope in common with the 25-
8 kDa protein. The localization of the protein on the surface of *C. albicans* cell walls was
9 confirmed by immunofluorescence staining and a cell ELISA using a specific antiserum.
10 These results suggest that the 25-kDa protein is an adhesin localized on the surface of *C.*
11 *albicans* cell walls and is in part responsible for the interaction with DMBT1.

12 The possible mechanism by which the 25-kDa protein inhibits *C. albicans* binding to
13 SRCRP2 may be via electrostatic interactions conferred by *N*-acetylneuraminic acid
14 residues. Since *N*-acetylneuraminic acids are acidic sugars rendering electronegativity
15 (Jones et al., 1995), their involvement in the binding of *C. albicans* to mammalian cells
16 and/or proteins is possible. Sweet *et al*. (Sweet et al., 1995) reported enhanced *C.*
17 *albicans* binding to buccal epithelial cells by sialidase-treated human saliva compared to
18 that with intact saliva, suggesting the involvement of electronegativity induced by sialic

acids. In our study, *N*-acetylneuraminic acid showed strong inhibitory effects on *C. albicans* binding to SRCRP2 at low concentrations, indicating that its electronegativity may be important to the interaction with positively-charged SRCRP2. The mechanism by which mannose inhibits *C. albicans* binding to SRCRP2 remains unclear. Cell surface hydrophobicity is an important factor for the binding of *C. albicans* to target surfaces, and an association of cell wall mannoprotein content and composition with surface hydrophobicity has been reported (Hazen et al., 2001; Masuoka & Hazen, 1997). Further studies on the association between sugar contents and surface hydrophobicity and on sugar structures, including glycosylation patterns, are necessary to elucidate the mechanism.

C. albicans adheres to host ligands, including extracellular matrix, laminin, fibronectin, and collagen, to facilitate its establishment on host tissues (Chaffin, 2008). Many adhesins, including Als, Hwp1, and Iff/Hyr family proteins, in *C. albicans* cell walls have been identified and functionally examined (de Groot et al., 2013; Desai, 2018). Regarding adhesins interacting with salivary proteins, Jeng *et al*. (Jeng et al., 2005) purified two polypeptides (97.4 and 35 kDa) from *C. albicans* cell walls, which possessed inhibitory activity against *C. albicans* binding to saliva-coated hydroxyapatite beads, and identified the 35-kDa protein as the 1,3-β glucosyltransferase Bgl2p by N-terminal

sequencing and immunoblotting. In the present study, peptide mass fingerprinting analysis revealed that the isolated 25-kDa protein had 56% protein sequence coverage for the *C. albicans* 60S ribosomal protein L10a. For better characterization, N-terminal sequencing of the 25-kDa protein was performed. However, no amino acids were identified, probably because the N-terminus was blocked. Ribosomal proteins play an essential role in ribosome assembly and protein translation, and their extraribosomal functions have been appreciated recently (Prakash et al., 2020; Zhou et al., 2015). Several ribosomal proteins were demonstrated to be localized on the surface of pathogens (Singh et al., 2002; Spence & Clark, 2000). Recently, Satala *et al*. (Satala et al., 2021) identified ribosomal protein L10a in the cell wall proteins of *C. albicans* by LC-MS/MS analysis, and their results support our findings. Further studies are necessary to identify it and clarify the function of surface localized L10a using recombinant version of it.

In conclusion, we demonstrated that *C. albicans* binds to DMBT1 and isolated a novel mannose-containing 25-kDa sialoprotein adhesin involved in the interaction with the binding domain of DMBT1. The data presented in this report suggest that the isolated adhesin is localized on the surface of *C. albicans* cell walls and that sialic acid and mannose residues in the adhesin play a significant role in the binding reaction.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

TO conceived and designed the experiments. DS and TO collected and analyzed the data. DS wrote the original draft. TO and EN validated the data and modified the manuscript. All authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data generated or analyzed during this study are included in this article.

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18

TABLE 1 Effects of cations on the binding of *C. albicans* to DMBT1[a]

Cation	Binding ($\times 10^5$ cells)[b]
Calcium	1.52 ± 0.08*
Magnesium	1.05 ± 0.03
Manganese	1.16 ± 0.02
EDTA	1.04 ± 0.04
PBS (no additives)	1.03 ± 0.04

1 [a]*C. albicans* cells (5×10^7 cells/ml) were allowed to react with DMBT1 immobilized on
2 microtiter plate wells (5 µg/ml) in the presence of $CaCl_2$, $MgCl_2$, $MnCl_2$, or EDTA (1
3 mM) and then analyzed by ELISA.

4 [b]Values represent the means ± SEMs of triplicate assays.

5 *$p < 0.05$ compared with the control (PBS), as determined by ANOVA with Dunnett's
6 test.

7

TABLE 2 Binding of *C. albicans* to DMBT1-derived peptides[a]

Peptide	Binding (×10^5 cells)[b]	
	Ca^{2+} (+)	Ca^{2+} (-)
SRCRP1	2.37 ± 0.31	1.97 ± 0.24
SRCRP2	9.33 ± 0.68	9.43 ± 0.28
SRCRP3	3.00 ± 0.25	2.50 ± 0.09
SRCRP4	2.10 ± 0.22	1.47 ± 0.15
SRCRP5	4.33 ± 0.52*	2.00 ± 0.25
SRCRP6	3.67 ± 0.50*	1.60 ± 0.13
SRCRP7	3.50 ± 0.66	2.63 ± 0.52
SID22	2.73 ± 0.14	1.97 ± 0.15

1 [a]*C. albicans* cells (5 × 10^7 cells/ml) were allowed to react with each peptide immobilized

2 on microtiter plate wells (10 nmol/ml) and then analyzed by ELISA.

3 [b]Values represent the means ± SEMs of triplicate assays.

4 *$p < 0.05$ compared with the control (without calcium), as determined by Student's *t*-test.

5

TABLE 3 Effects of sugars on the binding of *C. albicans* to SRCRP2[a]

Sugar	% inhibition[b]
Fucose	5.0 ± 2.2
Galactose	14.0 ± 2.6
Mannose	31.7 ± 0.3
N-Acetylgalactosamine	12.3 ± 5.2
N-Acetylglucosamine	11.0 ± 2.4
N-Acetylneuraminic acid	41.7 ± 1.5

1 [a]*C. albicans* cells (5 × 10^7 cells/ml) were allowed to react with SRCRP2 immobilized on
2 microtiter plate wells (20 µg/ml) in the presence of various sugars (10 mM).
3 [b]Percent inhibition was calculated as follows: percent inhibition = 100 × [(a - b)/a], where
4 a is the mean fungal number without inhibitor (control) and b is the mean fungal number
5 with inhibitor. Values represent the means ± SEMs of triplicate assays.
6

1 **Figure legends**

2

3 **FIGURE 1** Binding of *C. albicans* cells to DMBT1 (A) and the effect of soluble
4 DMBT1 on the *C. albicans* binding to DMBT1 (B). (A) Microtiter plate wells were
5 coated with DMBT1 (5 μg/ml) and *C. albicans* cells in various numbers were examined
6 for binding to DMBT1. The bound cell number was determined by ELISA. (B) *C.*
7 *albicans* cells (5×10^7 cells/ml) were allowed to react with DMBT1 immobilized on
8 microtiter plate wells (5 μg/ml) in the presence of various amounts of soluble DMBT1
9 and then analyzed by ELISA. % control indicates the ratio of the bound cell number to
10 that of the control (DMBT1 = 0). Values represent the means ± SEMs of triplicate
11 assays. $^{**}p < 0.01$ compared with the control (DMBT1 = 0), as determined by ANOVA
12 with Dunnett's test.

13

14 **FIGURE 2** Dose-dependent inhibition of the binding of *C. albicans* cells to SRCRP2
15 by the lectins Con A (●), MAM (▲), and SSA (■). *C. albicans* cells (5×10^7 cells/ml)
16 were allowed to react with SRCRP2 immobilized on microtiter plate wells (20 μg/ml) in
17 the presence of various amounts of lectins. % control indicates the ratio of the bound
18 cell number to that of the control (lectin = 0). Values represent the means ± SEMs of

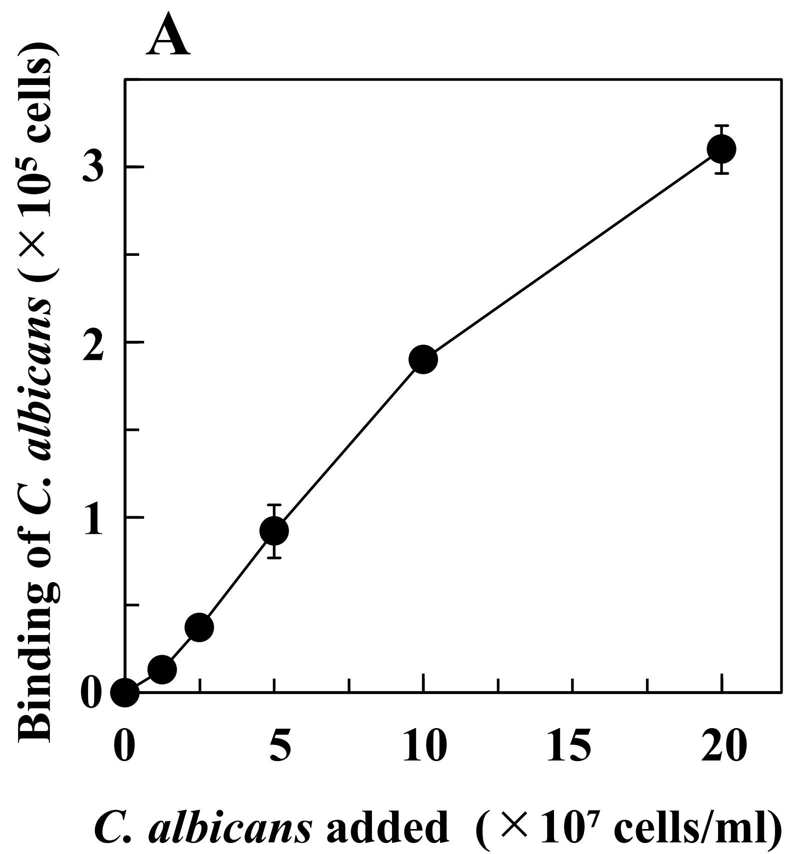
1 triplicate assays. **$p < 0.01$ compared with the control (lectin = 0), as determined by
2 ANOVA with Dunnett's test.
3

4 **FIGURE 3** SDS-PAGE (A), and lectin blotting (B) analyses of the binding inhibitory
5 component isolated by anion-exchange chromatography. (A) *C. albicans* samples were
6 suspended in SDS-PAGE nonreducing (1% SDS) buffer and heated at 100°C for 3 min.
7 The samples were then subjected to SDS-PAGE (12.5% polyacrylamide), and the gels
8 were stained with Coomassie Brilliant Blue R-250. Lanes: 1, precipitates of cell wall
9 extracts at 30% ammonium sulfate (40 μg); 2, isolated binding inhibitory component (1
10 μg). (B) The isolated component (1 μg) on the gel were electrophoretically transferred
11 to nitrocellulose membranes, and the membranes were reacted with biotinylated Con A
12 (lane 1), biotinylated MAM (lane 2), or biotinylated SSA (lane 3).
13

14 **FIGURE 4** Dose-dependent inhibition of *C. albicans* binding to SRCRP2 by the
15 isolated 25-kDa protein. *C. albicans* cells (5×10^7 cells/ml) were allowed to react with
16 SRCRP2 immobilized on microtiter plate wells (20 μg/ml) in the presence of various
17 amounts of the 25-kDa protein (●). BSA (○) was used as a control. % control indicates
18 the ratio of the bound cell number to that of the control (protein = 0). Values represent

1 the means ± SEMs of triplicate assays. $*p < 0.05$, $**p < 0.01$ compared with the control
2 (protein = 0), as determined by ANOVA with Dunnett's test.
3
4 **FIGURE 5** Surface localization of the isolated 25-kDa protein. The surface
5 expression of the isolated 25-kDa protein on *C. albicans* cell walls was examined by
6 immunostaining. *C. albicans* cells were incubated with mouse antiserum against the
7 isolated 25-kDa protein (a, b, c), followed by detection with Alexa Fluor 555-conjugated
8 goat anti-mouse IgG. Preimmune serum was used as a control (d, e, f). The images
9 correspond to fluorescence (a, d), bright light (b, e), and the merge (c, f). The 25-kDa
10 proteins are stained red (a, c). The bar represents 5 μm.
11
12 **FIGURE 6** Cell surface expression of the isolated 25-kDa protein. Microtiter plate
13 wells were coated with *C. albicans* cells (1×10^8 - 1.5×10^5 cells/well), and incubated
14 with mouse preimmune serum (○) or antiserum (●) against the isolated 25-kDa protein.
15 The antiserum reactivity was expressed by A_{405} determined using a cell ELISA. Values
16 represent the means ± SEMs of triplicate assays. $*p < 0.05$ compared with the control
17 (preimmune serum) at each cell number, as determined by Student's *t*-test.
18

FIGURE 7 SDS-PAGE (A) and Western blotting (B, C) analyses of cell wall and cytoplasmic proteins of *C. albicans*. (A) *C. albicans* samples were suspended in SDS-PAGE nonreducing (1% SDS) buffer and heated at 100°C for 3 min. The samples were then subjected to SDS-PAGE (12.5% polyacrylamide), and the gels were stained with Coomassie Brilliant Blue R-250. Lane: 1, isolated 25-kDa protein (1 μg); 2, cell wall proteins (30 μg); 3, cytoplasmic proteins (30 μg). (B, C) Proteins on the gel were electrophoretically transferred to nitrocellulose membranes, and the membranes were reacted with mouse antiserum against the isolated 25-kDa protein (B) or preimmune serum (C). Lane: 1, isolated 25-kDa protein (1 μg); 2, cell wall proteins (6 μg); 3, cytoplasmic proteins (6 μg).



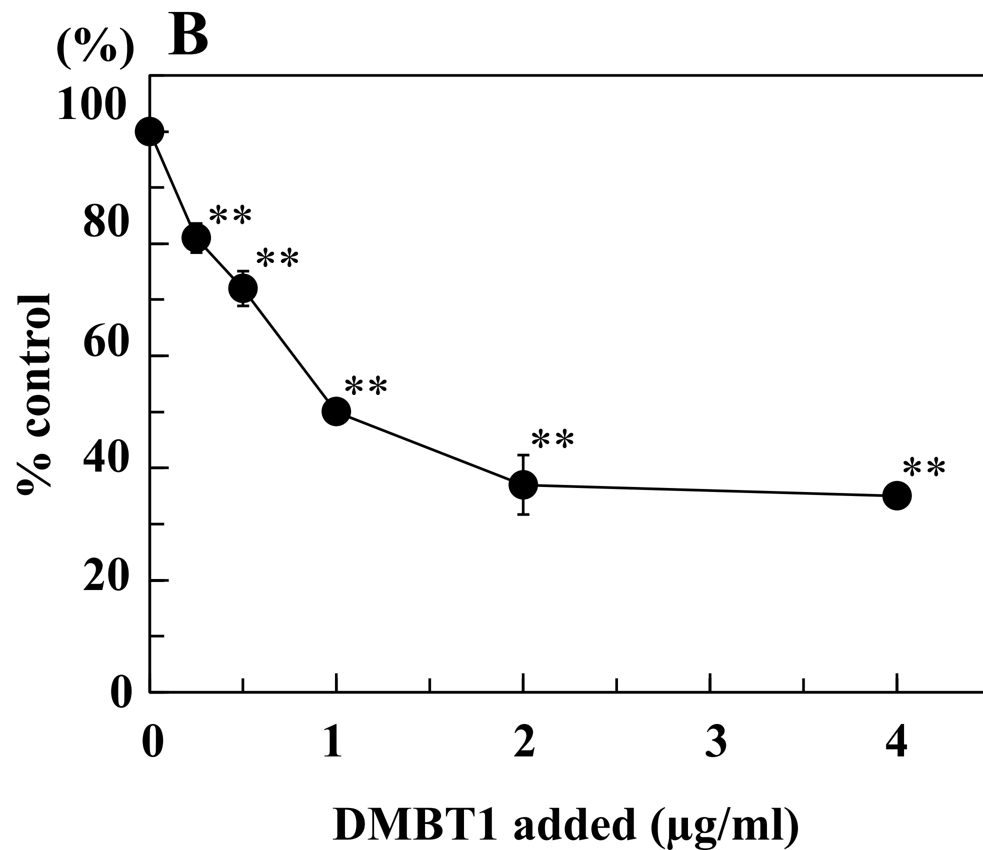


Fig. 1

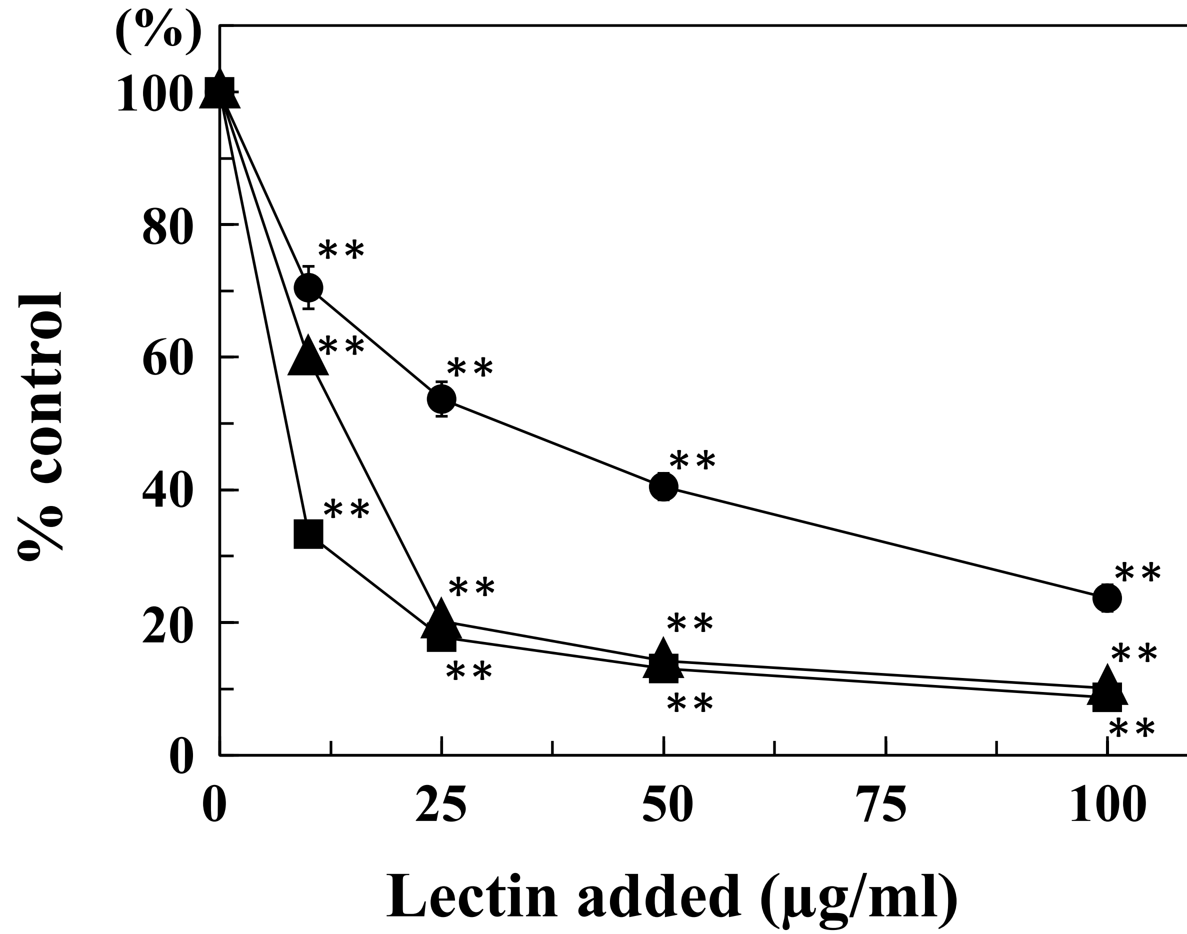


Fig. 2

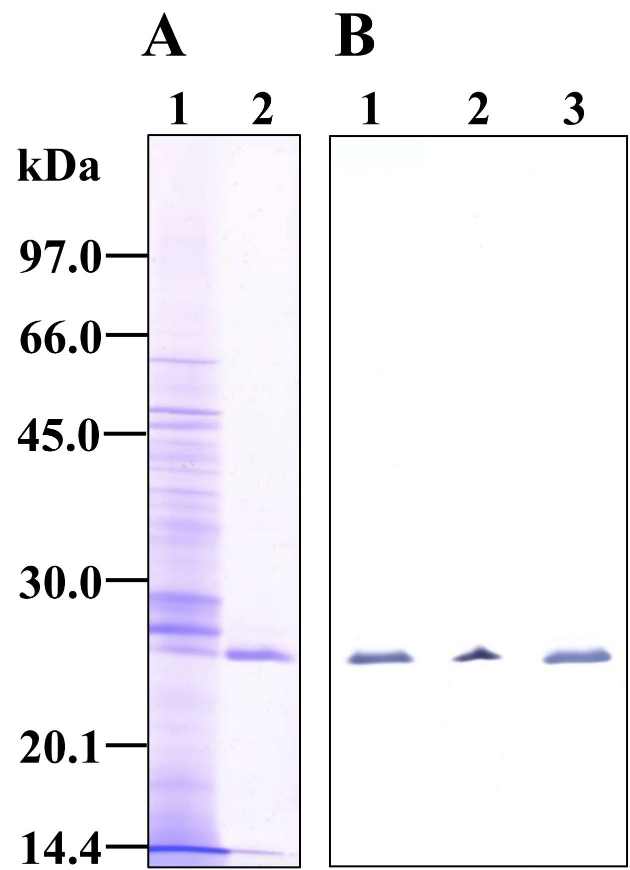


Fig. 3

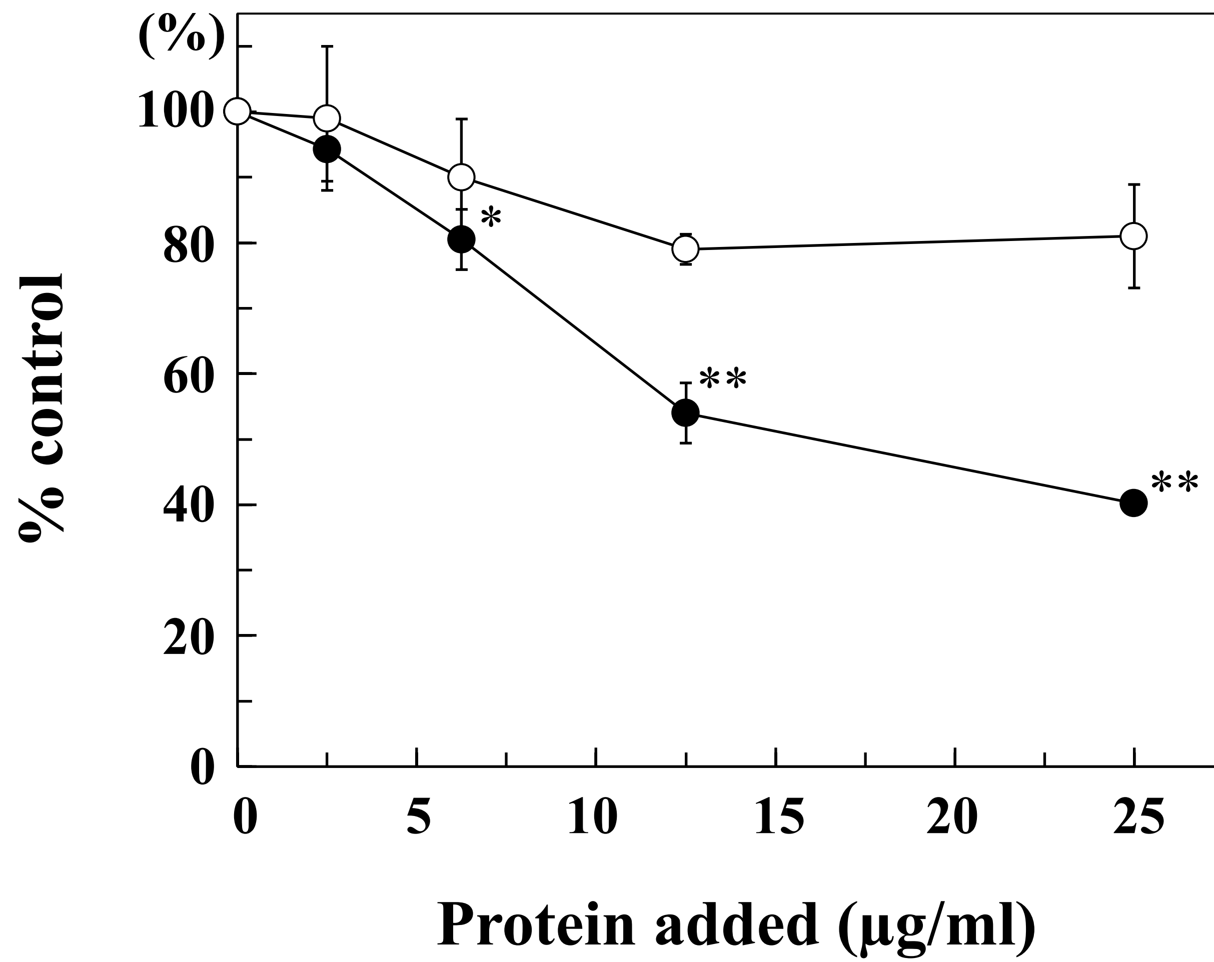
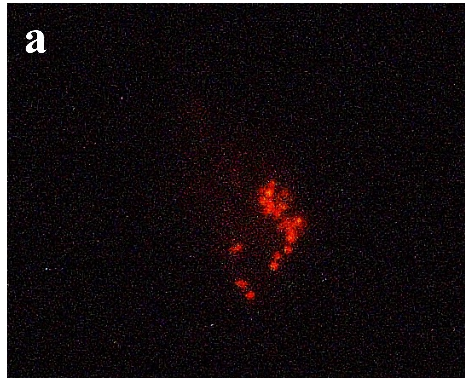
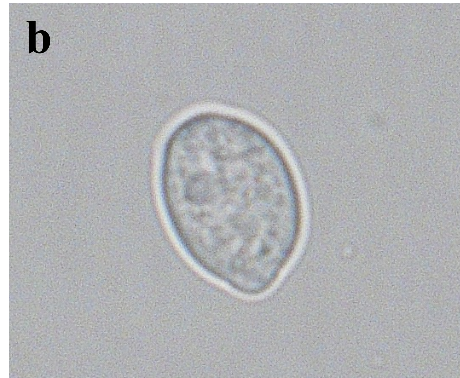
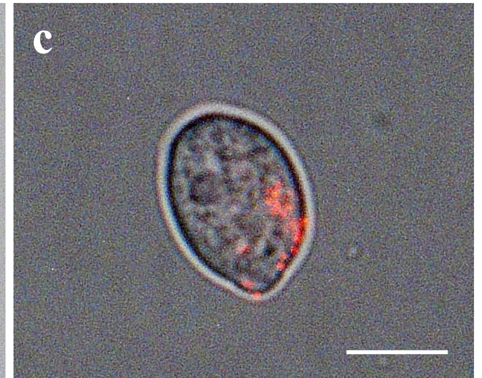


Fig. 4

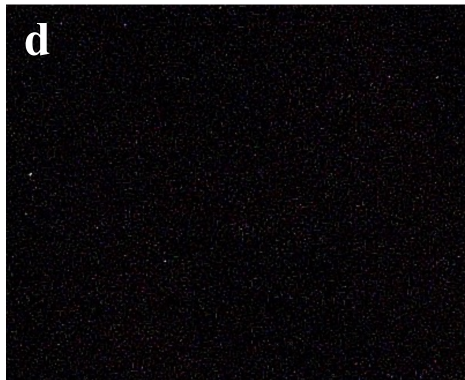
Antiserum

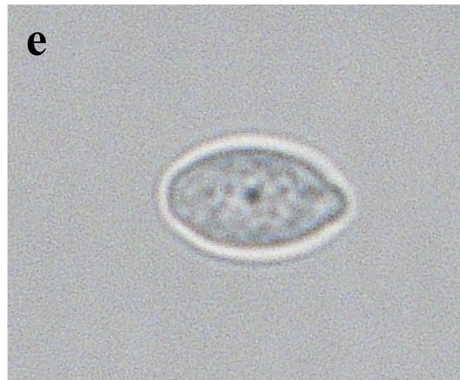






Preimmune serum





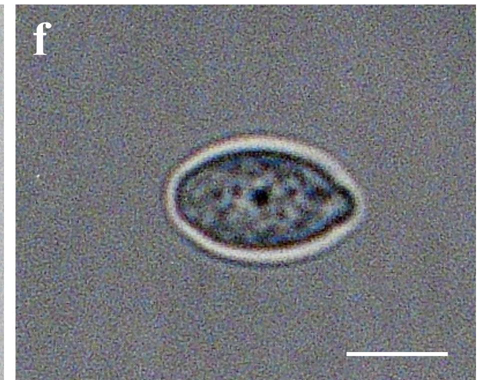


Fig. 5

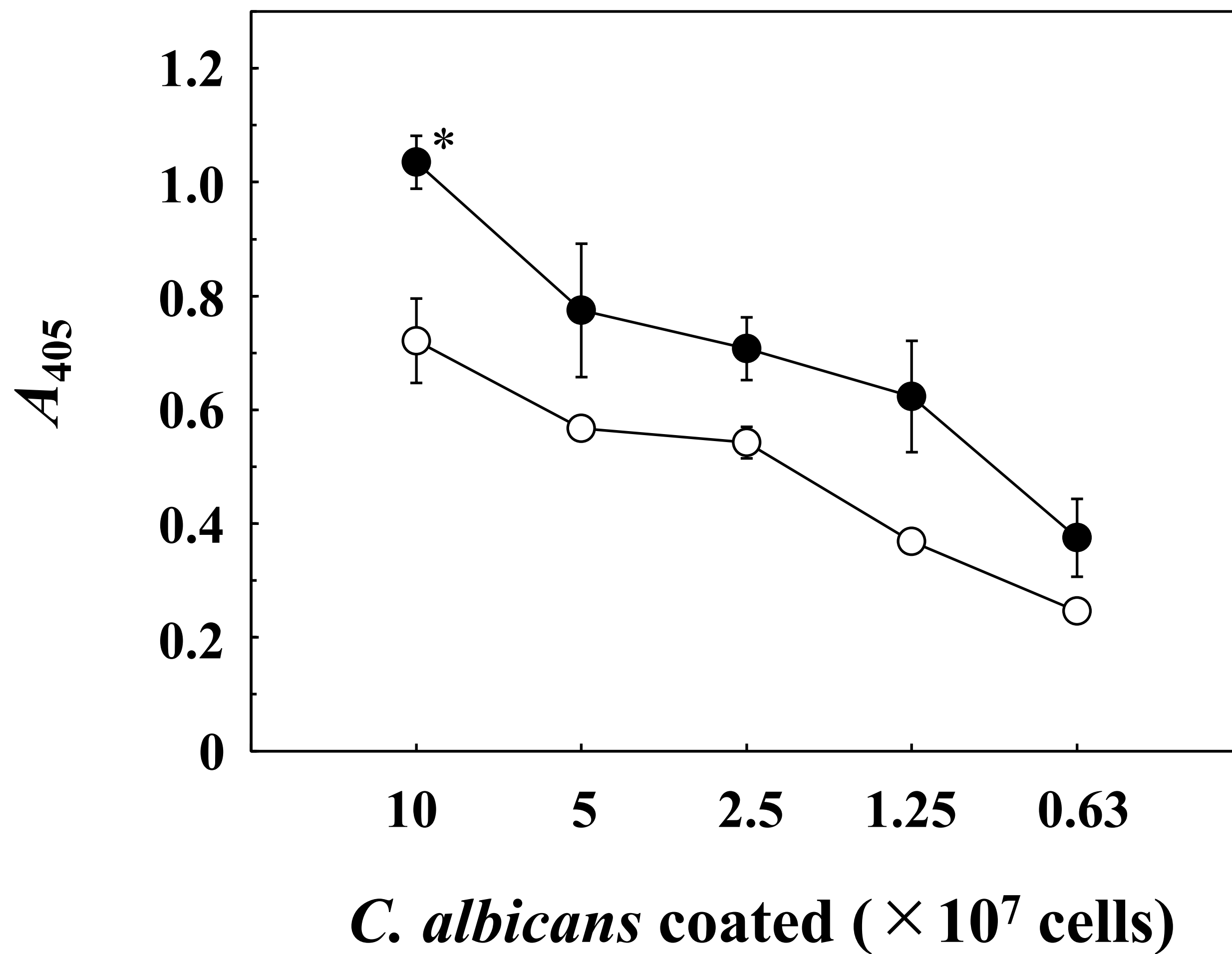


Fig. 6

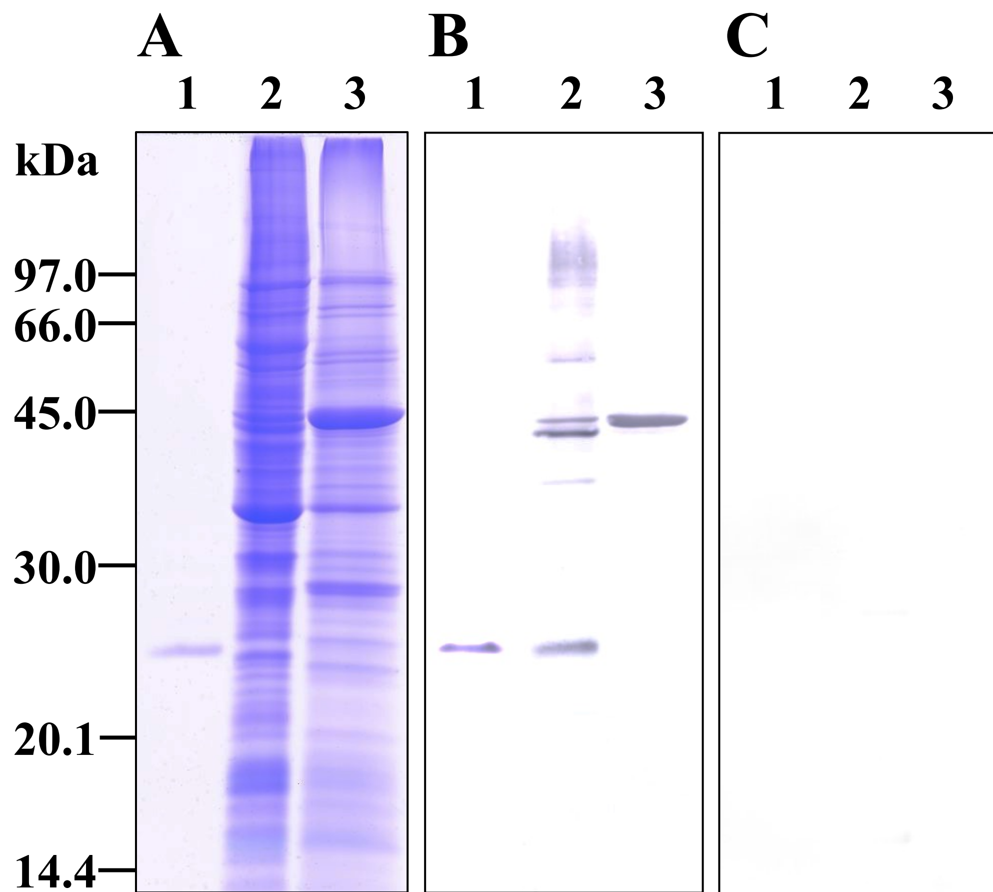


Fig. 7