Analysis of emulsification phenomenon caused by yeast cells with altered cell wall structure and development of novel antifungal drug screening method using the phenomenon

September 2023

Shinsuke Nerome

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細胞壁構造が変化した酵母細胞によって生じる乳化現象の解析とその現象を利用した新規抗真菌薬スクリーニング法の開発

September 2023

Graduate School of Engineering

Osaka City University

Shinsuke Nerome

根路銘　伸介

Contents

Chapter 1

General introduction

1-1 Emulsification phenomenon and biosurfactants from yeast

Emulsification is frequently performed in the food and cosmetics industries to stably mix oil and water. Emulsion attributes to the appearance, texture, mouthfeel, and flavor of food and cosmetical productions [1]. Emulsifiers, which are amphiphilic compounds, are used for the formation and stability of emulsions by adsorb to oil droplet surfaces as shown in Fig. 1-1. Especially, there are two types of emulsions formed by proteins. Flexible proteins, including both hydrophilic amino acid regions and hydrophobic amino acid regions in secondary structures such as α-helix or β-sheet, and change their conformation when they adsorb to oil droplet surfaces. Globular proteins normally have nonpolar and sulfhydryl residues in their inner structures. The hydrophobic structures are exposed to the outside by unfolding when globular proteins adsorb to the oil droplet surface, resulting in emulsion formation with hydrophilic residues in water [1, 2].

In general, chemically synthesized emulsifiers are often used. However, the demand for natural emulsifiers increases because of the health and safety preferences of consumers. Biosurfactants are natural emulsifiers of microbial origin [3]. For example, sophorolipid, rhamnolipid, and mannosylerythritol lipids are well known as glycolipid emulsifiers [1]. Sophorolipid is produced by fermentation using suitable yeast strains, which is composed of a sophorose group and a fatty acid chain. Rhamnolipid is produced by fermentation using certain microorganisms (e.g., *Pseudomonas*

aeruginosa), which is composed of one or two rhamonose units and a fatty acid chain. Mannosylerythritol lipids are produced by basidiomycetous yeast strains in the genera *Ustilago* and *Pseudozyma*, which is composed of mannose, erythritol, fatty acids, and acetyl groups [4]. As lipopeptide emulsifiers, surfactin and iturin are well known [5, 6]. Surfactin is produced by several *Bacillus* species, it is composed of a heptapeptide forming a lactone ring structure with a fatty acid chain. Iturin is produced by several *Bacillus* species, it is a cyclic heptapeptide like surfactin with a β-amino fatty acid chain. As high-molecular-weight biosurfactant, emulsan, mannoprotein and hydrophobin are known. Emulsan is produced by *Acinetobacter venetianus* RAG-1 strain, which is composed of a sugar backbone decorated by fatty acids [7]. Mannoprotein is derived from yeast cell wall, which is composed of a protein with mannan chain [1]. Hydrophobin is produced by filamentous fungi, which is composed of 70–350 amino acids and contains eight conserved cysteine residues that form four disulfide bridges [8]. In this study, I focused on yeast mannoprotein with emulsification activity.

Cell wall mannoproteins of *Saccharomyces cerevisiae* are one of the most effective biosurfactants. *S. cerevisiae* has been used in fermented foods such as bread, miso, and brewing, so it has the potential to be a safe natural emulsifier. These can be easily extracted by heating with sodium dodecyl sulfate (SDS), acid-alkaline treatments, and β-1,3-glucanase treatment [1]. However, in order to use them for emulsification in actual food and cosmetic products, it is necessary to overcome the cost barrier of preparation. Therefore, various techniques to improve the productivity of biosurfactants have been studied [3].

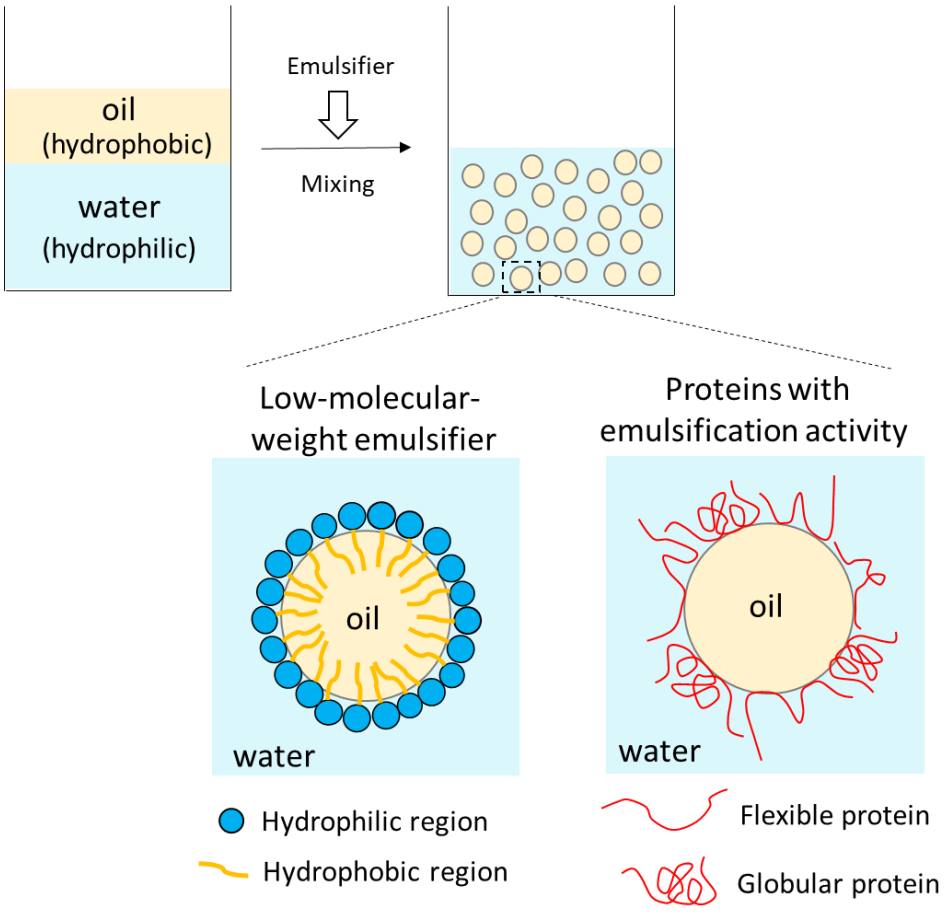

Fig. 1-1 State of the emulsifier at the oil-water interface.

1-2 Yeast cell wall

The yeast cell wall is needed to stabilize cells' internal osmotic conditions, protect cells against physical stress, maintain cell shape, and become a scaffold for proteins. The cell wall of *S. cerevisiae* comprises mannoproteins, β-1,3-glucan, β-1,6-glucan, and chitin, which account for approximately 30%–50%, 30%–45%, 5%–10%, and 1.5%–6% of the dry cell wall weight, respectively [9]. Yeast cell wall structure model is shown in Fig. 1-2. β-Glucan, the skeletal structure of the cell wall, is synthesized by a membrane enzyme using intracellular UDP-glucose as a substrate. Two kinds of β-1,3-glucan synthases, Fks1 and Fks2, are known in *S. cerevisiae*, and Fks2 functions when Fks1 does not work. β-1,3-Glucan chains are composed with a degree of polymerization of ~1500 glucose units per chain and have a coiled spring-like structure, resulting in the elasticity and tensile strength of the cell wall. Additionally, β-1,3-glucan is a scaffold to link the other cell wall components. β-1,6-Glucan chains are composed of ~350 glucose units per chain. β-1,6-Glucan covalently links to β-1,3-glucan, chitin, and mannoproteins. Chitin is a linear polymer with ~100 *N*-acetylglucosamine (GlcNAc) residues via β-1,4-bond, and the crystalline structure contributes to cell wall stretching resistance [10].

Mannoproteins are localized in the outermost layer of the cell wall. These proteins are extensively *N*-linked glycosylated and cover the cells with mannan without gaps. Mannoproteins limit wall permeability to solutions [9]. The majority of mannoproteins are divided into three types: glycosylphosphatidylinositol cell wall proteins (GPI-CWPs), covalently bound to the cell wall; alkali-sensitive linkage (ASL-) CWPs; and SDS-extractable (SE-) CWPs [11]. As shown in Fig. 1-3, GPI-CWPs are attached to β-1,6-glucan through a remnant of the GPI anchor [10, 11, 12, 13, 14]. From genome-wide

identification, there are 66 putative GPI-CWPs in *S. cerevisiae* [15]. ASL-CWPs, such as heat shock proteins and glycolytic enzymes, form alkali-soluble ester bonds between the γ-carboxyl groups of glutamic acid and β-1,3-glucan chains and are released from the cells by a reducing agent under weak alkaline conditions [10, 16, 17]. SE-CWPs link to the cell wall with non-covalent bonds or link to other types of mannoproteins by disulfide bonds and are extracted by heating cells with detergents and thiol reagents [11] (Fig. 1-2).

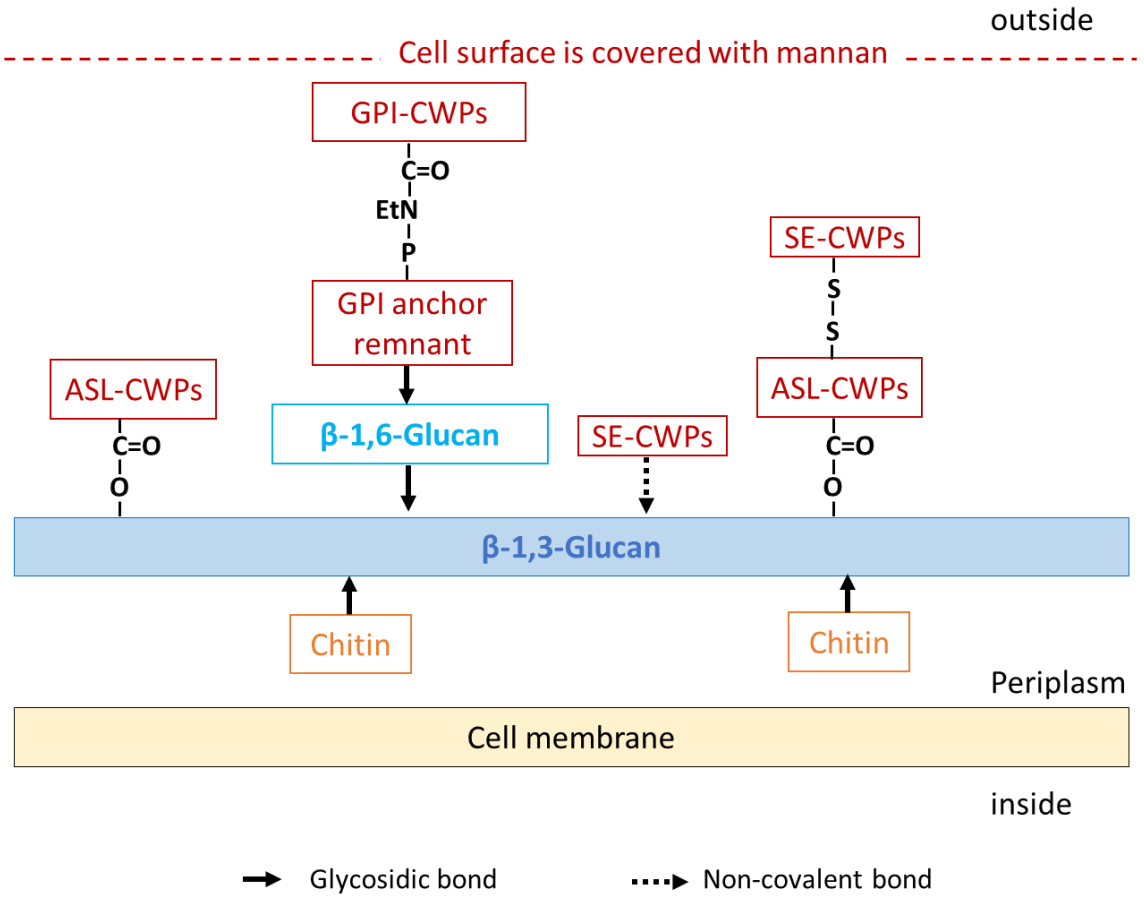

Fig. 1-2 Yeast cell wall structure model. β-1,3-Glucan is a scaffold to link the other cell wall components. β-1,6-Glucan covalently links to β-1,3-glucan, chitin, and mannoproteins. Chitin contributes to cell wall stretching resistance. Mannoproteins are localized in the outermost layer of the cell wall. These proteins are *O*- and *N*-linked glycosylated and cover the cells with mannan without gaps. Mannoproteins limit wall permeability to solutions.

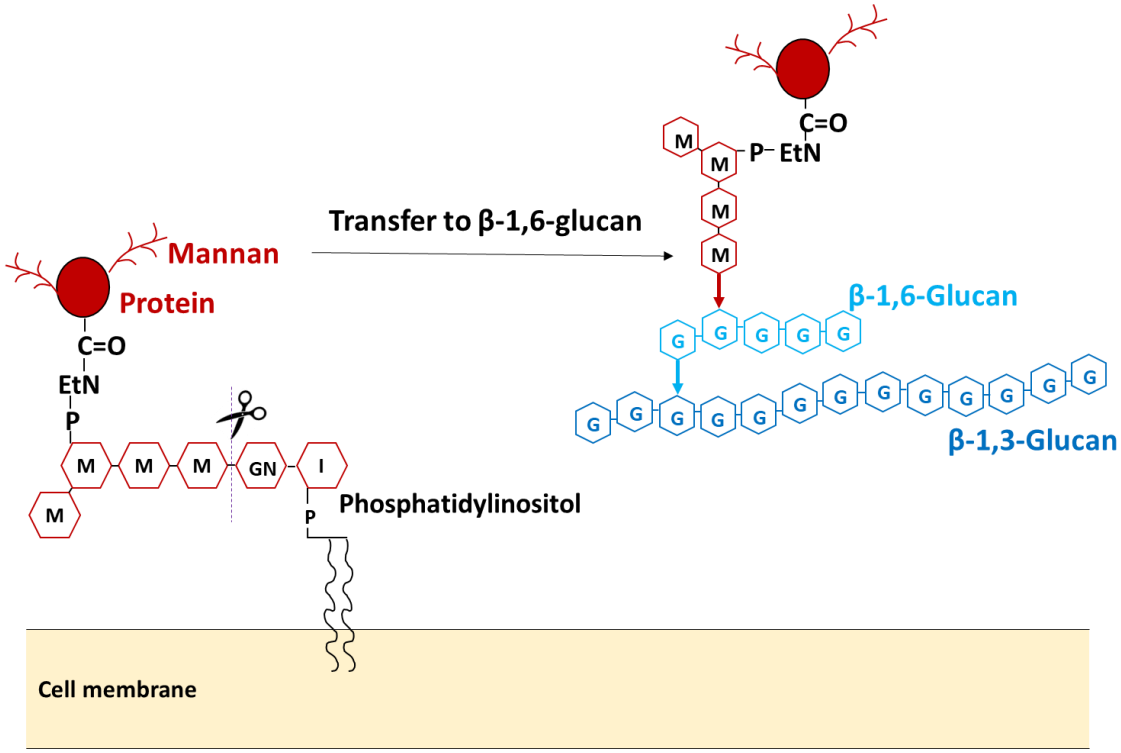

Fig. 1-3 Translation of GPI anchored protein to β-1,6-glucan. M: mannose, GN: glucosamine, I: inositol, P: phosphate, EtN: ethanolamine. GPI-anchored proteins attached to the cell membrane are transferred to the cell wall β-1,6-glucan and connected via the remnant of the GPI anchor.

1-3 Development of antifungal drugs

Recently, severe fungal diseases have affected over one billion people worldwide, with an estimated 1.6 million annual fatalities attributed to fungal infections. While antifungal drugs are essential for treatment, only five categories are used in humans: azoles, echinocandins, polyenes, pyrimidine analogs, and allylamines. As shown in Fig. 1-4, azoles, polyenes, and allylamines act on the cell membrane; pyrimidine analogs target nucleic acid synthesis [18, 19]; and echinocandins inhibit cell wall β-1,3-glucan synthesis [20]. Furthermore, tunicamycin inhibits *N*-linked protein glycosylation in fungi [21], and nikkomycin Z hinders chitin synthesis [22]; however, these cannot be used in humans. Recently, novel antifungal agents have emerged that target the pathway of GPI-anchor synthesis [23]. Developing antifungal agents poses challenges due to the shared cellular components between fungi and mammals, leading to potential side effects. Nonetheless, there is a need to improve the current therapeutic strategies for fungal diseases and develop novel antifungal drugs. The fungal cell wall, an absent component in mammals, represents a promising target for antifungal agents with reduced side-effects [24, 25, 26].

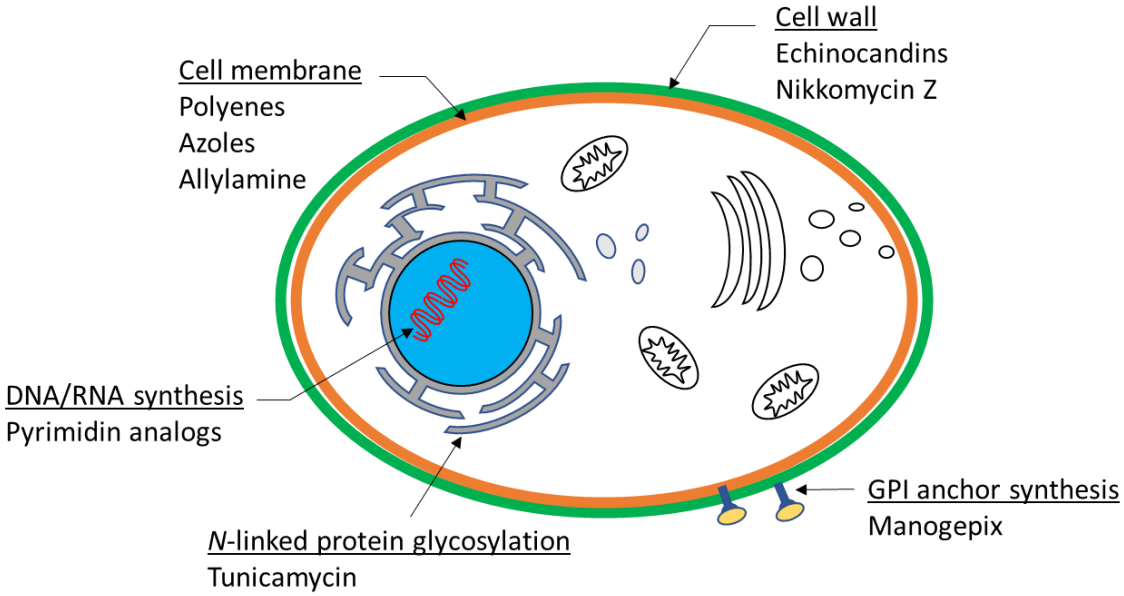

Fig. 1-4 Antifungal agents and their target components.

1-4 Outline of this study

There are two goals in this investigation. One goal is to better understand the link between yeast cell emulsification and yeast cell surface structure from analyses of yeast mutants. These results could promote the use of yeast derived emulsifier in the food and cosmetical productions via improvement of the cost barrier of preparation. The second goal is to utilize emulsification as an indication for identifying inhibition of cell wall formation because emulsification is readily assessed by mixing oil and water. Furthermore, the emulsifying proteins released by inhibition of cell wall formation was analyzed for understanding how emulsification is triggered.

In chapter 2, the emulsification activities of different mutant strains of *S. cerevisiae* were assessed. The results revealed that certain mutant strains with significant reductions in mannan content exhibited strong emulsification activity. These included the *mcd4*Δ strain, which has a defect in the synthesis of GPI anchors, as well as the *anp1*Δ, *och1*Δ, *mnn10*Δ, *mnn11*Δ, and *hoc1*Δ strains, all of which have defects in the formation of *N*-linked glycans.

In chapter 3, I examined whether emulsification was detected upon inhibition of cell wall synthesis. As a result, it was confirmed that proteins released from the cell surface and emulsification were detected when the pathogen *Candida albicans* β-1,3-glucan synthesis was inhibited under osmotic protection.

In chapter 4, I conducted an investigation to determine if inhibiting GPI-anchor synthesis would result in the detection of emulsification. This was based on the understanding that many cell wall proteins are linked to β-glucan through the GPI anchor. The findings revealed that weak emulsification was observed when GPI-anchor

synthesis was inhibited, in comparison to the inhibition of β-1,3-glucan synthesis. Additionally, the study identified several cell wall proteins, namely Phr2, Tkl1, Eno1, and Fba1, as efficient emulsifiers.

In chapter 5, I summarized this study and described the significance of this study and future studies.

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Chapter 2

Cell surface changes that advance the application of using yeast as a food emulsifier

2-1 Introduction

Emulsification is a procedure performed in the food and cosmetics industries. Yeast derived emulsifier is one of candidates of natural emulsifiers, which consumers prefer. In order to use yeast as an emulsifier in food and cosmetic, it is necessary to reduce the cost of preparation. Mannoproteins obtained from β-1,3-glucanase or physical treatment of cells have been reported to have an emulsifying effect. There are also a few reports on the ability of *Saccharomyces cerevisiae* cells to form emulsions [1, 2, 3, 4, 5]. However, there is no research on how to enhance the emulsification ability of the yeast.

In this chapter, with the result as a trigger that strong emulsifying activity in the cells of the yeast mutant *mcd4*Δ were found, I focused on gene mutations that enhance the emulsification ability and screened the gene deletion mutants with strong emulsification activity. Furthermore, the physiological functions of β-glucans, which is main component of the yeast cell wall, are well known [6], and the immunomodulatory effects of *S. cerevisiae* β-glucans have been reported [7, 8, 9]. In the studies performed by Maneesri *et al*. and Sakai *et al*., *mcd4*Δ cells, which is defective in the synthesis of GPI anchors necessary for fixing mannoproteins to β-glucans [10], strongly activated mouse macrophages through direct contact *in vitro* and induced high secretion of tumor

necrosis factor-α (TNF-α) [11]. Therefore, the immunostimulatory of mutants confirmed to have high emulsifying activity was also evaluated.

In this chapter, I focused on *anp1*Δ, *gup1*Δ, *mnn10*Δ, *mnn11*Δ, *hoc1*Δ, and *och1*Δ strains were used. Their functions are briefly described below. Och1, Anp1, Mnn10, Mnn11, and Hoc1 are related to protein *N*-glycosylation. The model of *N*-glycosylation in *S. cerevisiae* is shown in Fig. 2-1. At first, Och1, which is mannosyltransferase, attaches α-1,6-linked mannan residue to the core structure ($Man_8GlcNAc_2$). Subsequently, Mnn9, Van1, Anp1, Mnn10, Mnn11, and Hoc1, which are α-1,6-mannosyltransferase subunits, extend the mannan backbone [12,13]. Gup1 is related to GPI anchor remodeling. The model of GPI anchor remodeling in *S. cerevisiae* is shown in Fig. 2-2. Gup1 transfer a $C_{26:0}$ acyl chain to the *sn*-2 position after removal of the *sn*-2 acyl chain of diacylglycerol by Per1 [13,14].

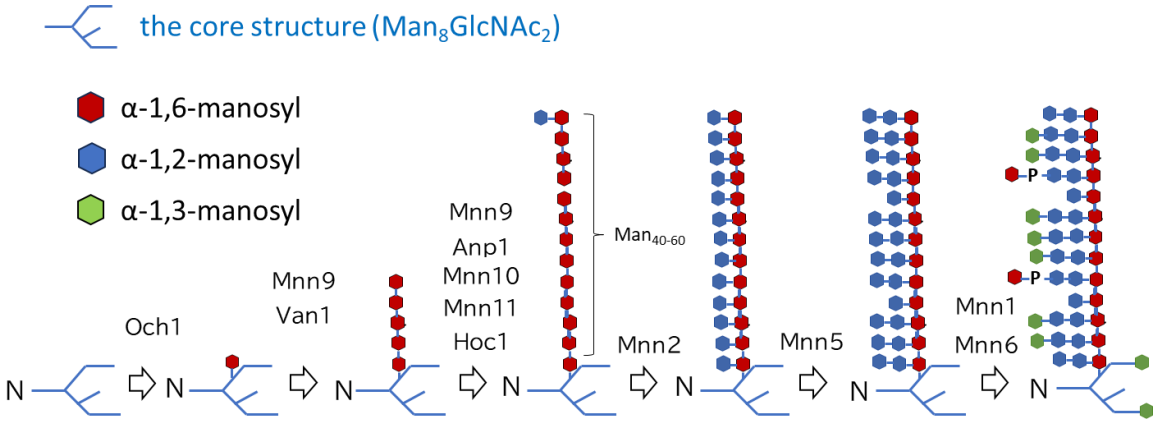

Fig. 2-1 The model of *N*-glycosylation in *S. cerevisiae*. Man: mannose, GlcNAc: *N*-acetylglucosamine. Och1 attaches α-1,6-linked mannan residue to the core structure (Man8GlcNAc2). Mnn9, Van1, Anp1, Mnn10, Mnn11, and Hoc1, which are α-1,6-mannosyltransferase subunits, extend the mannan backbone. Mnn2 and Mnn5 add the α-1,6-linked branches to the mannan backbone. Mnn1 and Mnn6 add the α-1,3-mannose and mannose phosphate, respectively.

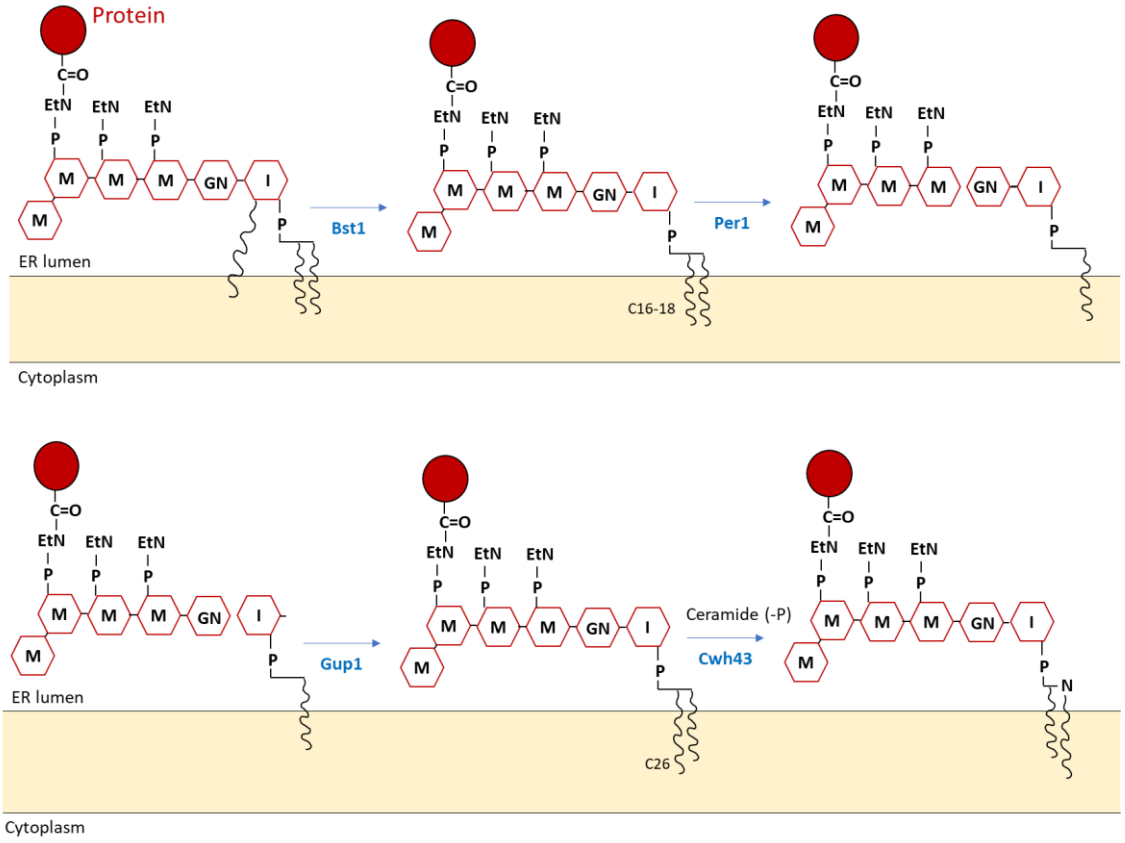

Fig. 2-2 GPI anchor remodeling model. M: mannose, GN: glucosamine, I: inositol, P: phosphate, EtN: ethanolamine, ER: endoplasmic reticulum. In the ER membrane, Bst1 remove the inositol acyl moiety, Per1 remove the *sn*-2 acyl chain of diacylglycerol, Gup1 transfer a $C_{26:0}$ acyl chain to the *sn*-2 position. Subsequently, the diacylglycerol of GPI anchor is replaced with ceramide by Cwh43.

2-2 Materials and methods

2-2-1 Strains and media

S. cerevisiae BY4741 (mating type a [MAT a], *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (mating type α [MAT α], *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was used as the parental strain [15]. Each knockout strains (BY4741 or BY4742, *orf*Δ::*kanMX4*) [16] were purchased from Dharmacon (now Horizon Discovery, Cambridge, UK). The *mcd4*Δ strain (MAT a) was obtained previously from the heterozygous strain [10].

YPD medium (1% [w/v] yeast extract, 2% [w/v] HIPOLYPEPTON, and 2% [w/v] glucose) was used to culture yeast strains. Sorbitol (0.6 M) was added to the YPD medium when osmotic support was necessary; this medium was denoted as YPDS. The yeast extract was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Sorbitol was purchased from Sigma-Aldrich Co. LCC (St. Louis, MO, USA), and other medium components were purchased from Fujifilm Wako Pure Chemical, Inc. (Osaka, Japan).

2-2-2 Emulsification by yeast cells

Each strain was cultured in 4 mL of YPDS medium at 30°C for 48 h, and the cells were harvested by centrifugation at 3000 × *g* for 5 min. The optical density at 600 nm (OD_{600}) was adjusted to 0.7 using phosphate-buffered saline (PBS) containing 0.6 M sorbitol (PBSS), the cell suspension (5 mL) was centrifuged at 3000 × *g* for 5 min, and the pellet was resuspended in PBS (5 mL). In order to evaluate emulsion form, 1 mL of either kerosene or tetradecane was added to the cell suspension, and vortex for 30 sec. After

incubation at 30°C for 1, 24, or 48 h, the formation of an emulsion was visually assessed, *i.e.*, the height and fineness of foams. The emulsification test was performed twice using samples from different cultures to confirm reproducibility.

2-2-3 Microscopic observation of micelles obtained after mixing oil and mcd4Δ cell suspension

The *mcd4*Δ strain was cultured in 4 mL of YPDS medium at 30°C for 48 h. After centrifugation at 3000 × g for 5 min, the OD_{600} value was adjusted to 0.7 using PBSS. Subsequently, the cell suspension (5 mL) was centrifuged at 3000 × *g* for 5 min, and the pellet was resuspended in PBS (5 mL). After mixing tetradecane with the cell suspension, the image of micelles immediately was observed under an Olympus BH50 microscope (Olympus, Tokyo, Japan).

2-2-4 Emulsion formation by substances released from yeast cells

Each strain was cultured in 4 mL of YPDS medium at 30°C for 48 h. After harvesting, the OD_{600} value was adjusted to 0.7 using PBSS. The cell suspension (5 mL) was then centrifuged at 3000 × *g* for 5 min, and the pellet was resuspended in 5 mL of PBS, and then vortexed. After centrifugation at 3000 × *g* for 5 min, the supernatant (5 mL) and 1 mL of kerosene were mixed, and emulsion formation was assessed as described in *section 2-2-2*. Additionally, the precipitate obtained after the last centrifugation was resuspended in 5 mL of PBS, mixed with 1 mL of kerosene, and emulsion formation was assessed.

2-2-5 Ratio of mannose to glucose in the cell wall

The amounts of glucose and mannose in the cell wall were evaluated according to a previously reported method [17, 18]. The BY4741 (wild type; WT), *anp1*Δ, and *gup1*Δ strains were cultured in 100 mL of YPDS medium at 30°C for 24 h, and the *mcd4*Δ strain was cultured in YPDS medium at 30°C for 48 h. After centrifugation at 3000 × *g* for 5 min, the cells were harvested and washed twice with PBSS. The cells were broken using glass beads on ice, and cell walls were collected by centrifugation at 3,800 × *g* for 5 min and then freeze-dried. Then, 3 mg of the collected cell walls was suspended in 75 µL of 72% [w/w] H_2SO_4 and kept at room temperature for 3 h. The obtained slurry was diluted to 1 mL with pure water and heated at 100°C for 4 h. The suspension was cooled on ice and neutralized with saturated $Ba(OH)_2$. After centrifugation at 3,800 × *g* for 5 min, the supernatant was collected and kept at 4°C overnight. The precipitate was removed by centrifugation, and the supernatant was freeze-dried. The amounts of glucose and mannose were measured using a high-performance liquid chromatograph equipped with an RID-6A detector (Shimadzu Corp., Kyoto, Japan) and a Rezex RPM-Monosaccharide column (300 × 7 mm; Phenomenex, Torrance, CA, USA). Milli-Q water (Millipore Corp., Bedford, MA, USA) was used for the elution. The flow rate and column temperature were 1.0 mL/min and 78°C, respectively.

2-2-6 Macrophage activation by contact with yeast cells

Macrophage activation by contact with yeast cells was measured according to a previously described method with some modifications [11]. The WT, *anp1*Δ, and *gup1*Δ

strains were cultured in 100 mL of YPDS medium at 30°C for 24 h, and the *mcd4*Δ strain was cultured for 48 h. The cultured cells were harvested and washed twice with PBSS. The cells were then fixed in 70% [v/v] ethanol and freeze-dried. The dried cells were resuspended in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% [v/v] heat-inactivated fetal bovine serum (ICN Biomedicals, Aurora, OH, USA). The mouse macrophage line RAW264.7 (ATCC TIB-71) was also precultured in RPMI-1640 in 5% CO_2 at 37°C, washed twice with medium, counted, and resuspended in fresh medium at 0.5×10^6 cells/mL. The cells were cultured for 24 h, the culture medium was removed, and then 0.5 mL of new medium containing yeast cells at a final concentration of 100 μg/mL was added. After adding the yeast samples to the macrophages, the solution was incubated in 5% CO_2 at 37°C for 6 h and the supernatant was recovered by centrifugation. The levels of mouse TNF-α in the supernatants were measured using enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA). As the positive control, baker's yeast-derived insoluble β-glucan (Oriental Yeast Co., Ltd.) was used at a final concentration of 50 μg/mL [11].

2-2-7 Emulsion formation by substances released from yeast cells after treatment with β-1,3-glucanase

The BY4741 strain was cultured in 4 mL of YPD medium at 30°C overnight. After centrifugation at 3000 × *g* for 5 min, the cells were harvested and washed with 66 mM phosphate buffer (pH 7.5) containing 1.2 M sorbitol. The OD_{600} value was adjusted to either 1.0 or 3.0 with the buffer. β-1,3-Glucanase (Zymolyase-100T) was added to the

cell suspension (4 mL) at a final concentration of 10 units/mL. The suspension was then incubated at 35°C for 2 h. After centrifugation at 700 × *g* for 5 min, the pellet was washed with the buffer and then resuspended in 4 mL of buffer. The supernatant or pellet suspension (2 mL) was mixed with 1 mL of kerosene, and emulsion formation was assessed as described in *section 2-2-2*.

2-2-8 Analysis of substances released from yeast cells

The *mcd4*Δ strain was cultured in 100 mL of YPDS medium at 30°C for 48 h, and the cultured cells were harvested by centrifugation at 3000 × *g* for 5 min. Subsequently, the OD_{600} value was adjusted to 15 using PBSS, the cell suspension (20 mL) was centrifuged at 3000 × *g* for 5 min, and the pellet was resuspended in 20 mL of PBS. After vortex and centrifugation at 3000 × *g* for 5 min, the supernatant (10 mL) was filtered through an Amicon® Ultra Centrifugal Filter (10,000 or 100,000 molecular weight cutoff, MWCO), and any residual material that did not pass through the filter was diluted to its original volume (10 mL) using PBS. The emulsifying activities of the unfiltered material (top chamber) and the filtrate (bottom chamber) were measured as described above.

2-2-9 Screening of yeast strains with emulsifying activity

First, deletion strains (MAT a) of the Yeast Knock Out Strain Collection were evaluated. In order to make the operation as easy as possible, the cells were cultured on YPDS agar at 30°C for 48 h, and approximately equal numbers were visibly chosen and gathered

using a toothpick from single colonies on the plates and suspended in PBS. A 0.5 mL suspension was mixed with 0.5 mL of kerosene in a 1.5 mL micro tube. After incubation at 30°C for 1 h, emulsion formation was observed. Emulsification similar to that of *mcd4*Δ was evaluated as ++, and the emulsification ability of the tested strains was divided into four categories ranging from − to ++. Strains that exhibited ++ emulsification ability were selected, and the effects of those deletion genes were confirmed from the emulsification ability of gene deletion strains of MATα.

Next, in order to further investigate the details of emulsion formation with the eight selected strains, the cells were cultured in 100 mL of YPDS medium at 30°C for 48 h, harvested by centrifugation at 3000 × g for 5 min, and the OD_{600} value was adjusted to 0.5 with 50 mL of PBSS. The cell suspension was centrifuged, and the pellet was resuspended in 7 mL of PBS. After vortex, the cell suspension was centrifuged again, and the supernatant was collected and lyophilized. The lyophilized material was resuspended in 1.1 mL of pure water; a 1 mL sample was mixed with 1 mL of kerosene, and emulsion formation was observed. Emulsification similar to that of *mcd4*Δ was assessed as +++, and the emulsification ability of mutants was divided into five categories ranging from − to +++. Mutants that showed an emulsification ability of +++ in both MAT a and MAT α were selected.

2-2-10 Statistical analysis

In order to measure the ratio of mannose to glucose in the cell wall as well as macrophage activation by contact with yeast cells, all results are shown as the mean ± standard deviation of independent experiments (n≥3). Welch's t-test was used to

compare two groups. Differences were assessed using a two-sided test with an α-level of 0.05. Significant differences from WT were indicated by an asterisk (*$p<0.05$).

2-3 Results and discussion

2-3-1 Emulsion formation by mcd4Δ cells

*S. cerevisiae mcd4*Δ cells have macrophage activation ability because the mannan layer decreases largely [10]. Then in order to analyze the degree of hydrophobicity on the *mcd4*Δ cell surface, the cell suspension was mixed with tetradecane, and emulsion form was observed. The emulsion formation was maintained for approximately 24 h, after which it weakened after 48 h (Fig. 2-3A). An equivalent emulsion was observed when kerosene was added instead of tetradecane. Such phenomena were not observed in WT cells. In the microscopic observation of the emulsion, yeast cells were dispersed, did not surround the micelles (Fig. 2-3B). This result referred that *mcd4*Δ cells were not directly involved in micelle formation. Emulsion formation by 10% [w/w, on a wet basis] of yeast cells solution and oil has previously been reported [5]; however, in that report, the amount of yeast used was greater than that used in this experiment, and considerable yeast precipitate was observed in the emulsifying test. So, we concluded that changes of cell surface due to the MCD4 deletion enhanced yeast emulsification ability.

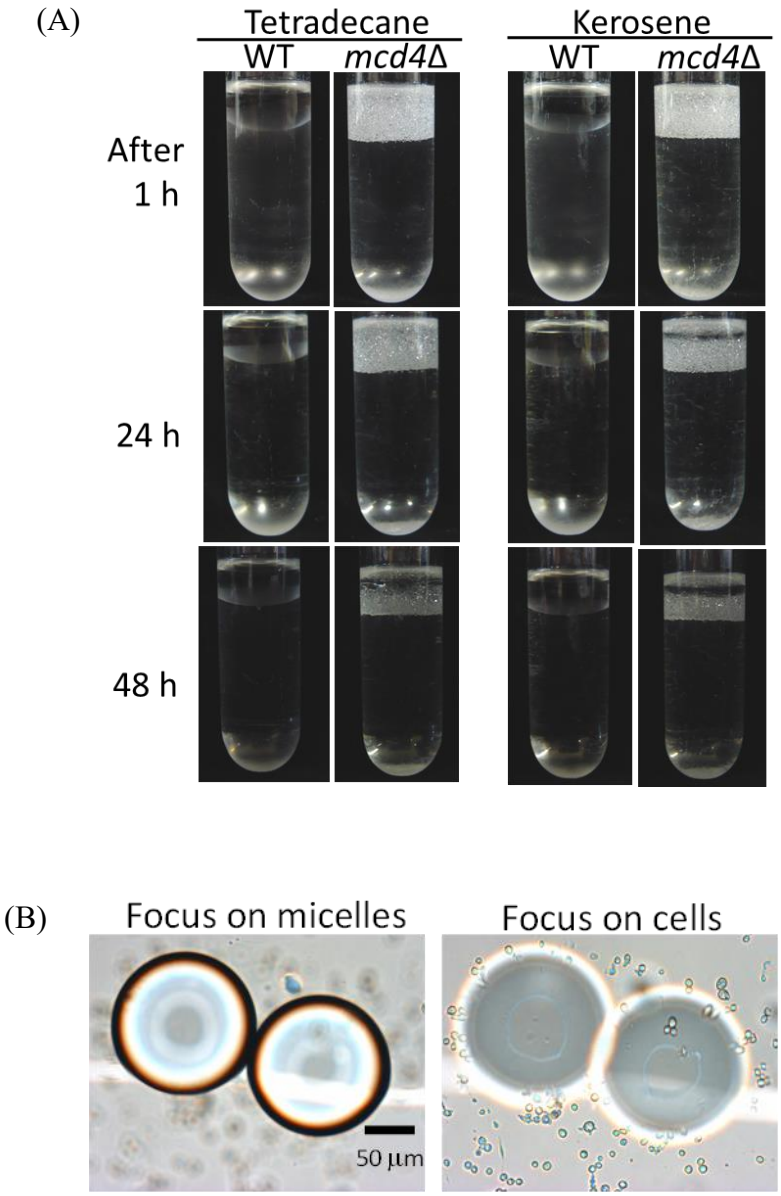

Fig. 2-3 (A) Emulsion formation by mixing oil with an *mcd4*Δ cell suspension and (B) a microscopic image of micelles formed by mixing tetradecane and a *mcd4*Δ cell suspension. Reproduced with permission from Nerome *et al.*, Food Chem. Copyright 2020 Elsevier.

2-3-2 Ratio of mannose to glucose in the cell wall of mcd4Δ, anp1Δ, and gup1Δ strains

Mannan staining using Con A-FITC showed that the amount of mannan in the *mcd4*Δ cell wall was extremely low [10]. However, no quantitative evaluation was performed. Hence, as β-glucans are derived from D-glucose, the ratio of mannose to glucose in the cell wall was examined (Fig. 2-4). As described in *section 1-2*, mannan is located in the outermost layer of the cell wall and covers β-glucans; therefore, it is believed that the ratio of mannose to glucose represents the degree of β-glucan exposure to the cell surface. In addition, the ratios of mannose to glucose in *anp1*Δ and *gup1*Δ strains, which are expected to have a low mannan content, were also measured. Anp1 is a subunit of the α-1,6-mannosyltransferase complex, which is responsible for the synthesis and initial branching of the long α-1,6-linked backbone of the hypermannose structure [12, 13]. Gup1 is involved in remodeling the GPI anchors that are required for the attachment of several CWPs to β-glucans [14]. As expected, the ratio of mannan to glucose in *mcd4*Δ was extremely low (< 0.1). The ratios in *anp1*Δ and *gup1*Δ were also reduced but were higher than those in *mcd4*Δ with an order of magnitude, as follows: *mcd4*Δ < *anp1*Δ < *gup1*Δ < WT.

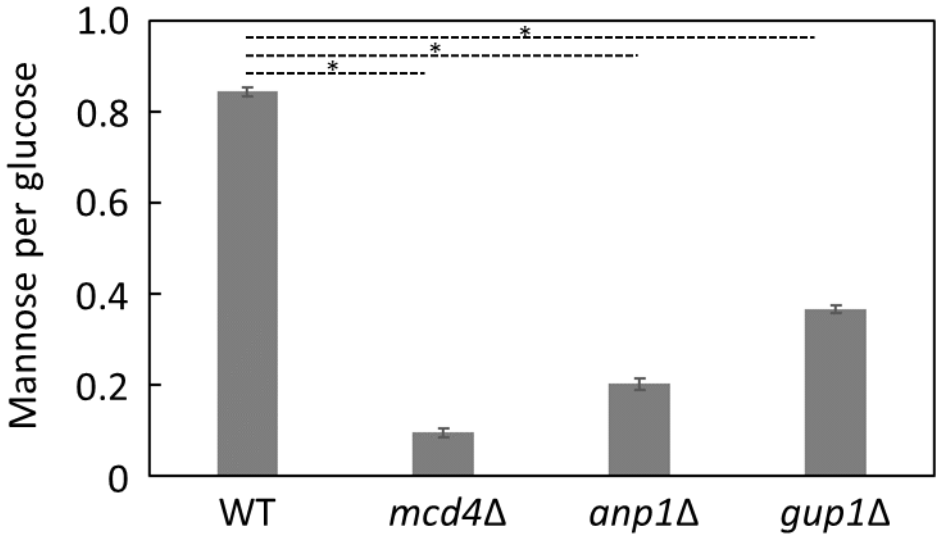

Fig. 2-4 Ratio of mannose to glucose in *mcd4*Δ, *anp1*Δ, and *gup1*Δ. Results are shown as the mean ± standard deviation of independent experiments (n≥3). Significant differences from WT were indicated by an asterisk (*p<0.05). Reproduced with permission from Nerome *et al.*, Food Chem. Copyright 2020 Elsevier.

2-3-3 Macrophage activation activities of mcd4Δ, anp1Δ, and gup1Δ strains

The macrophage activation ability was evaluated by measuring the amount of secreted TNF-α (Fig. 2-5). The activity of *mcd4*Δ was again high similar to previous results [11]. The activity of *anp1*Δ was as high as that of *mcd4*Δ, but the activity of *gup1*Δ was low, which might have been results of low exposure of β-glucans, as observed in the different ratios of mannose to glucose (Fig. 2-4).

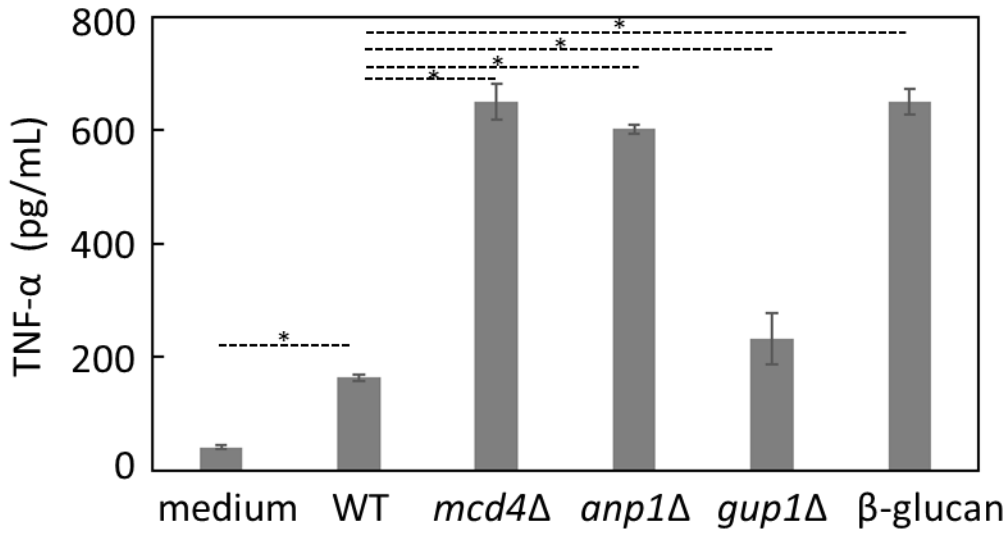

Fig. 2-5 Macrophage activation of the *mcd4*Δ, *anp1*Δ, and *gup1*Δ strains. Results are shown as the mean ± standard deviation of independent experiments (n≥3). Significant differences from WT were indicated by an asterisk (*p<0.05). Reproduced with permission from Nerome *et al.*, Food Chem. Copyright 2020 Elsevier.

2-3-4 Emulsification activity of anp1Δ and *gup1Δ strain*

The emulsification abilities of the *anp1*Δ and *gup1*Δ strains were evaluated (Fig. 2-6), and it was observed that *anp1*Δ strain could form emulsions, but not *gup1*Δ. In *gup1*Δ strain, some of the GPI anchor proteins leaked into the medium [13]. In contrast, *anp1*Δ showed activity similar to that of *mcd4*Δ, except that *mcd4*Δ was always stable and showed high activity, whereas *anp1*Δ occasionally showed weak activity under the same culture conditions, depending on the culture lot, suggesting that the activity of *anp1*Δ might be sensitive to differences in subtle culture conditions. From these results, remarkable decreasing of mannan content might enhance the release of emulsifying substances from cell surface.

There have been no reports on the relation between the yeast emulsification ability and genetic variation. These results of *mcd4*Δ and *anp1*Δ evaluations suggested that there are other strains with a high emulsification ability.

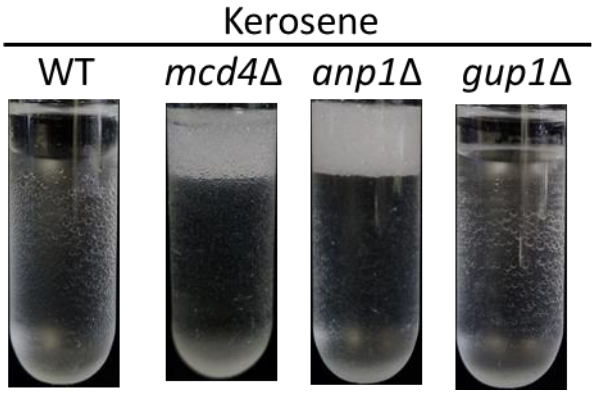

Fig. 2-6 Emulsion formation by mixing oil with *mcd4*Δ, *anp1*Δ, and *gup1*Δ cells suspension. Reproduced with permission from Nerome *et al.*, Food Chem. Copyright 2020 Elsevier.

2-3-5 Emulsification activity of the cells defecting in the formation of N-linked glycans

Results of *section 2-3-4* indicated that high-mannose *N*-linked glycans might be involved in the emulsifying ability of cells. In order to confirm this effect, we evaluated the emulsifying abilities of several mutants with a defect in *N*-linked glycans (Fig. 2-7).

At first, as Mnn10, Mnn11, Hoc1, and Anp1 are subunits of the α-1,6-mannosyltransferase complex [13], the effects of these deletions were examined. As a result, weak emulsion formations were detected in *mnn10*Δ, *mnn11*Δ, and *hoc1*Δ. These results support that a decrease in mannan resulting from an abnormality in the α-1,6-mannosyltransferase complex might enhance the emulsification ability of the cells.

Next, as Och1 is involved in the first stages of *N*-linked glycan formation (i.e., the stage before the action of the α-1,6-mannosyltransferase complex) and the defect makes it impossible to form *N*-linked glycans [13], the effect of the deletion was examined. As a result, the *och1*Δ strain showed high emulsifying activity similar to that of *mcd4*Δ.

From the above results, it was suggested that high-mannose *N*-linked glycans are not directly involved in emulsion formation, but the decrease in mannan from a defect in *N*-linked glycans induces this formation. Glycans might play a role in physically suppressing the release of CWPs involved in emulsion formation by cells.

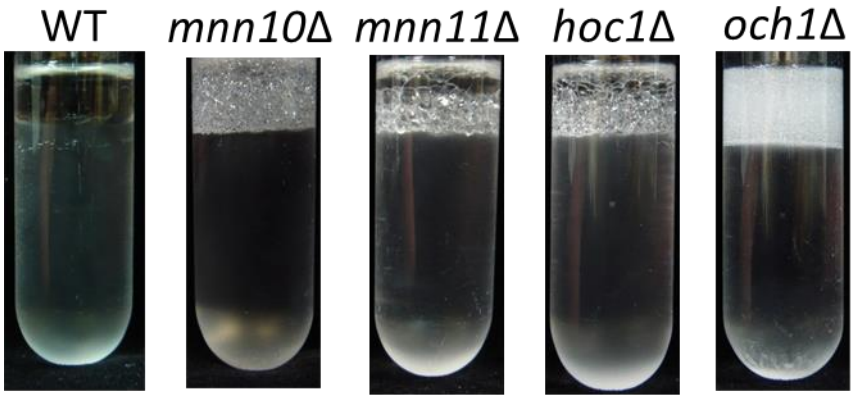

Fig. 2-7 Emulsion formation by mixing oil with *mnn10*Δ, *mnn11*Δ, *hoc1*Δ, and *och1*Δ cells suspension. Reproduced with permission from Nerome *et al.*, Food Chem. Copyright 2020 Elsevier.

2-3-6 Emulsifying activity of CWPs released from cells

S. cerevisiae cell wall mannoproteins is released from cell surface and show efficient emulsification activity when β-1,3-glucan is hydrolyzed with β-1,3-glucanase [1]. As shown in Fig. 2-6A, the released substances in the supernatant exhibited strong emulsion formation. These results and microscopic image in Fig. 2-1B refer that the emulsification ability of *mcd4*Δ cells results from the release of proteins with an emulsification ability from the cell wall.

As described in *section 1-2*, CWPs are localized to the outermost layer of the cell in several ways; and in addition to binding using GPI anchors, there are noncovalently bound proteins [19, 20]. Most CWPs are released from the cells by the above-mentioned β-1,3-glucanase treatment, resulting in emulsification activity (Fig. 2-8A). However, it was presumed that it is difficult to release covalently bound proteins from cells by mixing cells with oil. Therefore, the noncovalently bound proteins might be important for emulsion formation. Then we evaluated the emulsification ability of proteins easily released from the cell wall by washing the cells. The emulsifying activity of the centrifuged supernatants containing proteins released after washing *mcd4*Δ cells cultured in a YPDS medium was measured. When *mcd4*Δ cells were washed with PBSS, the supernatant created by centrifugation showed no emulsifying activity; however, when the cells of the pellet were resuspended in PBS and centrifuged, the supernatant induced emulsion formation. Morphological microscopic observations did not reveal any cell rupture when the cells were resuspended in PBS. The emulsion formation induced by the supernatant was also observed in *och1*Δ. However, it was not observed in similarly prepared supernatants of WT cells (Fig. 2-8B). These results refer that an

emulsion might have been formed by proteins weakly bound to the cell wall, which were released from mutant cells in response to changes in the osmotic pressure when the cells were suspended in PBS.

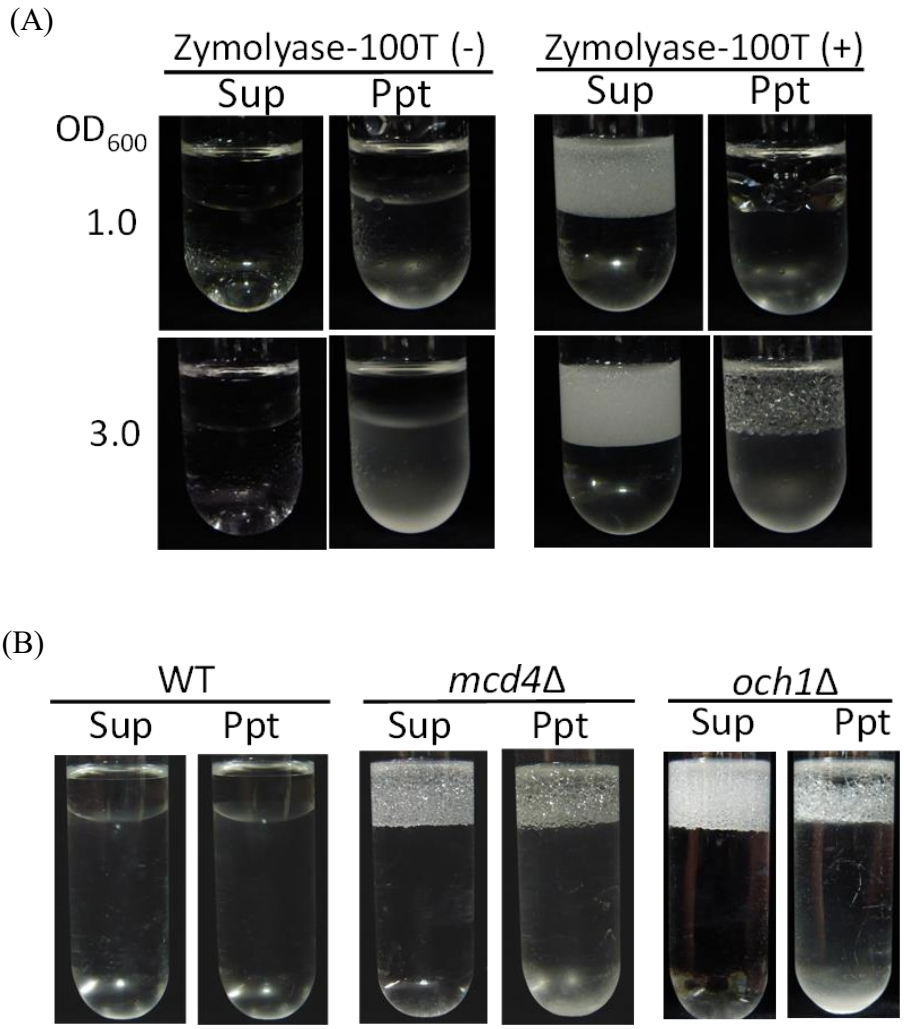

Fig. 2-8 (A) Emulsion formation by mixing kerosene with substances released from WT cells by Zymolyase-100 T treatment. (B) Emulsion by the supernatant and pellet suspension after washing *mcd4*Δ and *och1*Δ cells with PBS. Adapted with permission from Nerome *et al.*, Food Chem. Copyright 2020 Elsevier.

2-3-7 Analysis of released CWPs with emulsifying activity

In order to estimate the molecular weight of the emulsifying substance released from *mcd4*Δ cells, the cell supernatants after resuspension in PBS were filtered using Amicon® Ultra Centrifugal Filters. Strong emulsifying activity was observed in the fractions that did not pass through the 10,000 MWCO filter and in those that did pass through the 100,000 MWCO filter (Fig. 2-9). These results refer that the substances with emulsification activity are not low-molecular-weight materials but have a high molecular weight in the order of tens of thousands. In recent study performed by Onishi *et al.*, GPI-anchor protein Gas1 was identified as an efficient emulsifier [21]. The proteins with emulsification activity should be identified in future studies.

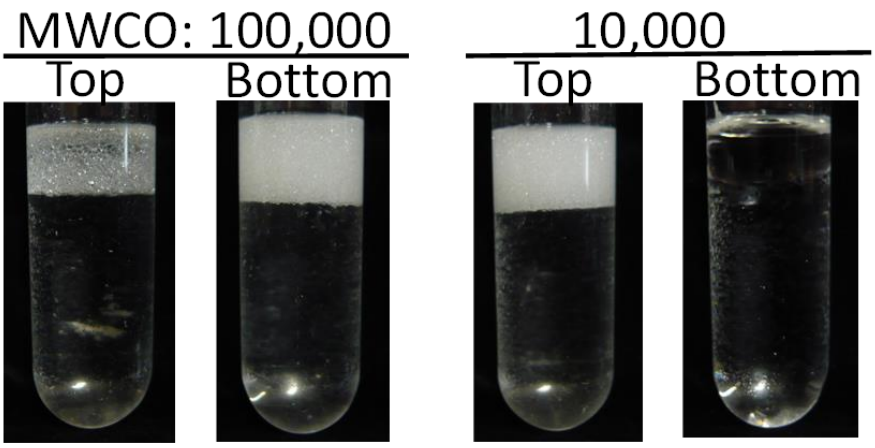

Fig. 2-9 Emulsion formation by mixing kerosene with substances after filtering the *mcd4*Δ supernatant using Amicon® Ultra Centrifugal Filters. MWCO; molecular weight cutoff. Adapted with permission from Nerome *et al.*, Food Chem. Copyright 2020 Elsevier.

2-3-8 Screening yeast strains with high emulsifying activity

In *section 2-3-5*, it was confirmed that the strains with significantly reduced mannan have a strong emulsifying ability. In order to examine other factors that induce emulsion formation, the Yeast Knock Out Strain Collection (5,154 strains) constructed from the BY4741 parental strain (MAT a) was screened. In this screening the emulsifying activity of the cells was used, because measuring cell emulsion formation is very simple. To simplify the experiment, the cells cultured on YPDS solid medium were picked up, and suspended in PBS, and emulsion formation was evaluated using a micro tube, as shown in Fig. 2-10A.

At first, about 5,076 strains showed no activity (evaluated as −). Strains that formed an emulsion similar to that of *mcd4*Δ (evaluated as ++) were selected, and the reproducibility of the effect of those gene deletions was confirmed using strains of MAT α lacking the same gene. In the screening, eight strains (*arp1*Δ, *asc1*Δ, *scp160*Δ, *pop2*Δ, *clc1*Δ, *hpr1*Δ, *ymr001c-a*Δ, and *rvs161*Δ) whose emulsification activity was categorized as ++ in both MAT a and MAT α were selected.

Next, about the selected eight strains, the emulsifying activity of the supernatants obtained after centrifugation of the cells resuspended in PBS was measured. The criteria for the evaluation are shown in Fig. 2-10B. Finally, it was concluded that *asc1*Δ and *scp160*Δ showed strong emulsifying activity equivalent to that of *mcd4*Δ in both MAT a and MAT α (Fig. 2-10C). Scp160 is an RNA-binding protein, and Scp160-dependent mRNA trafficking is known to be involved in pheromone-gradient sensing and chemotropism [22]. Scp160 is not known to be directly involved in cell wall formation; however, the relationship between RNA

metabolism by Scp160 and the cell wall has been described previously [23]. Asc1 is a core component of the small (40S) ribosomal subunit, G-protein β-subunit, and guanine dissociation inhibitor for Gpa2. The direct involvement of Asc1 in the cell wall is also not known; however, the disruption of Asc1 results in increased chitin deposition within the cell wall [24]. Considering their emulsification activities, *asc1*Δ and *scp160*Δ are expected to result in changes in the cell wall structure.

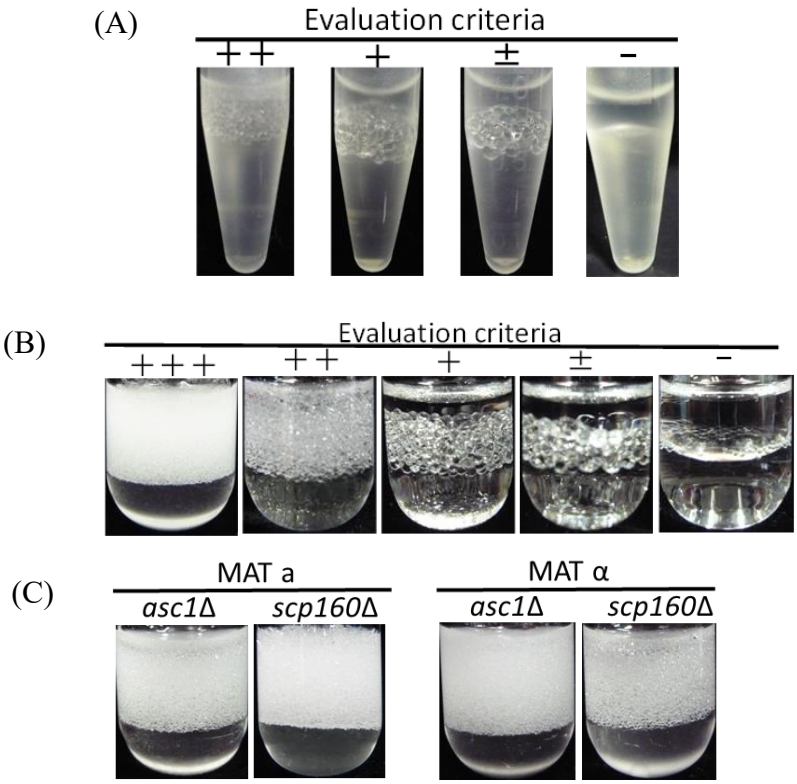

Fig. 2-10 Emulsion criteria for screening and emulsion formation by substances released after washing the cells of the selected strains with PBS. (A) Criterion for cell suspensions, (B) criterion for substances released from cells, and (C) emulsion formed by substances released from cells. Reprinted with permission from Nerome *et al.*, Food Chem. Copyright 2020 Elsevier.

2-3-9 Characterization of asc1Δ and scp160Δ

In *section 2-3-8*, the *asc1*Δ and s*cp160*Δ strains showed strong emulsification activity, then it was examined whether the macrophage activation activity and mannan amount of the strains were similar to *mcd4*Δ and *anp1*Δ strains.

Macrophage activation of *asc1*Δ and s*cp160*Δ was evaluated by measuring the amount of secreted TNF-α (Fig. 2-11A). Although the macrophage activity of *scp160*Δ cells was statistically increased compared to that of WT ($p<0.05$), the activation ability was not as strong as that of *mcd4*Δ (Fig. 2-5). While, there was no statistically significant difference between *asc1*Δ and WT cells.

Furthermore, the ratio of mannose to glucose was examined (Fig. 2-11B). In *asc1*Δ and *scp160*Δ cells, remarkable decrease in the ratio of mannose to glucose was not shown compared to that of *mcd4*Δ and *anp1*Δ (Fig. 2-4). Although the direct effect of these defective genes on cell wall synthesis is unknown, they do affect the cell wall structure [22, 23, 24]. The above results refer that there are also other changes of cell wall structure involved in the emulsification ability, differ from remarkable decreasing of mannan layer.

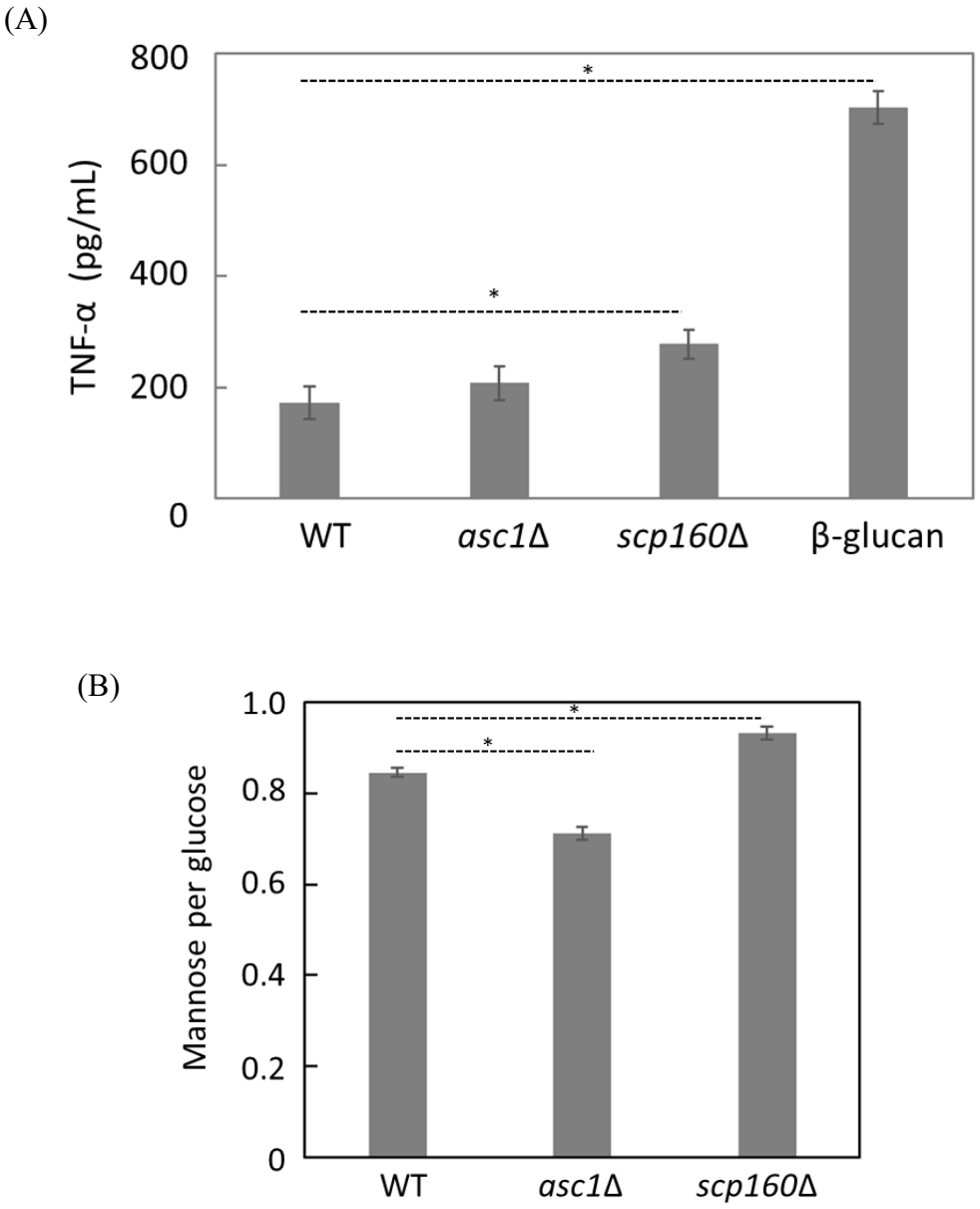

Fig. 2-11 (A) Macrophage activation by contact with *asc1*Δ and *scp160*Δ cells and (B) the ratio of mannose to glucose in *asc1*Δ and *scp160*Δ. Significant differences from WT were indicated by an asterisk (*$p<0.05$). Reproduced with permission from Nerome *et al.*, Food Chem. Copyright 2020 Elsevier.

2-4 Conclusion

The *mcd4*Δ strain which has a defect in the cell wall has strong emulsifying activity. Furthermore, it was confirmed that the substances with emulsifying ability can release from cells by washing with PBS, and they have a high molecular weight in the order of tens of thousands. Additionally, the strains that have a defect in the formation of *N*-linked glycans (*anp1*Δ, *och1*Δ, *mnn10*Δ, *mnn11*Δ, and *hoc1*Δ) have an emulsification ability. After screening the strains that have emulsifying activity, two strains (*asc1*Δ and *scp160*Δ) with a strong emulsification ability were found. However, in *asc1*Δ and *scp160*Δ cells, a large decrease in the mannan content were not shown. Although the direct effect of these defective genes on cell wall synthesis is unknown, they do affect the cell wall structure, and cell wall changes are considered to be important factors that influence the emulsifying activity. There have been no reports that such mutations affect emulsion formation. The findings from this chapter about cell wall mutation and release of emulsifying protein could be useful to improve the productivity of yeast derived emulsifier.

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Chapter 3

Evaluating inhibition of β-1,3-glucan synthesis using emulsion formation as an indicator

3-1 Introduction

As described in chapter 2, the cell wall proteins of *S. cerevisiae* released from cells by β-1,3-glucanase treatment and worked as an efficient emulsifier. Furthermore, cell wall mutant cells, such as *mcd4*Δ and *och1*Δ strain whose mannan remarkably decrease [1, 2, 3, 4], had a strong emulsification activity. From such results, the emulsification was presumed to be caused by the released cell wall proteins. From a different point of view, these results indicated that cell wall changes in *S. cerevisiae* could be evaluated based on the emulsification phenomenon.

Candida albicans is a major pathogen that causes candidiasis infections on the skin, mucous membranes, bloodstream, and deep organs in immunocompromised patients [5]. Antifungal drugs are necessary for the treatment of fungal infections. Five main classes of antifungal drugs are used: azoles, echinocandins, polyenes, pyrimidine analogs, and allylamines [6]. The structures and actions of these drugs are summarized in Table 2-1. Additionally, other antifungal agents used in this chapter is summarized in Table 2-2. Drug side-effects and drug-resistant strains are significant concerns with these drugs; therefore, new antifungal agents are desired [7]. The fungal cell wall is a necessary and unique structure that is absent in mammalian cells, therefore cell wall represents a

promising target for antifungal drugs with low side-effects.

The cell walls of *S. cerevisiae* and *C. albicans* are composed of β-1,3-glucan, β-1,6-glucan, mannoproteins, and chitin, and their composition ratios are very similar [8]. The main component, β-1,3-glucan, is synthesized by a membrane enzyme using intracellular UDP-glucose as a substrate. Two kinds of β-1,3-glucan synthases, Fks1 and Fks2, are known in *S. cerevisiae*, and Fks2 functions when Fks1 does not work. Meanwhile, three types of similar proteins have been reported in *C. albicans*; among them, Fks1 is considered essential for β-1,3-glucan synthesis [9]. From those background, in this chapter, it was examined whether emulsification functions as an indicator of compounds acting on the formation of the major scaffold (β-1,3-glucan) for cell wall proteins. Specifically, it was evaluated whether the emulsion phenomenon occurs using cell wall proteins obtained by β-1,3-glucanase treatment and whether emulsification occurs using the culture supernatant brought about by treating with bioactive compounds, including echinocandins (micafungin and caspofungin) in *C. albicans*.

Table 2-1 Five types of antifungal drugs used in human treatment

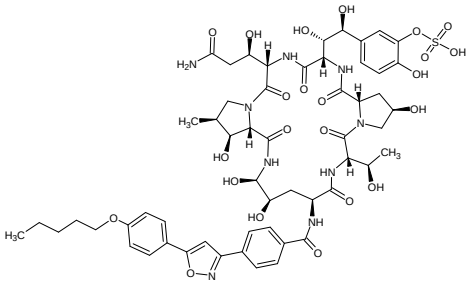
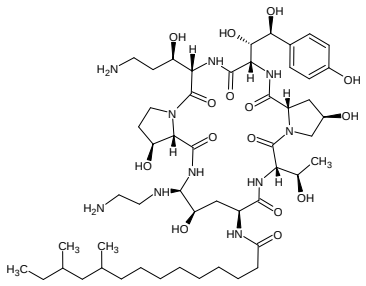
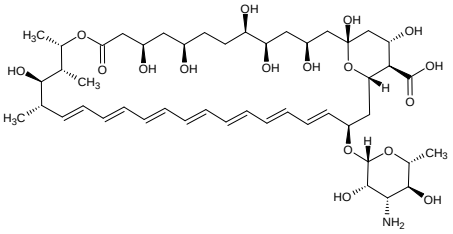
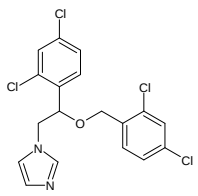
Name, type, and structure	Action mechanism
Micafungin (MCF): echinocandins (Used in this study) 	Inhibition of β-1,3-glucan synthase Fks1 or Fks2, resulting in cell burst by osmotic pressure [10].
Caspofungin (CSPF): echinocandins (Used in this study) 	Inhibition of β-1,3-glucan synthase Fks1 or Fks2, resulting in cell burst, by osmotic pressure [10].
Amphotericin B (AMHB): polyenes (Used in this study) 	Amphotericin B binds to ergosterol on the cell membrane and makes ionophores, resulting in disruption of cell membrane integrity [11].
Miconazole (MCZ): azoles (Used in this study) 	Miconazole inhibits fungal ergosterol synthesis, affecting the cell membrane, resulting in disruption of cell membrane integrity [11].

Table 2-1 Five types of antifungal drugs used in human treatment (Continued)

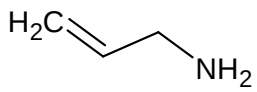
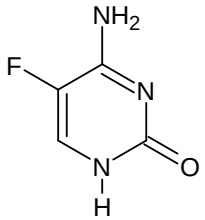
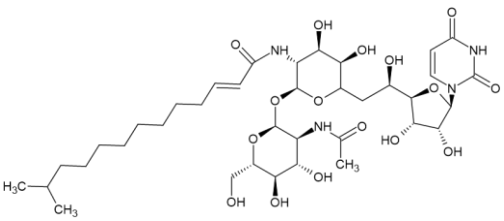
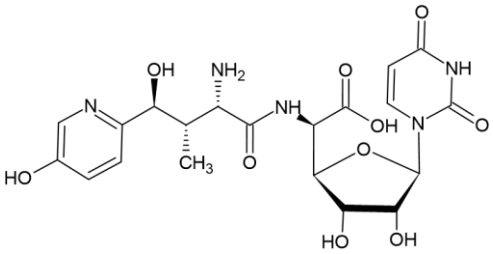
Name, type and structure	Action mechanism
Allylamine 	Allylamine inhibits squalene epoxidase (Erg1). Erg1 catalyzes the conversion of squalene into 2,3-squalene epoxide, resulting in squalene accumulation that may increase permeability, leading to disruption of cellular organization [12].
5-Flucytosine (5FC): pyrimidine analog (Used in this study) 	5-Flucytosine is inserted in the mRNA instead of uracil and inhibits mRNA and protein synthesis [11].

Table 2-2 Other bioactive compounds used in this study

Name and structure	Action mechanism
Tunicamycin (TCM) 	Tunicamycin inhibits *N*-linked protein glycosylation [13].
Nikkomycin Z (NKMZ) 	Nikkomycin Z inhibits chitin synthesis [14].

3-2 Materials and methods

3-2-1 Strains, media, and reagents

The *C. albicans* IFO1061 strain obtained from the Institute for Fermentation Osaka (Osaka, Japan) and the *S. cerevisiae* BY4741 strain [15] were used in this chapter. YPD medium and YNBP medium (0.17% [w/v] Difco yeast nitrogen base without amino acids, 0.5% [w/v] ammonium sulfate, 2% [w/v] HIPOLYPEPTON, 2% [w/v] glucose, 0.06 mg/mL Leu, 0.02 mg/mL His, 0.02 mg/mL Ura, 0.03 mg/mL Lys, and 0.02 mg/mL Met) were used to culture yeast cells. Especially, YNBP medium was used to evaluate emulsification after culturing yeast cells with antifungal agents. Sorbitol (0.6 M) was added to the YNBP medium when osmotic support was necessary; this medium was termed YNBPS. The yeast extract was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Sorbitol was purchased from Sigma-Aldrich Co. LCC (St. Louis, MO, USA), and other medium components were purchased from Fujifilm Wako Pure Chemical, Inc. (Osaka, Japan).

Zymolyase-100T (Mitsubishi Corporation Life Sciences Ltd., Inc., Tokyo, Japan) was used after dissolution in 66 mM phosphate buffer (pH 7.5). Micafungin (MedChemExpress, Monmouth Junction, NJ, USA) was used after dissolution to 5 mg/mL in dimethyl sulfoxide (DMSO). Caspofungin (Abcam plc, Cambridge, UK) was used after dissolution to 5 mg/mL in distilled water. Tunicamycin (Fujifilm Wako Pure Chemical, Inc.) was used after dissolution to 5 mg/mL in methanol. Nikkomycin Z (Sigma-Aldrich Co. LCC) was used after resolution to 5 mg/mL in distilled water. Amphotericin B (Fujifilm Wako Pure Chemical) was used after dissolution to 1 mg/mL

in DMSO. Miconazole (Fujifilm Wako Pure Chemical) was used after dissolution to 10 mg/mL in DMSO. 5-Flucytosine (Fujifilm Wako Pure Chemical) was used after dissolution to 10 mg/mL in distilled water.

3-2-2 Emulsion formation after β-1,3-glucanase treatment in C. albicans

The *C. albicans* IFO1061 strain was cultured in 4 mL of YPD medium at 37°C for 14 to 16 h. After centrifugation at 3000 × *g* for 2 min, the precipitate was washed with 66 mM phosphate buffer (pH 7.5) including 1.2 M sorbitol. The optical density at 600 nm (OD_{600}) was adjusted to either 1.0 or 3.0 with the buffer. β-1,3-Glucanase (Zymolyase-100T) was added to the cell suspension (4 mL) to a final concentration of 10 U/mL. Subsequently, the suspension was incubated at 35°C for 2 h. After centrifugation at 700 × *g* for 5 min, the supernatant was collected, and the precipitate was washed with the buffer and then resuspended in 4 mL buffer. The supernatant and the cell suspension (2 mL) were mixed with 1 mL oil (kerosene). After incubation at 30°C for 1 h, the emulsion formation was visually assessed regarding the height and fineness of foams. The emulsification test was performed three times using samples of different cultures to confirm reproducibility.

3-2-3 Emulsion formation by the culture supernatant after treatment of cells with β-1,3-glucan synthesis inhibitors

The *C. albicans* IFO1061 strain was cultured in 4 mL of YNBP or YNBPS medium at 37°C overnight. The OD_{600} value was adjusted to 0.5 with fresh medium. Micafungin

was added to 4 mL cell suspensions at a concentration of 0.002, 0.01, 0.05, or 0.25 μg/mL. Caspofungin was added to 4 mL cell suspensions at a concentration of 0.01, 0.05, 0.25, or 1.25 μg/mL. Subsequently, the cell suspensions were incubated at 37°C for 24 h. Cell growth was evaluated by the OD_{600} value. After centrifugation at 700 × *g* for 5 min, 2 mL of the obtained supernatant were mixed with 1 mL kerosene, and emulsion formation was assessed as described in *section 3-2-2.*

S. cerevisiae was cultured in 4 mL of YNBP or YNBPS medium at 30°C overnight. The OD_{600} value was adjusted to 0.5 with fresh medium. Micafungin was added to 4 mL cell suspensions at a concentration of 0.01, 0.1, 0.3, or 0.5 μg/mL. Cell growth and emulsion formation were assessed as described above. The emulsification test was performed three times using samples of different cultures.

3-2-4 Emulsion formation by the culture supernatant after treatment of cells with compounds act on N-glycosylation, chitin, cell membrane, and nucleic acid synthesis

The *C. albicans* IFO1061 strain was cultured in 4 mL of YNBPS medium at 37°C overnight. The OD_{600} value was adjusted to 0.5 with fresh medium. Tunicamycin (at a concentration of 0.08, 0.4, 2.0, and 10 μg/mL), nikkomycin Z (0.2, 1.0, 5.0, and 25 μg/mL), miconazole (0.02, 0.1, 0.5, and 2.5 μg/mL), amphotericin B (0.008, 0.04, 0.2, and 1.0 μg/mL), and 5-flucytosine (0.2, 1.0, 5.0, and 25 μg/mL) were added to 4 mL cell suspensions. As a positive control of emulsion detection, micafungin (0.002, 0.01, 0.05, and 0.25 μg/mL) was added to 4 mL of the cell suspension. Subsequently, the cell suspensions were incubated at 37°C for 24 h. Cell growth and emulsion formation were assessed as described in *section 3-2-2*. Regarding emulsification evaluation, emulsions

formed with 0.05 and 0.25 μg/mL micafungin treatment were denoted as +++, and the absence of emulsion formation was denoted as −.

3-2-5 SDS-PAGE analysis of cell wall proteins released after treatment with β-1,3-glucan synthesis inhibitors and other bioactive compounds

As described in section *3-2-3* and *3-2-4*, the *C. albicans* IFO1061 strain was cultured in YNBPS medium including micafungin, caspofungin, tunicamycin, miconazole, amphotericin B, and 5-flucytosine at concentrations of 0.25, 1.25, 10, 2.5, 1.0, and 25 μg/mL, respectively. After incubation, the culture supernatants were collected by centrifugation at 3000 × *g* for 5 min. After second centrifugation at 10,000 × *g* for 5 min, the fine particles were removed. Subsequently, 200 μL of 100% [w/v] trichloroacetic acid was added to the supernatant (1 mL) to a final concentration of 16.6% [v/v], and the pellet obtained after centrifugation at 12,000 × *g* for 5 min was rinsed with acetone to remove trichloroacetic acid and dissolved in 20 μL sodium dodecyl sulfate (SDS) denaturation buffer (62.5 mM Tris-HCl buffer (pH6.8) including 5% [w/v] 2-mercaptoethanol, 2% [w/v] SDS, 5% sucrose, and 0.002% [w/v] bromophenol blue) and treated at 95°C for 10 min. The solution was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% [w/v] acrylamide, and the proteins were detected by staining with Coomassie brilliant blue (CBB).

3-2-6 A simpler assessment method for detecting inhibition of β-1,3-glucan synthesis

C. albicans was cultured in YNBPS medium at 37°C overnight, and the OD_{600} value was adjusted to 0.5 with fresh medium. The cell suspension (500 μL) was dispensed into a small test tube (inner diameter, 8 mm; height, 75 mm; Nichiden-Rika Glass Co., Ltd., Tokyo, Japan), and micafungin was added to the suspensions at a concentration of 0.01, 0.1, 1.0, or 2.0 μg/mL. The suspensions were then incubated in static culture at 37°C for 24 h. The culture broth was directly mixed with 250 μL kerosene. After incubation at 30°C for 30 min, emulsification was visually assessed.

3-3 Results and discussion

3-3-1 Emulsion formation by cell wall proteins released from C. albicans cells after β-1,3-glucanase treatment

As the results of *section 2-3-6*, the cell wall proteins of *S. cerevisiae* released from the cell surface by β-1,3-glucanase treatment worked as an effective emulsifier. As described in *section 3-1*, the cell walls of *S. cerevisiae* and *C. albicans* are very similar [8]. In this section, *C. albicans* cells were treated with Zymolyase-100T as a β-1,3-glucanase and it was examined whether the same phenomenon occurs. *C. albicans* cells were cultured and treated with Zymolyase-100T under osmotic protection (to avoid cell bursting), and emulsion formation was assessed after mixing the centrifugal supernatant or precipitate with oil (Fig. 3-1). When the OD_{600} value of cells was

adjusted to 1.0, emulsification was detected in the supernatant (Sup) but not in the cell suspension after centrifugation (Ppt). When the OD_{600} value was 3.0, strong emulsification was detected in the supernatant, and only very weak emulsification could be detected in the cell suspension. Emulsion formation was not observed without Zymolyase-100T treatment, and furthermore no emulsification was observed in Zymolyase-100T in phosphate buffer. These results strongly supported the contention that cell wall proteins released from *C. albicans* cells by β-1,3-glucanase treatment also have emulsification activity.

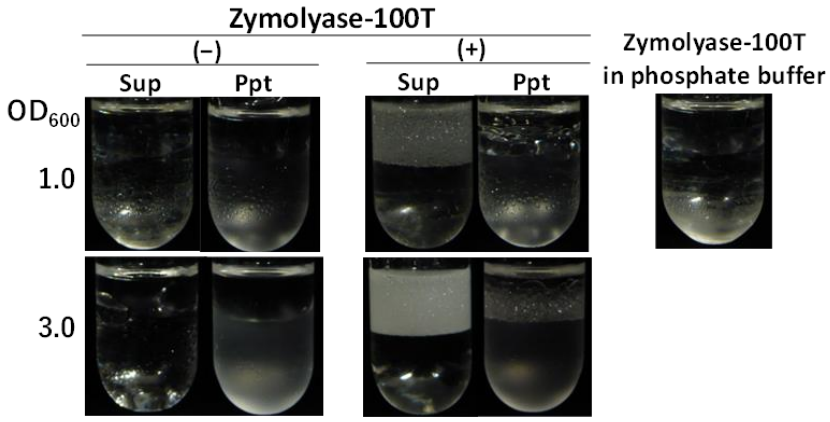


Fig. 3-1 Emulsion formation upon mixing oil with the supernatant (Sup) or suspension of precipitate (Ppt) after centrifugation of cell suspensions treated with and without Zymolyase-100T treatment. Emulsification with a solution of Zymolyase-100T at the concentration used for the treatment was also examined (Zymolyase-100T in phosphate buffer). Reprinted with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2021 Elsevier.

3-3-2 Emulsion formation by the centrifugal supernatant obtained after culturing C. albicans cells in a medium containing β-1,3-glucan synthesis inhibitors under osmotic protection

Micafungin and caspofungin are echinocandin family antifungal agents that inhibit β-1,3-glucan synthase, and the growth inhibition activity is complemented by osmotic protection [16, 17]. Therefore, in this chapter, it was evaluated whether emulsification was also detected in the centrifugal supernatant after cultivation in a medium containing micafungin or caspofungin under osmotic protection.

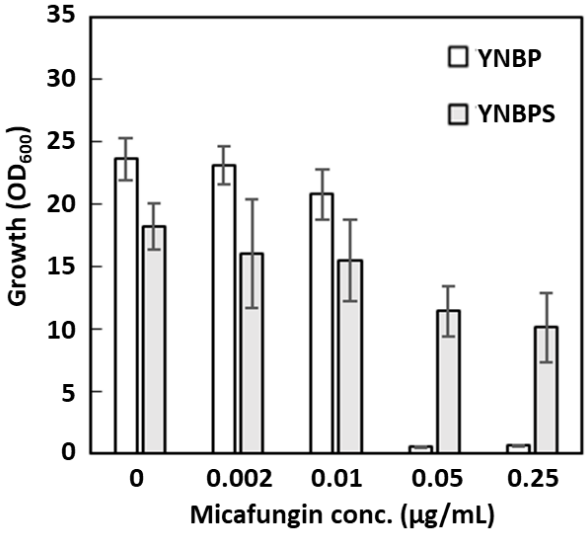
At first, *C. albicans* was cultured at various concentrations of micafungin, and the growth and emulsification activity in the centrifugal supernatants were evaluated. In the culture with osmotic support (YNBPS), *C. albicans* was able to grow even in a medium containing 0.05 and 0.25 μg/mL micafungin (Fig. 3-2A), and remarkable emulsification was detected at these concentrations (Fig. 3-2B). Meanwhile, 0.25 μg/mL micafungin solution without cells did not show emulsification. In contrast, in the culture without osmotic support (YNBP), *C. albicans* could not grow in a medium containing 0.05 and 0.25 μg/mL micafungin, and emulsification was observed only at 0.01 μg/mL. This concentration does not inhibit growth but may have caused some damage to the cell wall, resulting in emulsification. Emulsification was not observed in culture with YNBPS medium at the same concentration, probably because this value is the minimum concentration that damages the cell wall, and the difference may be caused by subtle differences in culture conditions. Further, emulsification was not observed at ≥0.05 μg/mL in the YNBP medium because cells did not grow due to the lack of osmotic protection. The above results under osmotic support suggested that cells with an

incomplete cell wall grew in the medium containing micafungin and that cell wall proteins with emulsification activity were released from cells as they grew.

Next, the effect of caspofungin was examined. Under osmotic protection (YNBPS), *C. albicans* could grow under 0.25 and 1.25 μg/mL caspofungin (Fig. 3-3A), and strong emulsification was detected in the centrifuged culture supernatant (Fig.3-3B). Furthermore, 1.25 μg/mL caspofungin solution without cells did not show emulsification. Without osmotic protection (YNBP), cell growth was completely inhibited under the same concentration, and emulsification was not detected. Emulsification was detected in YNBP medium with 0.01 μg/mL caspofungin and YNBPS medium with 0.05 μg/mL caspofungin. These concentrations showed weak growth inhibition and may have caused some damage to the cell wall. Subtle differences in culture conditions may have affected emulsification near the lowest concentration of caspofungin, affecting the cell wall.

The above results using micafungin and caspofungin indicated that the release of cell wall proteins that cause emulsification is largely dependent on the inhibition of β-1,3-glucan.

(A)



(B)

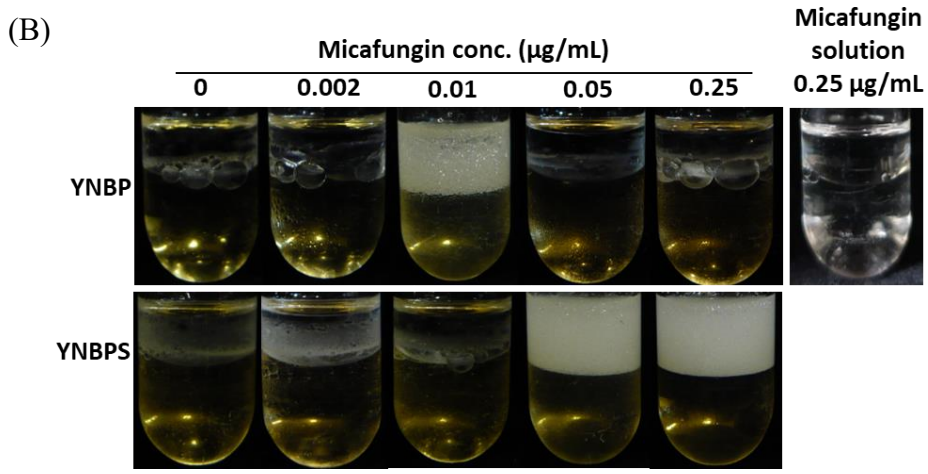


Fig. 3-2 Effect of micafungin on growth and emulsion formation. (A) Growth of *C. albicans* in YNBP and YNBPS media with and without micafungin. (B) Emulsion formation by the centrifugal supernatant obtained after the cultivation of *C. albicans* in those media and micafungin dissolved in distilled water. Growth is shown as the mean ± standard deviation of independent experiments (n=3). Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2021 Elsevier.

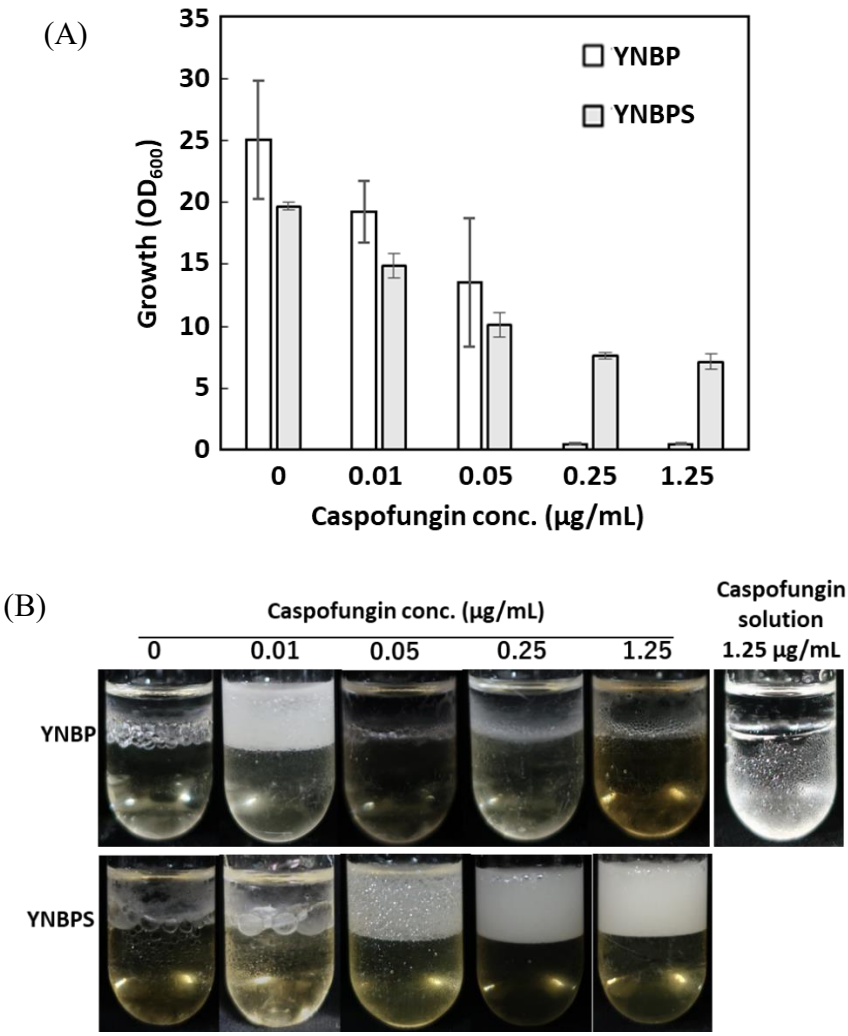


Fig. 3-3 Effect of caspofungin on growth and emulsion formation. (A) Growth of *C. albicans* in YNBP and YNBPS media with and without caspofungin. (B) Emulsion formation by the centrifugal supernatant obtained after cultivation of *C. albicans* in those media and caspofungin dissolved in distilled water. Growth is shown as the mean ± standard deviation of independent experiments (n=3). Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2021 Elsevier.

3-3-3 Emulsion formation by the centrifugal supernatant obtained after culturing S. cerevisiae cells in a medium containing micafungin under osmotic protection

S. cerevisiae also has a similar β-1,3-glucan synthesis mechanism to *C. albicans,* as described in *section 3-1*. Therefore, the emulsification phenomenon by micafungin observed in *C. albicans* was also examined in *S. cerevisiae*. Consequently, emulsification was also detected in *S. cerevisiae*, however this required higher concentrations of micafungin than those for *C. albicans* (Fig. 3-4A, B). This is because that it may be different affinity of the drug for the target enzymes or different levels of expression of the enzymes, between the two species. In addition, the emulsification observed for *S. cerevisiae* was weaker than that for *C. albicans.* This is considered to be due to the difference in growth recovery by osmotic support.

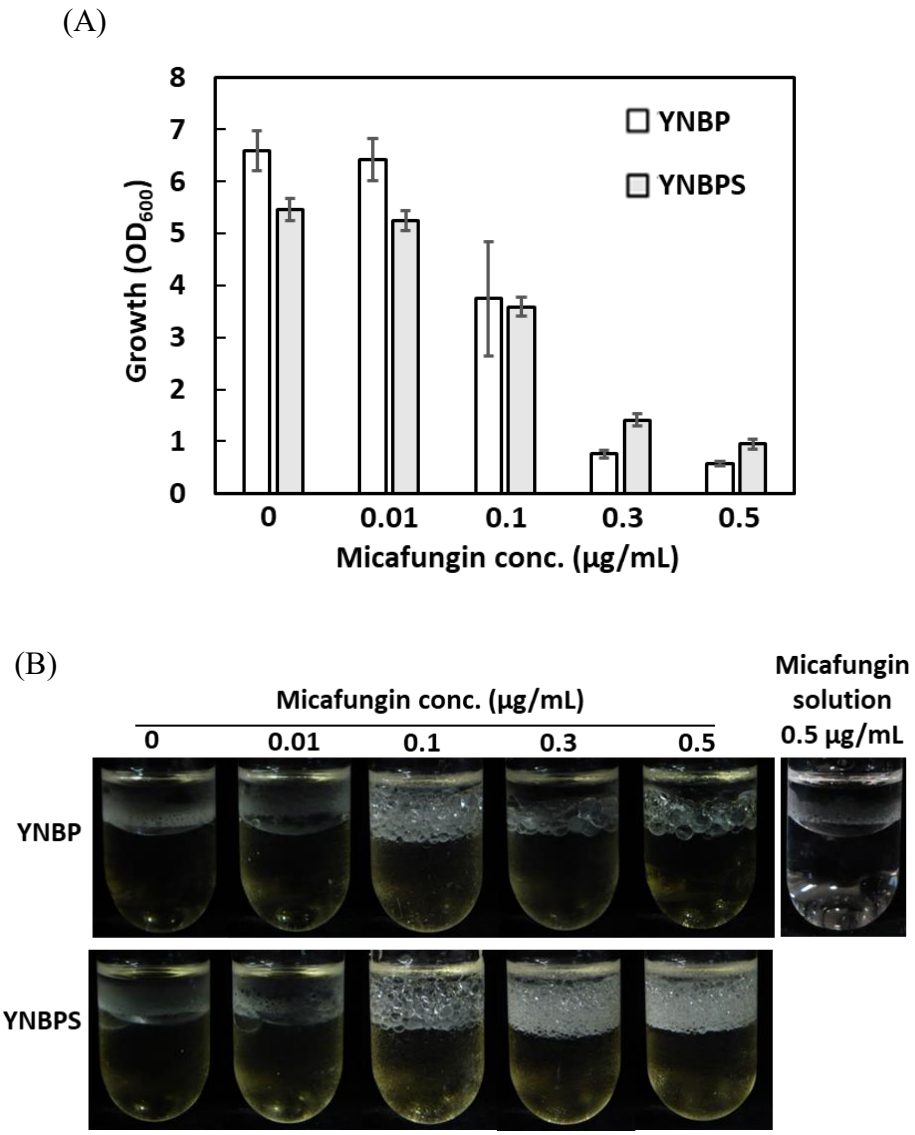


Fig. 3-4 Effect of micafungin on growth and emulsion formation. (A) Growth of *S. cerevisiae* in YNBP and YNBPS media with and without micafungin. (B) Emulsion formation by the centrifugal supernatant obtained after the cultivation of *S. cerevisiae* in those media and micafungin dissolved in distilled water. Growth is shown as the mean ± standard deviation of independent experiments (n=3). Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2021 Elsevier.

3-3-4 Effects of the addition of other bioactive compounds on emulsion formation; inhibitors act on N-linked glycosylation, chitin, cell membrane, and nucleic acid synthesis

Strong emulsification by the culture supernatant was detected after the addition of β-1,3-glucan synthesis inhibitors (micafungin and caspofungin) into the osmotic stabilized medium. In order to confirm whether this emulsion phenomenon is peculiar to β-1,3-glucan synthesis inhibition, the effects of other compounds on the emulsification were examined. Five compounds of different class were used. Tunicamycin, which was identified and isolated by Takatsuki *et al*. (1971) [18], inhibits *N*-linked protein glycosylation [13]. Nikkomycin Z inhibits chitin synthesis, affecting the cell wall [14]. Amphotericin B binds to ergosterol on the cell membrane and makes ionophores but does not affect cell wall components. Miconazole inhibits fungal ergosterol synthesis, affecting the cell membrane. 5-Flucytosine is inserted in the mRNA instead of uracil and inhibits mRNA and protein synthesis [10, 11]. Regarding each compound's effects on cell growth, partial growth inhibition was observed at a specific concentration range (Fig. 3-5B). However, 25 μg/mL nikkomycin Z did not show growth inhibition under this culture condition although inhibition was expected from the literature [14]. Emulsification by the culture supernatant of *C. albicans* cultured in YNBPS medium, including each compound, was evaluated. The criteria of the emulsification from – to +++ are shown in Fig. 3-5A. However, no emulsification was observed for the other compounds.

In chapter 2, emulsification was observed in cell suspensions and cell washes in the *S. cerevisiae och1*Δ strain, which has a defect in *N*-linked protein glycosylation and

lacks the hypermannose structure [7]. Therefore, emulsification was expected after treatment of tunicamycin, which inhibits *N*-linked glycosylation. However, no emulsification was observed. The reason is unclear, but it may be attributed to the differences between *C. albicans* and *S. cerevisiae* or the differences between gene mutation and addition of the compound. Furthermore, no emulsification was observed with compounds that act on the cell membrane or inside the cells.

From the above results, it was confirmed that emulsification was specifically detected when β-1,3-glucan synthesis was inhibited in an osmotic stabilized medium.

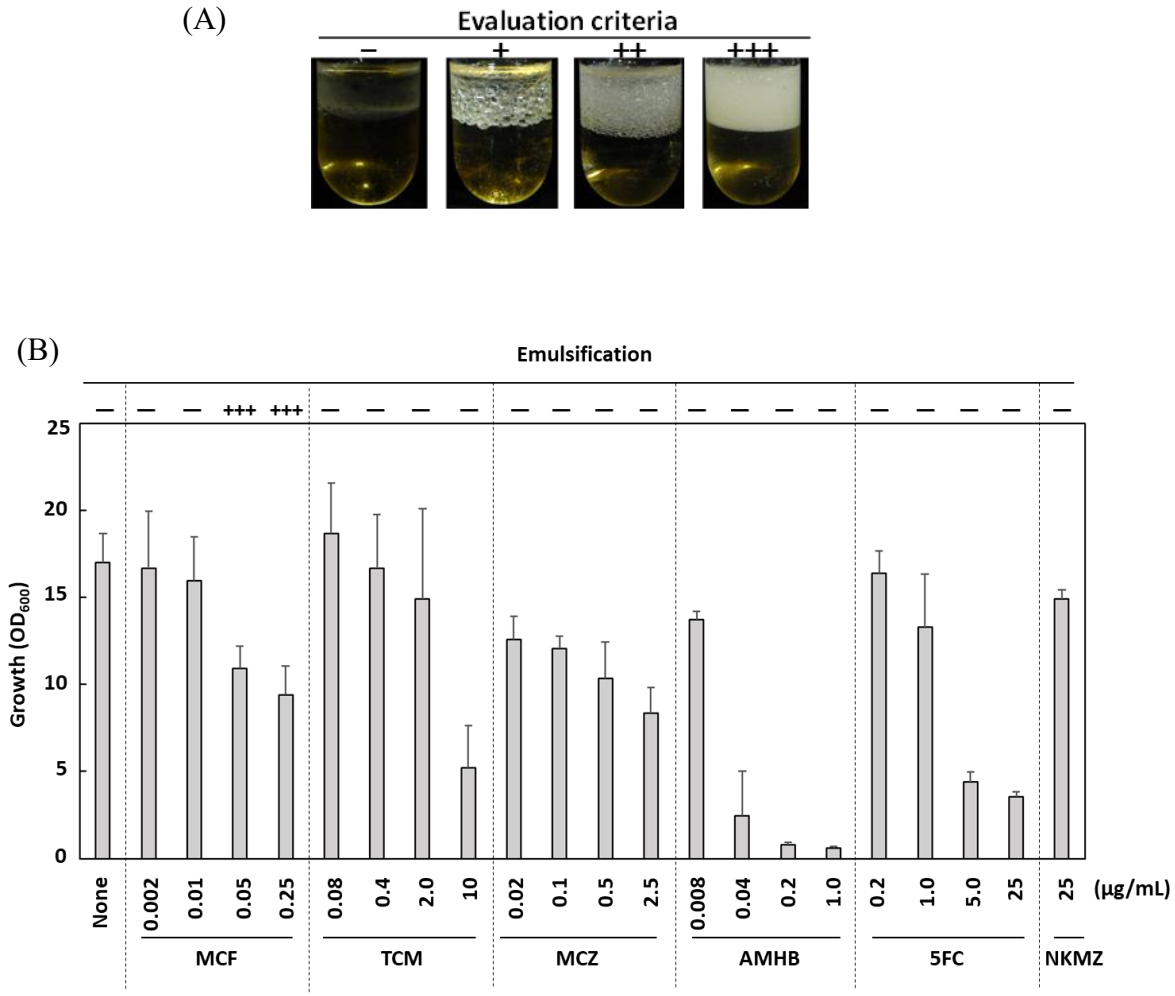


Fig. 3-5 Effects of the addition of compounds on emulsion formation by centrifugal supernatant after cultivation. (A) The criterion used for emulsification. (B) The emulsification test was performed thrice using samples of different cultures. Similar results were obtained in the three tests. Only miconazole (0.02, 0.1, and 0.5 μg/mL) showed subtle results (−, −, and +), which were interpreted as −. None refers to the culture solution without the addition of drugs. MCF, micafungin; TCM, tunicamycin; MCZ, miconazole; AMHB, amphotericin B; 5FC, 5-flucytosine; NKMZ, nikkomycin Z. Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2021 Elsevier.

3-3-6 SDS-PAGE analysis of cell wall proteins released after treatment with β-1,3-glucan synthesis inhibitors and other bioactive compounds

In order to clarify the factors of emulsification caused by β-1,3-glucan synthesis inhibition, the culture supernatant after treatment of micafungin and caspofungin was analyzed by SDS-PAGE. As shown in Fig. 3-6A, a remarkable protein release was observed in the samples treated with micafungin and caspofungin, and there was no apparent difference in released protein profile between the two treatments. Osmoprotectants were added to the culture, and cells can grow in the presence of micafungin and caspofungin. Therefore, intracellular protein leakage due to cell rupture is unlikely to occur. Furthermore, almost no protein was observed in the untreated case.

Next, the release of protein into the medium by treating with other drugs, except for nikkomycin Z which did not inhibit the growth, was compared. As shown in Fig. 3-6B, no remarkable protein release was observed for tunicamycin as well as miconazole, amphotericin B, and 5-flucytosine treatment compared to micafungin and caspofungin treatment.

These results strongly suggested that emulsification is a peculiar phenomenon that occurs when the functions necessary for fixing cell wall proteins to the cell wall, such as inhibition of β-1,3-glucan, are lost.

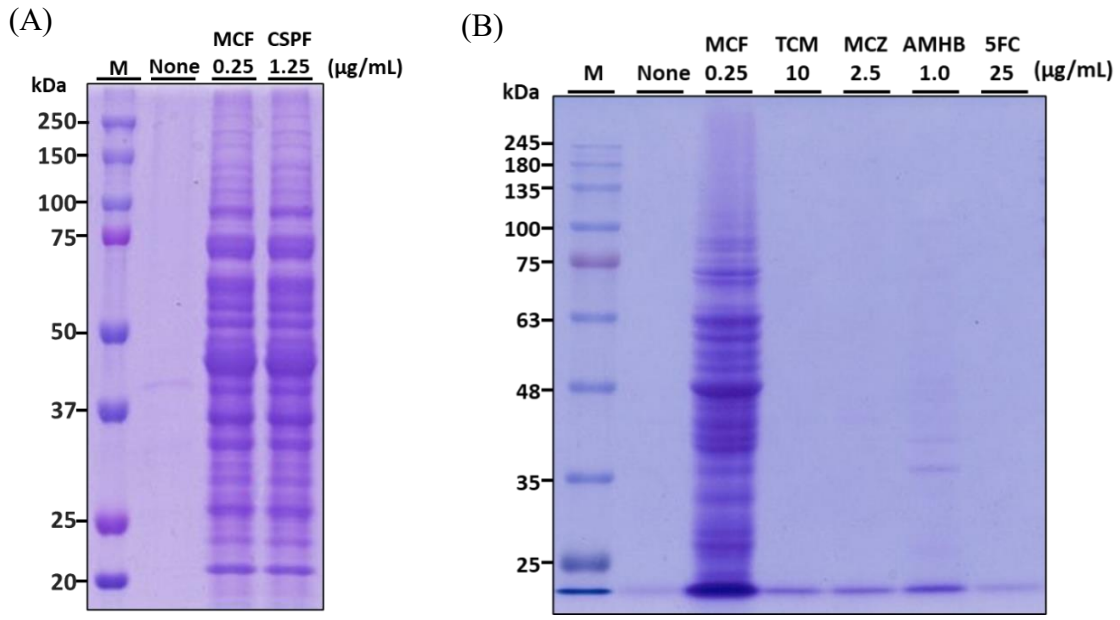


Fig. 3-6 SDS-PAGE analyses of the centrifugal supernatant after treating cells with MCF, CSPF, TCM, MCZ, AMHB, and 5FC. (A) M: Precision Plus Protein Dual Color Standards (Bio-Rad, Hercules, CA, USA) and (B) M: WIDE-VIEW Prestained Protein Size Marker III (Fujifilm Wako Pure Chemical). None refers to the culture solution without the addition of drugs. MCF, micafungin; CSPF, caspofungin; TCM, tunicamycin; MCZ, miconazole; AMHB, amphotericin B; 5FC, 5-flucytosine. Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2021 Elsevier.

3-3-7 A simpler assessment method for detecting inhibition of β-1,3-glucan synthesis

From above results, it was considered that the emulsification phenomenon could be applied not only to the confirmation of the effect on β-1,3-glucan synthesis but also to large-scale screening. However, it would be necessary to reduce the culture scale and simplify the evaluation to evaluate more samples in the actual screening. Therefore, the culture scale was reduced to 500 μL, the culture method was changed to static culture, and emulsification was evaluated without removing the cells after centrifugation. Although the addition of micafungin yielded slightly weaker results than the emulsion formation as shown in Fig. 3-2B, a significant emulsion was confirmed even under the new screening conditions (Fig. 3-7). Hence, this screening system is considered effective as an initial screen for compounds that inhibit β-1,3-glucan synthesis.

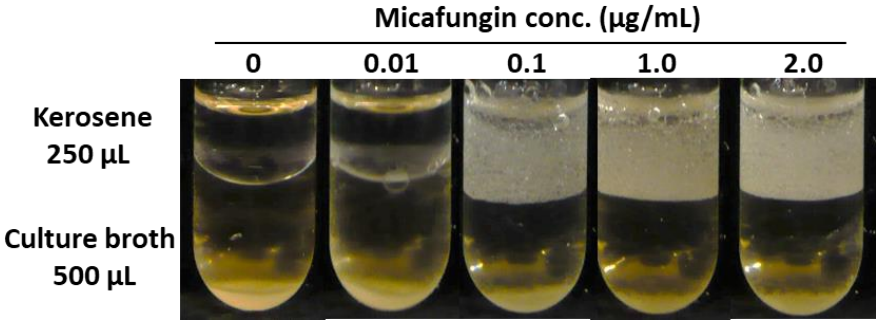


Fig. 3-7 Simpler emulsification assessment for detecting inhibition of β-1,3-glucan synthesis. Static culture of *C. albicans* was performed, and emulsion formation evaluated by mixing oil with the culture broth containing cells at a small scale. Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2021 Elsevier.

3-4 Conclusion

In this chapter, it was confirmed that remarkable emulsification was detected by mixing oil and the cell wall components obtained by treating *C. albicans* cells with β-1,3-glucanase. Furthermore, it was found that a similar phenomenon was detected by the centrifugal supernatant obtained after culture in the osmotic supported medium containing an inhibitor of β-1,3-glucan synthesis. Under osmotic support, yeast cells with an incomplete cell wall grew in the medium containing β-1,3-glucan synthesis inhibitor and cell wall proteins with emulsification activity were released from cells as they grew, resulting in the detection of strong emulsion in culture medium. Conversely, such emulsification was not observed for compounds with other action mechanisms. These results strongly supported the contention that emulsification serves as an indicator of the inhibition of normal β-1,3-glucan synthesis. Emulsification is readily assessed by mixing oil and water, so the phenomenon can be used for the first screening of antifungal agents inhibiting β-1,3-glucan synthesis.

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Chapter 4

Identification of emulsification proteins released from the cells by inhibiting the synthesis of GPI-anchor or β-1,3-glucan in *Candida albicans*

4-1 Introduction

In chapter 3, I found that a strong emulsification activity was detected in the culture broth when the pathogen *Candida albicans* was cultured with a β-1,3-glucan synthesis inhibitor under osmotic protection. This is presumed to be caused by cells with decreased β-1,3-glucan levels that grow under osmotic protection, and then many cell wall mannoproteins with emulsification activity were released from the cell surface. The emulsion phenomenon was not detected with other antifungal agents, which act on cell membrane synthesis or nucleic acid metabolism. As described in chapter 1, many cell wall proteins are linked to cell wall β-glucan via the carbohydrate moiety of the GPI-anchor, which remains when detached from the cell membrane [1]. Furthermore, *Saccharomyces cerevisiae* Gas1, which is GPI-anchor protein and responsible for β-1,3-glucanosyltransferase in the cell, was identified as an efficient emulsifier [2]. Considering above information, the emulsion phenomenon might be detected by inhibiting GPI-anchor synthesis. Additionally, which proteins have a strong emulsification ability was unclear. Then in this chapter, I evaluated whether emulsification was obtained by inhibition of GPI-anchor synthesis, and analyzed

proteins included in the culture supernatant after culturing *C. albicans* with inhibitor of β-1,3-glucan or GPI-anchor synthesis.

4-2 Materials and methods

4-2-1 Strains, media, and reagents

The *C. albicans* IFO1061 strain from the Institute for Fermentation Osaka (Osaka, Japan) and *C. albicans* SC5314/ATCC MYA-2876 strain [3] from the American Type Culture Collection was used in this chapter. Also, *S. cerevisiae* BY4741 strain (MAT a *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and *S. cerevisiae* double knockout strain *anp1*Δ*gup1*Δ (MAT a *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) were used to obtain recombinant proteins as a host. The *anp1*Δ strain (BY4741, *anp1*Δ:kanMX2) and *gup1*Δ strain (BY4742, MAT α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*, *gup1*Δ:kanMX2) [4] from Dharmacon (now Horizon Discovery, Cambridge, UK) were used to construct the *anp1*Δ*gup1*Δ strain. Mating sporulation and tetrad analysis were performed according to a previously described method [5]. The tester *S. cerevisiae* strains SH682 (MAT a *ura1 ura2 trp3 lys1 pho3 pho5*) and SH683 (MAT α *ura1 ura2 trp3 lys1 pho3 pho5*) were used to determine the mating type. The *S. cerevisiae* strains were transformed by a one-step method to derive strains with plasmids [6].

YPD medium, YNB medium (0.67% [w/v] Difco yeast nitrogen base without amino acids, 0.5% [w/v] ammonium sulfate, 2% [w/v] glucose, and necessary nutrients, such as amino acid), and YNBP medium were used to culture yeast cells. Especially, YNB

medium was used to culture the *S. cerevisiae* cells with plasmids, and YNBPS medium was used to culture the *C. albicans* cells with micafungin or manogepix. Sorbitol (0.6 M) was added to the YPD, YNB, and YNBP media when osmotic support was essential; these media were termed YPDS, YNBS, and YNBPS, respectively. The yeast extract was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Sorbitol was procured from Sigma-Aldrich Co. LCC (St. Louis, MO, USA), and other medium components were obtained from Fujifilm Wako Pure Chemical, Inc. (Osaka, Japan). The plasmid DNA was prepared using *Escherichia coli* strain DH5α. The bacterial cells were cultured and transformed using standard media and methods [7].

Micafungin and manogepix (MedChemExpress, Monmouth Junction, NJ, USA) were used after dissolving 5 mg/mL in dimethyl sulfoxide as inhibitors of β-1,3-glucan and GPI-anchor synthesis, respectively. Micafungin inhibits β-1,3-glucan synthase [8]. Manogepix inhibits Gwt1 required for inositol acylation, the first step of GPI synthesis [9]. The structure of manogepix is shown in Fig. 4-1.

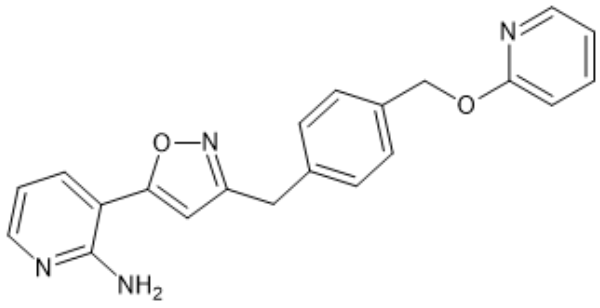


Fig. 4-1 Structure of manogepix.

4-2-2 *Emulsion formation by the culture supernatant after treatment of cells with GPI-anchor synthesis inhibitor*

The *C. albicans* IFO1061 strain was cultured in 4 mL of YNBP or YNBPS medium at 37°C overnight. The optical density at 600 nm (OD_{600}) value was adjusted to 0.5 with fresh medium. Manogepix was added to 4 mL of the cell suspensions at a concentration of 0.002, 0.01, 0.05, or 0.25 μg/mL. As a positive control of emulsion detection, micafungin was added to 4 mL of the cell suspension at a final concentration of 0.25 μg/mL. Subsequently, the cell suspensions were incubated at 37°C for 24 h. Cell growth was evaluated by the OD_{600} value. After centrifugation at 3000 × *g* for 5 min, the supernatant (500 μL) was mixed with 250 μL of kerosene (oil). After incubation at 30°C for 1 h, the formation of an emulsion was visually assessed. The emulsification test was performed three times using samples from different cultures to confirm reproducibility.

4-2-3 *SDS-PAGE analysis of cell wall proteins released by the addition of manogepix*

As described in *section 4-2-2*, the *C. albicans* IFO1061 strain was cultured in YNBPS medium including 0.002, 0.01, 0.05, or 0.25 μg/mL of manogepix. After centrifugation at 3000 × *g* for 5 min, the cells were removed. After the second centrifugation at 10,000 × *g* for 5 min, the fine particles were removed. The supernatants (500 μL) were concentrated to 25 μL by using VIVASPIN 500 (10,000 molecular weight cutoff, Sartorius Japan, Inc., Tokyo, Japan), to which was added same volume of 2 × SDS denaturation buffer and treated at 95°C for 10 min. A part of the denatured solution (25 μL) was subjected to SDS-PAGE (10% [w/v] acrylamide), and the proteins were

detected by CBB staining.

4-2-4 Endoglycosidase H treatment of cell wall proteins released by the addition of manogepix

As described in *section 4-2-2*, the *C. albicans* IFO1061 strain was cultured in a YNBPS medium including 0.002 μg/mL of manogepix. After incubation at 37°C for 24 h, the cells were removed by centrifugation at 3000 × *g* for 5 min. After the second centrifugation at 10,000 × *g* for 5 min, the fine particles were removed. The supernatants (100 μL) were concentrated to 20 μL by using VIVASPIN 500 (10,000 molecular weight cutoff, Sartorius Japan, Inc., Tokyo, Japan). A part of the solution (9 μL) was treated with 500 units of Endoglycosidase H (Endo H), which cleaves *N*-linked glycans from proteins (New England Biolabs Japan, Inc., Tokyo, Japan) at 37°C overnight. The treated solution (20 μL) was analyzed by SDS-PAGE (10% [w/v] acrylamide), and the proteins were detected by silver staining performed with Silver stain KANTO III (Kanto Chemical Co., Inc., Tokyo, Japan).

4-2-5 Fractionation and analysis of proteins released by the addition of micafungin

The *C. albicans* IFO1061 strain was precultured in the YNBPS medium at 37°C overnight. The OD_{600} value was adjusted to 0.5 with fresh medium. Micafungin was added to 50 mL of the cell suspension at a final concentration of 0.25 μg/mL. Subsequently, the cells were cultured at 37°C for 24 h. After centrifugation at 3000 × g for 5 min, the cells were removed, and the fine particles were removed by filtration

(0.22 μm). The filtrate was dialyzed against 20 mM phosphate buffer (pH 6.0) at 5°C overnight and concentrated to half of the initial volume with Amicon® Ultra Centrifugal Filters (molecular weight cutoff 10,000, Merck KGaA, Darmstadt, Germany). Subsequently, ammonium sulfate was added to the concentrated solution to a saturated concentration of 70%, stirred at 5°C for 30 min, and allowed to stand at 5°C for 24 h. After centrifugation at 12000 × *g* for 10 min, the supernatant and precipitate were collected. The centrifugal supernatant was filtered (0.22 μm). The precipitate was dissolved with the same volume of 20 mM phosphate buffer (pH 6.0) as the supernatant. The supernatant and precipitate solution were dialyzed against phosphate buffer at 5°C overnight. Finally, their volumes were adjusted to the initial volume with phosphate buffer. These protein solutions equivalent to 0.5 mL were concentrated using VIVASPIN 500, and all of the concentrates were subjected to SDS-PAGE (10% [w/v] acrylamide), and the proteins were stained with CBB. Subsequently, the protein solution (1.0 mL) from the centrifugal supernatant was concentrated to 20 μL using VIVASPIN 500. Part of the concentrate (9 μL) was treated with 500 units of Endo H at 37°C overnight. The treated solution was subjected to SDS-PAGE (8% [w/v] acrylamide), and the proteins were stained with CBB.

Finally, the precipitate solution (500 μL) obtained after salting out by ammonium sulfate and the solution adjusted to the initial volume after dialysis of the supernatant (500 μL) were mixed with 250 μL of kerosene, and emulsion formation was evaluated as described in *section 4-2-2*.

4-2-6 Emulsification using the culture supernatant after culturing in a medium containing micafungin

C. albicans IFO1061 strain was cultured in 4 mL of YNBPS medium at 37°C overnight. The OD_{600} value was adjusted to 0.5 with fresh medium. Micafungin was added to 4 mL of the cell suspension to a final concentration of 0.25 μg/mL. Subsequently, the cell suspension was incubated at 37°C for 3, 6, 9, or 24 h. The OD_{600} value evaluated the cell growth. After centrifugation at 3000 × *g* for 5 min, the cells were removed. The culture supernatant (500 μL) was mixed with kerosene (250 μL). Emulsification activity was assessed as described in *section 4-2-2*.

4-2-7 Analysis of cell wall proteins released by the addition of micafungin

C. albicans IFO1061 strain was cultured in the YNBPS medium, including 0.25 μg/mL of micafungin at 37°C for 3, 6, 9, or 24 h. After centrifugation at 3000 × *g* for 5 min, the cells were removed. After the second centrifugation at 10,000 × *g* for 5 min, the fine particles were removed. The centrifuged supernatants (500 μL) were concentrated to 25 μL by VIVASPIN 500, a part of the concentrate (10 μL) was subjected to SDS-PAGE (10% [w/v] acrylamide), and the proteins were stained with CBB.

4-2-8 Construction of expression plasmids

To obtain *C. albicans* Phr2, Tkl1, Hsp71, Kpyk, Eno1, and Fba1 with 6 × histidines at the C-terminal position, the plasmids expressing these genes under a PGK1 promoter

were constructed. The pSP-G1 plasmid [10] was provided by the National BioResource Project in Japan. The genomic DNA from the *C. albicans* IFO1061 strain or SC5314 strain was used as a template; the target genes were amplified with a combination of primers, as shown in Table 4-1. The CUG codon, which encodes an amino acid that differs between *C. albicans* and *S. cerevisiae*, was not included in the original ORF; therefore, the sequence was unaltered in expression in *S. cerevisiae*. The fragment of PHR2 with restriction enzyme sites of XmaI and XhoI was introduced into those restriction enzyme sites of the pSP-G1 plasmid. The constructed plasmid was named pSP-G1(1-544His6Phr2). The plasmid expressing PHR2 without the signal sequence required for binding to the GPI-anchor was constructed by inverse PCR using pSP-G1(1-544His6Phr2) as a template. The constructed plasmid was named pSP-G1(1-515His6Phr2). The fragments of TKL1, HSP71, KPYK, ENO1, and FBA1, which have restriction enzyme sites of BamHI and XhoI, were introduced into those restriction enzyme sites of the pSP-G1 plasmid. These insertions were confirmed by DNA sequencing. The constructed plasmids were named as shown in Table 4-1.

Table 4-1 The sequences of forward (F) and reverse (R) primers were used to construct expression plasmids of recombinant proteins and source of template DNA. Reprinted with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

Constructed plasmid	DNA source	Primers	
pSP-G1 (1-544His6Phr2)	SC5314	F	5'-ATGCCCCGGGATGTTGTTGAAATCTTTATTCC-3'
		R	5'-ATGCCTCGAGTTAGTGGTGGTGGTGGTGGTGCATGGTAATTAAACCGAAAC-3'
pSP-G1 (1-515His6Phr2)	SC5314	F	5'-AGTGGTAGTAAATCTCACCACCACCACCACCACTA-3'
		R	5'-AGATTTACTACCACTTGAACCAGATGA-3'
pSP-G1 (1-677His6Tkl1)	SC5314	F	5'-ATCGGGATCCATGCCTTCTCTTGATGAATT-3'
		R	5'-ATGCCTCGAGTTAGTGGTGGTGGTGGTGGTGGAAAGCTCTGTCTAAAGGAGA-3'
pSP-G1 (1-656His6Hsp71)	SC5314	F	5'-ATCGGGATCCATGTCTAAAGCTGTTGGTAT-3'
		R	5'- ATCGCTCGAGTTAGTGGTGGTGGTGGTGGTGATCAACTTCTTCAACAGT-3'
pSP-G1 (1-504His6Kpyk)	SC5314	F	5'- ATCGGGATCCATGTCTCACT CATCTTTATC-3'
		R	5'-ATGCCTCGAGTTAGTGGTGGTGGTGGTGGTGAGCTTGGACGATTCTAACAGTG-3'
pSP-G1 (1-440His6Eno1)	SC5314	F	5'- CGGGGATCCATGTCTTACGCCACTAAAATCCA-3'
		R	5'-CGCCTCGAGTTAGTGGTGGTGGTGGTGGTGCAATTGAGAAGCCTTTTGGAA-3'
pSP-G1 (1-359His6Fba1)	IFO1061	F	5'-ATGCGGATCCATGGCTCCTCCAGCAGTTTT-3'
		R	5'-ATGCCTCGAGTTAGTGGTGGTGGTGGTGGTGCAATTGTCCTTTAGTGTGGA-3'

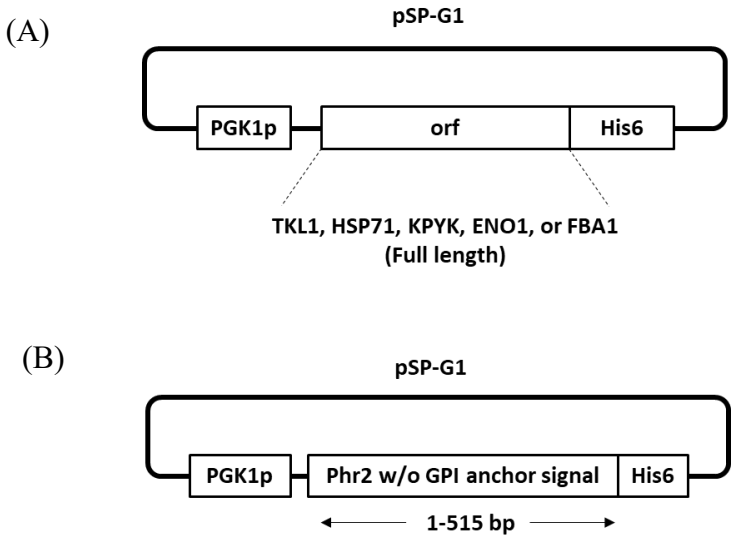


Fig. 4-1 Constructed plasmids

4-2-9 Purification of recombinant Phr2 without signal sequence

S. cerevisiae BY4741 cells with pSP-G1(1-515His6Phr2) were cultured in 4 mL of YNB medium without uracil at 30°C overnight. The precultured cells were inoculated into 100 mL YPD medium, and the OD_{600} value was adjusted to 0.1. The cell suspension was cultured at 30°C for 48 h. After centrifugation at 3000 × *g* for 5 min, the culture supernatant was collected, and fine particles were removed by filtration. The culture supernatant was applied to a Ni-NTA affinity column, HisTrapHP-5 mL, washed with 60 mL of 20 mM phosphate buffer (pH 7.4) containing 30 mM imidazole, and eluted with 20 mL of phosphate buffer containing 500 mM imidazole. The elute was fractionated to obtain 8 fractions (1 mL each), and No. 5 and 6 fractions (7.5 μL) were analyzed by SDS-PAGE and western blotting, and the proteins were detected by the CBB staining. Western blotting was performed with a standard method described in the kits and reagents by using polyvinylidene fluoride membrane (Immobilon-P, Merck Millipore, Billerica, MA, USA), anti-His-tag mAb, IgG/mouse as a first antibody (Medical & Biological Laboratories, Nagoya, Japan), anti-mouse IgG-alkaline phosphatase antibody produced in goat as a second antibody (Sigma-Aldrich, St. Louis, MO, USA), Can Get Signal® as an immunoreaction enhancer solution (Toyobo, Osaka, Japan), and BCIP-NBT Solution Kit for coloring (Nacalai Tesque, Kyoto, Japan).

In addition, the fractions including recombinant Phr2 without signal sequence were dialyzed with pure water, freeze-dried, and used for evaluation of emulsification activity. The protein concentration was measured by the BCA Protein kit (Thermo Fisher Scientific, Waltham, MA, USA). The purified recombinant protein and casein solutions were prepared with pure water at a final concentration of 10, 20, 30, 40, and 50 μg/mL,

and these protein solutions (500 μL) were mixed with 250 μL of kerosene. Emulsification activity was assessed as described in *section 4-2-2*.

4-2-10 Purification of recombinant Tkl1, Kpyk, Eno1, and Fba1

*S. cerevisiae anp1*Δ*gup1*Δ cells with the constructed plasmid were cultured in 4 mL of YNBS medium without uracil at 30°C overnight. The precultured cells were inoculated in 100 mL YPDS medium, and the OD_{600} value was adjusted to 0.1. The cell suspension was cultured at 30°C for 48 h. After centrifugation at 3000 × *g* for 5 min, the cells were collected and rinsed with phosphate-buffered saline (PBS), including 0.6 M sorbitol. After the second centrifugation at 10,000 × *g* for 5 min, the cells were suspended with PBS to adjust the OD_{600} value to 50. From the centrifugal supernatant at 10,000 × *g* for 5 min, the recombinant proteins were purified using a Ni-NTA affinity column as described in *section 4-2-9*. The fraction (No.5) was analyzed by SDS-PAGE and western blotting. The fractions, including recombinant Tkl1, Kpyk, and Eno1, were dialyzed with pure water, freeze-dried, and used for evaluating the emulsification activity. As for recombinant Fba1, the eluted fraction (No. 5) in the Ni-NTA affinity column was used for evaluating the emulsification activity without dialysis and freeze-dried. A BCA Protein kit measured the protein concentration. Emulsification activity was assessed as described in *section 4-2-2*.

4-2-11 Mass spectrometry of proteins

The target protein band on SDS-PAGE was cut out, decolorized with acetonitrile or Silver stain KANTO gel washing solution for MS (Kanto Chemical Co., Inc.), treated with trypsin, and analyzed by a mass spectrometer, MALDI-FT-ICR MS (solariX XR, Bruker Daltonics, Billerica, MA, USA). The obtained results were subjected to Mascot's MS/MS Ion Search or peptide mass fingerprinting analysis for identifying the proteins. The search parameters are mentioned in each figure legend.

4-2-12 Statistical analysis

Growth results are presented as means ± the standard deviation of three independent experiments. Welch's t-test was used to compare the two groups. Differences were assessed using a two-sided test with a 0.05 α-level. Asterisks indicated significant differences (*and**: $p<0.05$).

4-3 Results and discussion

4-3-1 Emulsion formation by the culture supernatant after treatment of cells with GPI-anchor synthesis inhibitors

Manogepix, a commercially available Gwt1 inhibitor, was used as an inhibitor of GPI-anchor synthesis [11]. GWT1 is required for inositol acylation, the first step of GPI synthesis, and is necessary for yeast cell wall integrity [12, 13]. Additionally, micafungin was used as a positive control of emulsion detection. In the medium without osmotic protection (YNBP), including 0.25 μg/mL of micafungin, cell growth was inhibited entirely, and no emulsification was detected in the culture supernatant. In the medium with osmotic protection (YNBPS), the OD_{600} value increased from 0.5 to 8.3 after 24 h of cultivation, and strong emulsification was detected in the culture supernatant. Such a tendency was similar to that described in *section 3-3-2*.

C. albicans was cultured with manogepix, and cell growth (Fig. 4-2A) and emulsification activity of culture supernatant (Fig. 4-2B) were evaluated. In the YNBPS medium, including 0.002, 0.01, 0.05, and 0.25 μg/mL of manogepix, the OD_{600} values increased to 9.5, 6.4, 3.8, and 3.2, respectively, and the culture supernatants showed emulsification activities. In the YNBP medium, including 0.002 and 0.01 μg/mL of manogepix, the OD_{600} values increased to 11.0 and 4.2, respectively, and the culture supernatants showed emulsification activities. Meanwhile, in the YNBP medium, including 0.05 and 0.25 μg/mL of manogepix, the OD_{600} values increased to about 2.0, and the results of emulsification activity varied, and emulsification was observed in only one out of three trials. Such subtle results might be because the cells did not grow

sufficiently, and the amount of emulsified substance released from the cells was small. Meanwhile, in YNBP and YNBPS media without antifungal treatment, the OD_{600} value increased to 22.4 and 16.7, respectively, but the culture supernatants showed no emulsification activity. Additionally, no emulsification activity was detected in 0.25 μg/mL of manogepix solution.

In chapter 3, I proposed emulsion formation as an indicator of inhibition of β-1,3-glucan synthesis, but the results obtained in this section showed that inhibition of GPI-anchor synthesis induces emulsion formation. However, we can distinguish inhibitions of β-1,3-glucan and GPI-anchor synthesis by comparing the cell growths with and without osmotic protection. The cell growth with β-1,3-glucan synthesis inhibitor recovered by osmotic protection, but not with GPI-anchor synthesis inhibitor. In addition, the inhibition of GPI-anchor synthesis tended to induce weaker emulsification than the inhibition of β-1,3-glucan.

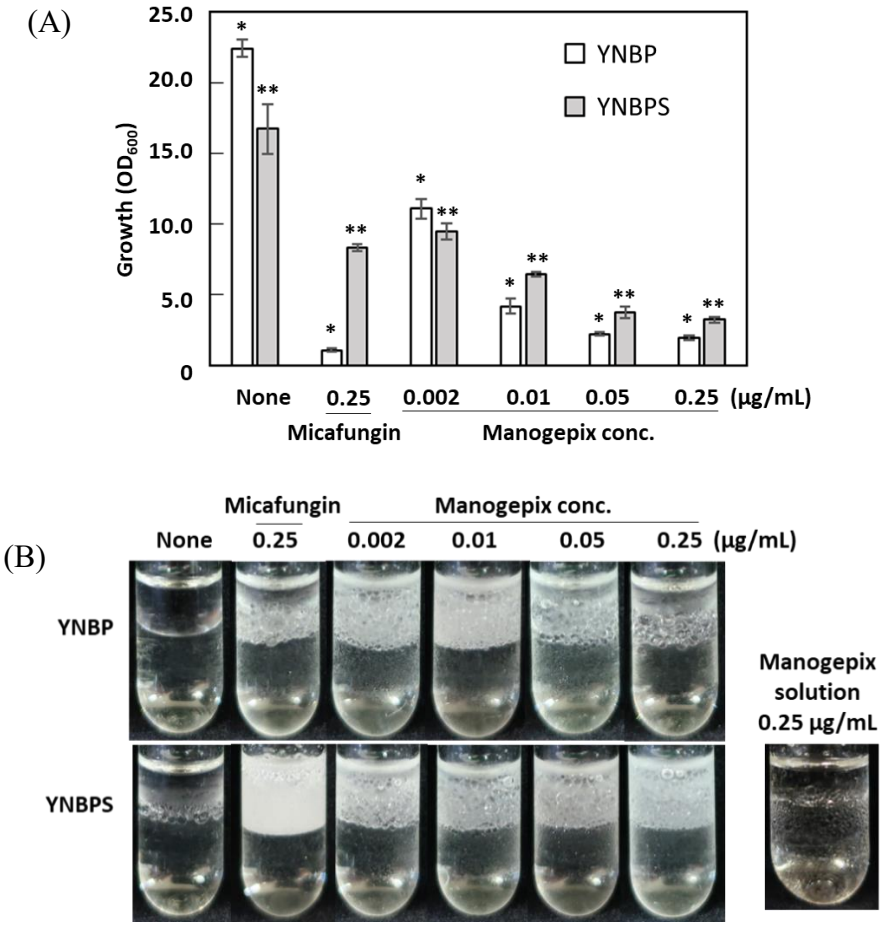


Fig. 4-2 The effects of adding manogepix on cell growth and emulsion formation. "None" indicates the culture supernatant without adding micafungin and manogepix. Micafungin was used as a positive control for emulsion detection. (A) Growth of *C. albicans* in YNBP and YNBPS media with and without manogepix or micafungin. (B) Emulsion formation by the centrifugal supernatant obtained after the cultivation of *C. albicans* in those media and manogepix dissolved in distilled water. Results are presented as means ± the standard deviation of three independent experiments. Growth after manogepix treatment at all concentrations differed significantly from non-treatment (None). Asterisks indicated significant differences (*and**: $p<0.05$). Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

4-3-2 Analysis of cell wall proteins released by the addition of manogepix

In chapter 3, it was confirmed that cell wall proteins were released from the cells due to inhibition of β-1,3-glucan synthesis. Therefore, it was expected that similar protein release was also caused by inhibiting GPI-anchor synthesis. Then, the proteins present in the culture supernatant after manogepix treatment were examined by SDS-PAGE (Fig. 4-3A). In micafungin treatment, many proteins were detected in the culture supernatant similar to the result shown in Fig. 3-6. Meanwhile, after manogepix treatment, a few proteins were detected in the culture supernatants. Especially in 0.002 μg/mL manogepix treatment, which showed emulsification activity, two bands that were not seen without additive were detected. One is a band between 25 and 37 kDa, and the other is a broad band above 75 kDa. The detected proteins were expected to have a high emulsification activity.

The released proteins might be modified by sugar chains because many cell wall proteins have them. Therefore, the culture supernatant was treated with Endo H, which cut off the *N*-linked sugar chain. In SDS-PAGE analysis after Endo H treatment, six protein bands were detected and named Ca1, Ca2, Ca3, Ca4, Ca5, and Ca6 (Fig. 4-3B). By using mass spectrometry analysis, the proteins of Ca2, Ca3, Ca5, and Ca6 were identified as Phr2, Pga4, Exg1, and Bgl2, respectively. The search parameters and each mass spectrums were shown in Fig. 4-4. The characteristics are summarized in Table 4-2. The bands of Ca2, Ca3, and Ca5 on SDS-PAGE after Endo H treatment did not match the molecular weight estimated from the amino acid sequence. This might be due to residual *O*-linked glycans or proteolytic degradation. Unfortunately, the Ca1 and Ca4 proteins could not be identified because their evaluation scores were less than the

individual ions scores 28 and 31, respectively, which indicate identity or extensive homology ($p<0.05$). The evaluation scores might be lower because the bands consisted of multiple proteins.

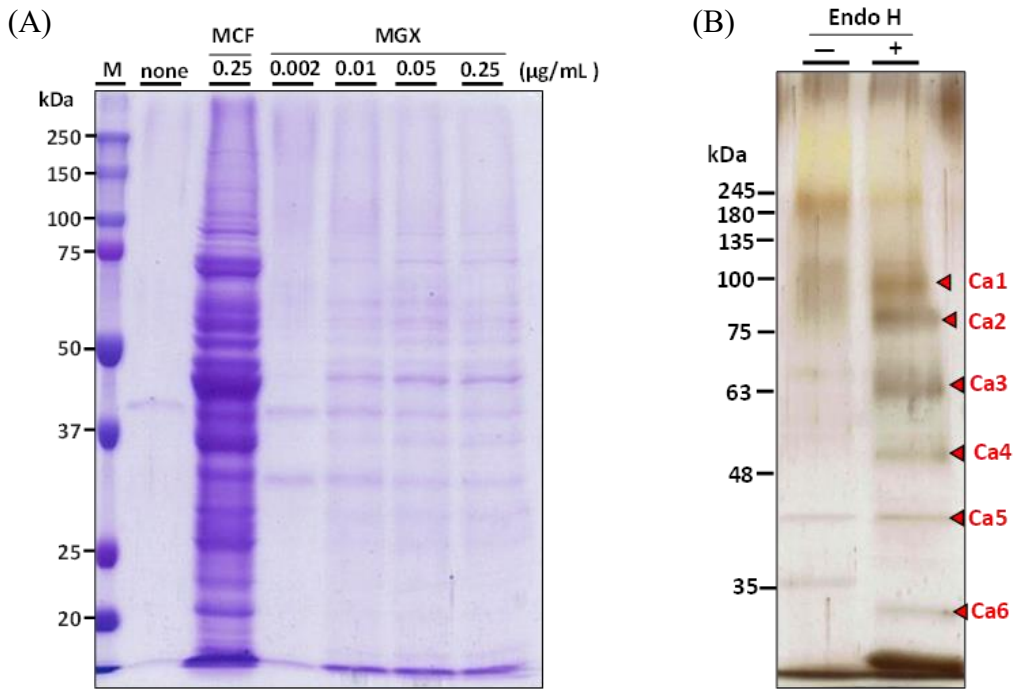
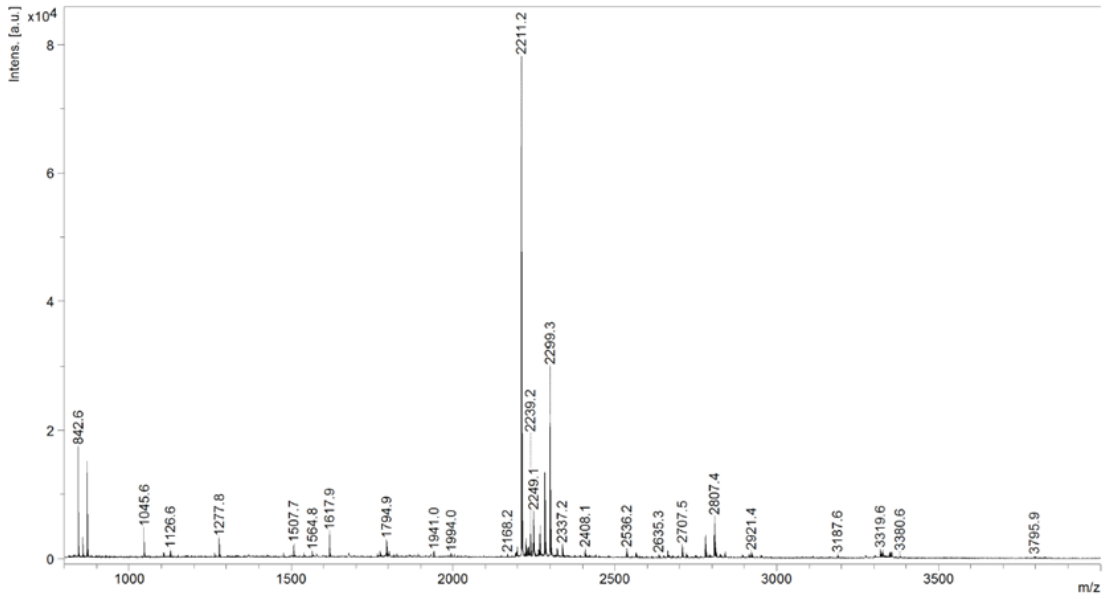


Fig. 4-3 The analysis of supernatant after culturing *C. albicans* IFO1061 strain in YNBPS with manogepix (MGX) or micafungin (MCF). (A) SDS-PAGE of supernatant after culturing for 24 h. M: Precision Plus Protein Dual Color Standards (Bio-Rad, Hercules, CA, USA) was used as a molecular weight marker. (B) The effects of Endo H treatment on proteins in the supernatant were obtained by culturing in a YNBPS medium with 0.002 mg/L MGX. M: WIDE-VIEW Prestained Protein Size Marker III (Fujifilm Wako Pure Chemical) was used as a molecular weight marker. Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

Table 4-2 The characteristics of proteins identified by mass spectrometry after manogepix treatment. Reprinted with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

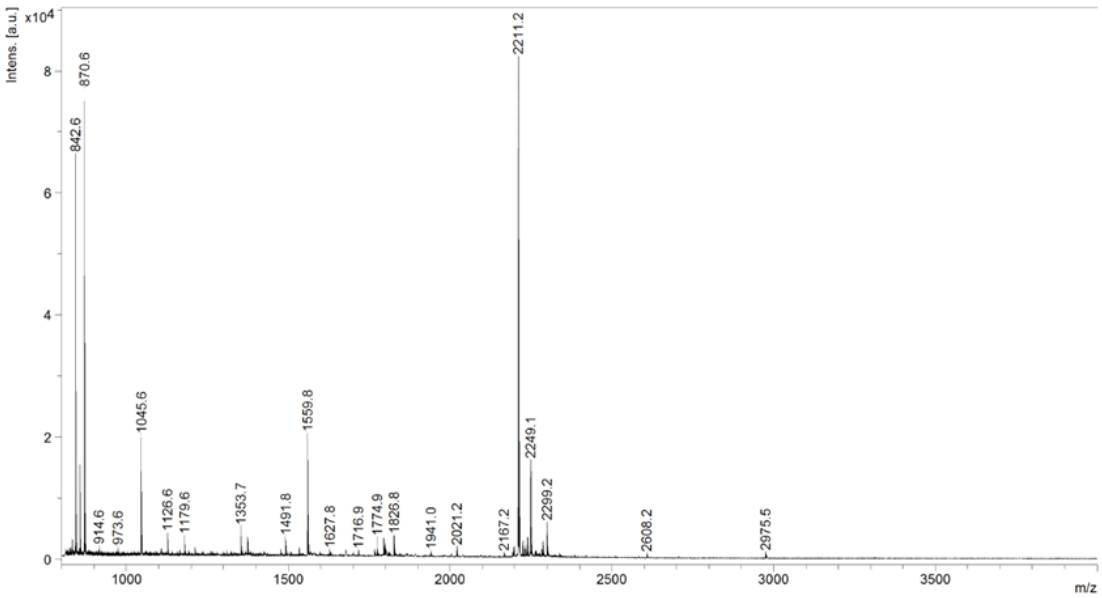
	Protein	MW [kDa]	Score	Localization (referred to CGD*)	Function
Ca2	Phr2	59.4	116	Cell wall, cell membrane, and extracellular vesicle	β-1,3-Glucanosyltransferase
Ca3	Pga4	49.4	49	Cell wall, cell membrane, and extracellular vesicle	β-1,3-Glucanosyltransferase
Ca5	Exg1	50.2	106	Cell surface	Exo-β-1,3-glucanase
Ca6	Bgl2	33.8	171	Cell wall and cell surface	β-1,3-Glucosyltransferase

MW is the molecular weight estimated from the amino acid sequence. The mean values of the scores were calculated using the Mascot database search, and a higher score indicates a more confident match. The scores of Ca2, Ca3, and Ca5 greater than 30 and scores of Ca6 greater than 29 were considered significant ($p<0.05$). *Candida Genome Database (http://www.candidagenome.org/).

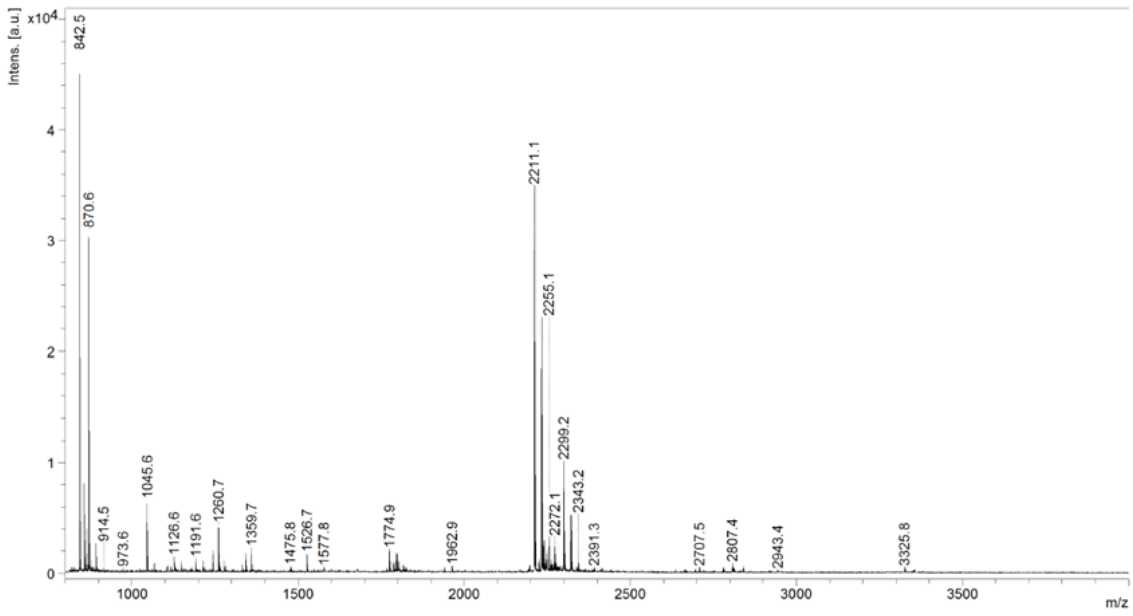


Mass spectrum of protein band Ca2

Fig. 4-4 The mass spectrum of protein bands detected by SDS-PAGE (Fig. 4-3B). The search parameters were as follows: type of search, MS/MS Ion Search; database, SwissProt 2022_03; taxonomy, Fungi; enzyme, trypsin; fixed modification, carbamidomethyl (C); variable modifications, oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ±50 ppm; peptide charge state, 1+; maximum missed cleavages, 2. The ions score was calculated as –10*Log(P), where P is the probability that an observed match is a random event. Individual ions scores of Ca2, Ca3, and Ca5 greater than 30 and individual ions scores of Ca6 greater than 29 indicate identity or extensive homology ($p<0.05$). Reprinted with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

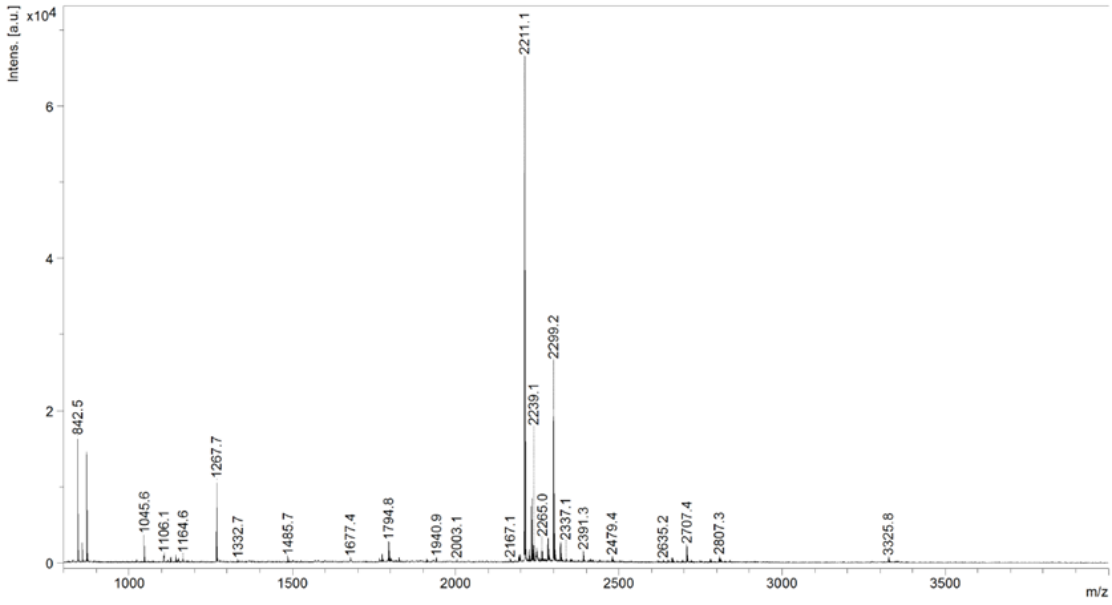


Mass spectrum of protein band Ca3



Mass spectrum of protein band Ca5

Fig. 4-4 The mass spectrum of protein bands detected by SDS-PAGE (Fig. 4-3B). (Continued)



Mass spectrum of protein band Ca6

Fig. 4-4 The mass spectrum of protein bands detected by SDS-PAGE (Fig. 4-3B). (Continued)

4-3-2 Emulsification activity of recombinant Phr2

C. albicans Phr2 is a homolog of *S. cerevisiae* Gas1 [3], which is known to have high emulsification activity [2]. The analysis with the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that Phr2 is 62% identical to *S. cerevisiae* Gas1. Therefore, Phr2 might have high emulsification activity. In order to confirm the emulsification activity of Phr2, a plasmid for *S. cerevisiae* to express recombinant Phr2 without a GPI-anchor signal sequence at the C-terminus and tagged it with 6 × histidines as shown in Fig 4-1B was constructed. After cultivation of *S. cerevisiae* carrying the plasmid, the recombinant Phr2 was purified by a Ni-NTA affinity column from the culture supernatant. The purity of the purified protein after column chromatography was analyzed by SDS-PAGE and western blotting. As shown in Fig. 4-5A, 4-5B, no bands other than the target protein were detected. The purified protein was confirmed to be highly pure. Furthermore, as shown in Fig. 4-5C, recombinant Phr2 exhibited as strong emulsification activity as casein, one of the protein emulsifiers. These results referred that *C. albicans* Phr2 contributed to emulsification caused by GPI-anchor synthesis inhibition.

Additionally, it is known that *S. cerevisiae* Gas5, similar to Gas1, also has emulsification activity [2]. Pga4 is an *S. cerevisiae* Gas homolog [14] and has 60% of identities to Gas5 according to the Basic Local Alignment Search Tool analysis. Although the activity was not confirmed, it is expected that Pga4 is highly likely to have an emulsifying activity. Pga4, in addition to Phr2, may also be one of the factors of emulsification caused by GPI-anchor synthesis inhibition.

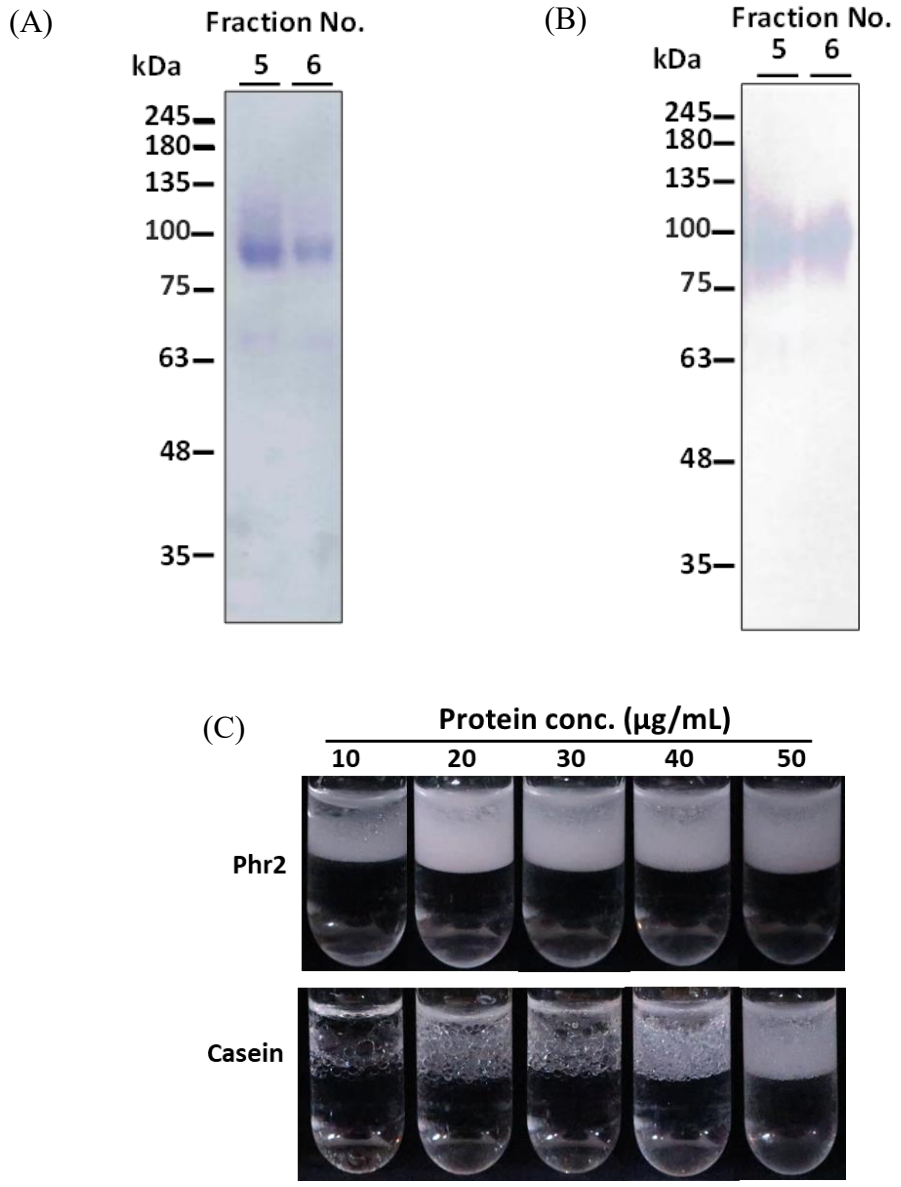


Fig. 4-5 The analysis of recombinant Phr2 expressed in the *S. cerevisiae* BY4741 strain. (A) SDS-PAGE and (B) western blotting of recombinant Phr2 purified using Ni-NTA affinity column. Recombinant Phr2 was detected with anti-His-tag mAb. M: WIDE-VIEW Prestained Protein Size Marker III (Fujifilm Wako Pure Chemical) was used as a molecular weight marker. (C) Emulsification using recombinant Phr2 and casein. Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

4-3-3 Detection of Phr2 and Pga4 from cell wall proteins released by the addition of micafungin

As described in chapter 1, many GPI-anchored proteins are linked to β-1,3-glucan via β-1,6-glucan. Therefore, Phr2 and Pga4 should lose the scaffold and be released from the cells and contribute to the emulsification activity even when β-1,3-glucan synthesis is inhibited by micafungin treatment. In this section, the proteins in the culture supernatant obtained after micafungin treatment were examined.

As shown in Fig. 4-3A, a large number of proteins were released when *C. albicans* cells were cultured for 24 h in an osmotically stabilized medium containing 0.25 μg/mL of micafungin. Then, the obtained culture supernatant was fractionated by ammonium sulfate precipitation. In SDS-PAGE analysis (Fig. 4-6A), a broad band was detected on the high molecular side of 75 kDa or more in the supernatant fraction after ammonium sulfate precipitation. Subsequently, Endo H treatment was performed and the products were analyzed by SDS-PAGE (Fig. 4-6B). As a result, four bands of Ca7-10 identified by mass spectrometry were detected, and it was found that Ca7 and Ca10 were Phr2 and Pga4, respectively. The search parameters and the mass spectrums were shown in Fig. 4-7. The characteristics are summarized in Table 4-3. While the Ca8 and Ca9 proteins could not be identified, because Ca8 had three candidates of proteins and the evaluation scores of Ca9 was less than the individual ions scores 28, which indicate identity or extensive homology ($p<0.05$). Furthermore, the supernatant after salting out treatment demonstrated emulsifying activity. (Fig. 4-6C).

From above results, Phr2 and Pga4 are considered to contribute to the emulsification activity of the culture supernatant obtained by micafungin treatment. However, strong

emulsification was detected by treating *C. albicans* cells with micafungin compared to treating them with manogepix, and the precipitate after salting out treatment also showed emulsifying activity, suggesting that other proteins in addition to Phr2 and Pga4 are expected to contribute to emulsification in micafungin treatment.

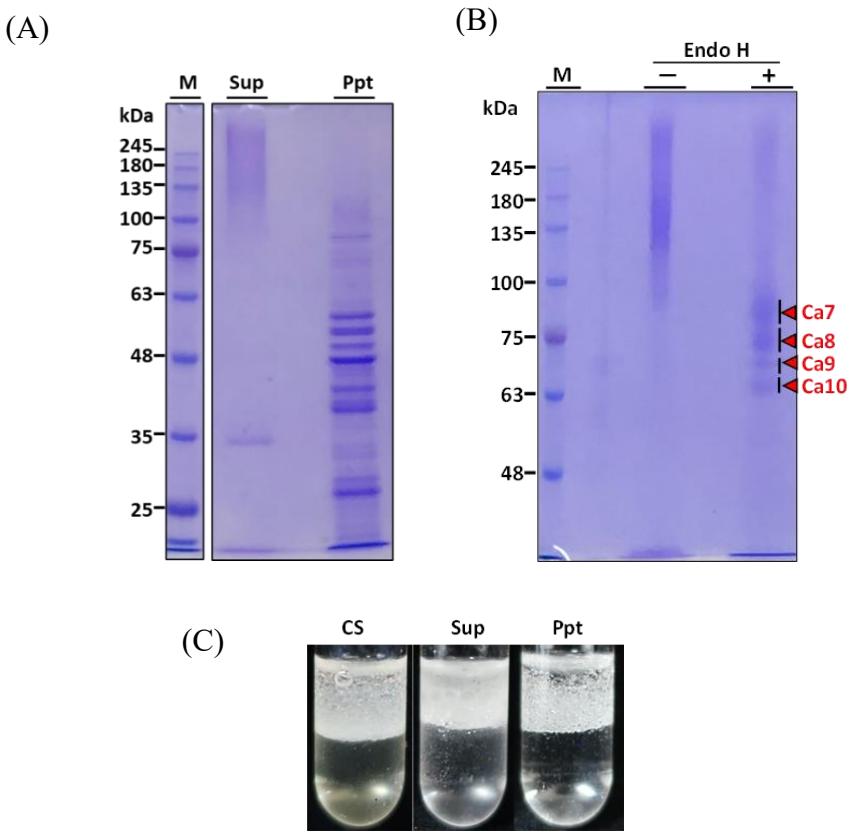
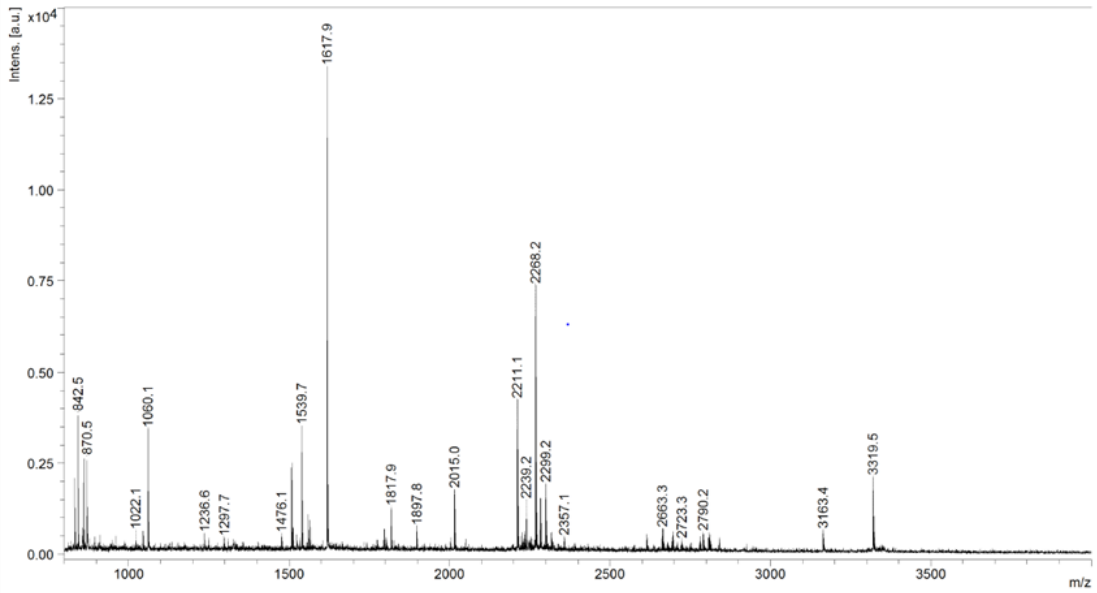


Fig. 4-6 The analysis of proteins fractionated by salting out with ammonium sulfate obtained from the culture supernatant after culturing *C. albicans* IFO1061 strain in YNBPS with micafungin. (A) SDS-PAGE of the supernatant (Sup) and precipitate (Ppt) after salting out with ammonium sulfate. (B) The changes in proteins in Sup upon Endo H treatment. (C) Emulsification using culture supernatant (CS) and Sup and Ppt after salting out with ammonium sulfate. M: WIDE-VIEW Prestained Protein Size Marker III (Fujifilm Wako Pure Chemical) was used as a marker. Reprinted with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

Table 4-3 The characteristics of proteins identified by mass spectrometry after micafungin treatment and salting out by ammonium sulfate. Reprinted with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

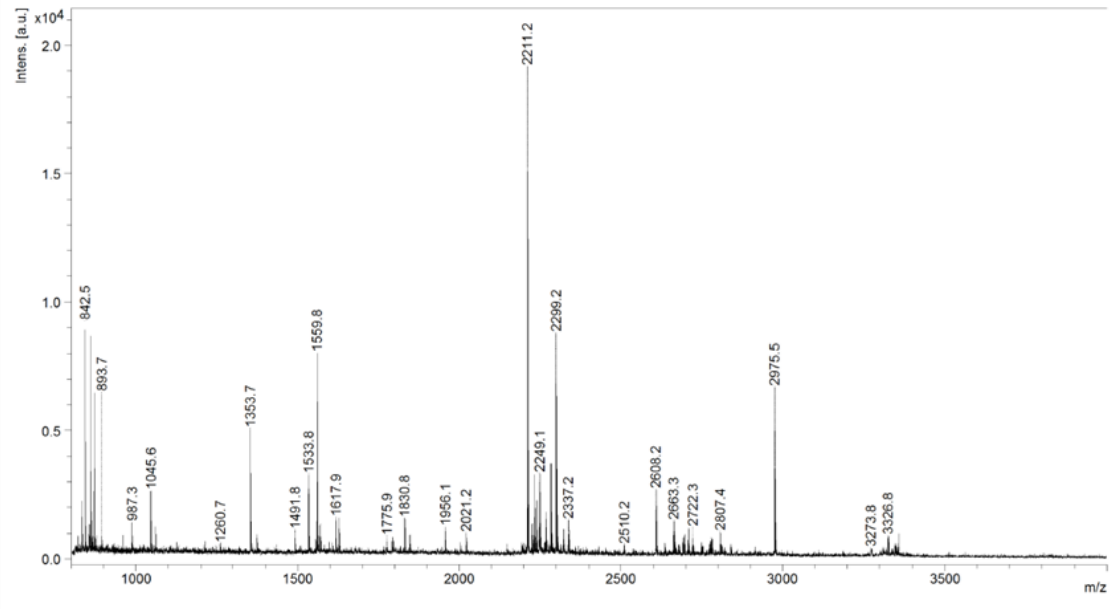
	Protein	M.W [kDa]	Score	Localization (referred to CGD)	Function
Ca7	Phr2	59.4	98	Cell wall, cell membrane, extracellular vesicle	β-1,3-Glucanosyltransferase
Ca10	Pga4	49.4	83	Cell wall, cell membrane, extracellular vesicle	β-1,3-Glucanosyltransferase

MW is the molecular weight estimated from the amino acid sequence. The scores of Ca7 and Ca10 greater than 28 are significant ($p<0.05$).



Mass spectrum of protein band Ca7

Fig. 4-7 The mass spectrum of protein bands detected by SDS-PAGE and characteristics of identified proteins (Fig. 4-6B). The search parameters were as follows: type of search, MS/MS Ion Search; database, SwissProt 2021_04; taxonomy, Other Fungi; enzyme, trypsin; fixed modification, carbamidomethyl (C); variable modifications, oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ±50 ppm; peptide charge state, 1+; maximum missed cleavages, 2. The ions score was calculated as –10*Log(P), where P is the probability that an observed match is a random event. The individual ions scores greater than 28 indicate identity or extensive homology ($p<0.05$). Reprinted with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.



Mass spectrum of protein band Ca10

Fig. 4-7 The mass spectrum of protein bands detected by SDS-PAGE and characteristics of identified proteins (Fig. 4-6B). (Continued)

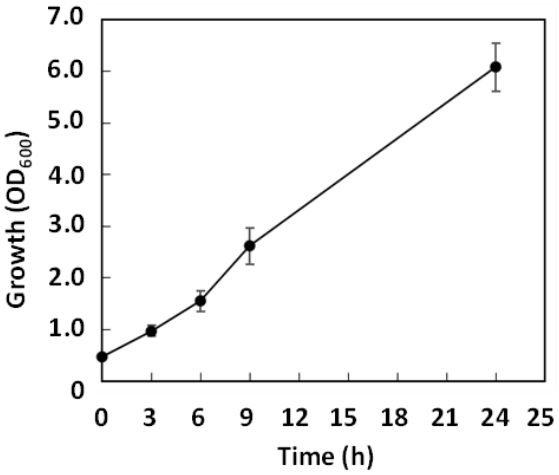
4-3-4 Analysis of cell wall proteins released by the addition of micafungin

Strong emulsification was detected when *C. albicans* cells were treated with 0.25 μg/mL of micafungin under osmotic protection because the cells with decreased β-1,3-glucan levels could grow under osmotic protection (Fig. 4-8A). Many cell wall proteins were released from the cells during 24 h incubation. As described in *section 4-3-3*, the proteins other than Phr2 and Pga4 are expected to contribute to emulsification. In this section, we examined the relationship between the released proteins and emulsification activity in a culture medium obtained after 0.25 μg/mL of micafungin treatment.

After 6 h incubation, the OD_{600} value increased from 0.5 to 1.5 (Fig. 4-8A), and a weak emulsion form was observed (Fig. 4-8B). Furthermore, after 9 and 24 h, the OD_{600} value increased to 2.6 and 6.1, respectively, and the culture supernatants showed strong emulsification. Subsequently, the samples of proteins in the culture medium were analyzed by SDS-PAGE (Fig. 4-8C). Without micafungin treatment, few proteins were included in the culture medium. Meanwhile, many proteins were detected after 24 h treatment with micafungin. Especially, five proteins were strongly detected and named Ca11, Ca12, Ca13, Ca14, and Ca15, as shown in Fig. 4-8C. These five proteins were also detected after 6 h and 9 h. Considering the emulsification activity shown in Fig. 4-8B, some of the five proteins were expected to have an emulsification activity. Therefore, we analyzed the bands (9 h) by mass spectrometry, and Ca11, Ca12, Ca13, Ca14, and Ca15 were identified as Tkl1, Hsp71, Kpyk, Eno1, and Fba1, respectively. The search parameters and mass spectrums were shown in Fig. 4-9. The characteristics of proteins are summarized in Table 4-4. The bands on SDS-PAGE almost matched the

molecular weight estimated from the amino acid sequence. Although these proteins exist in the cytoplasm and cell membrane, they are also reported to localize to the cell wall [15, 16]. In pathogen fungi including *C. albicans*, Tkl1, Hsp71, Kpyk, Eno1, and Fba1 are intercellular proteins with housekeeping functions and without conventional signal sequences, however these proteins also localized to cell surface and have pathogenic functions, such as binding to plasminogen, macrophage and monocyte activation [17]. From these reports, the emulsion obtained after micafungin treatment was supposed to be formed by these proteins released from cell surface.

(A)



(B)

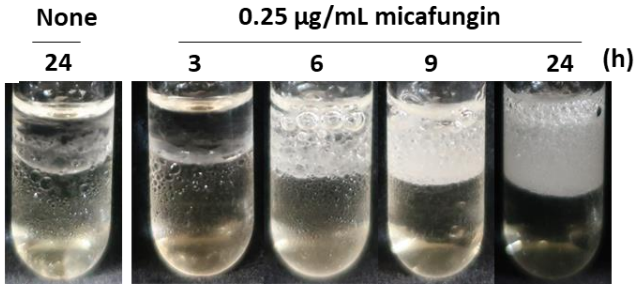


Fig. 4-8 The analysis over time of supernatants after culturing the *C. albicans* IFO1061 strain in YNBPS with 0.25 μg/mL micafungin. (A) Growth results are presented as means ± the standard deviation of three independent experiments. (B) Emulsification using culture supernatants. Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

(C)

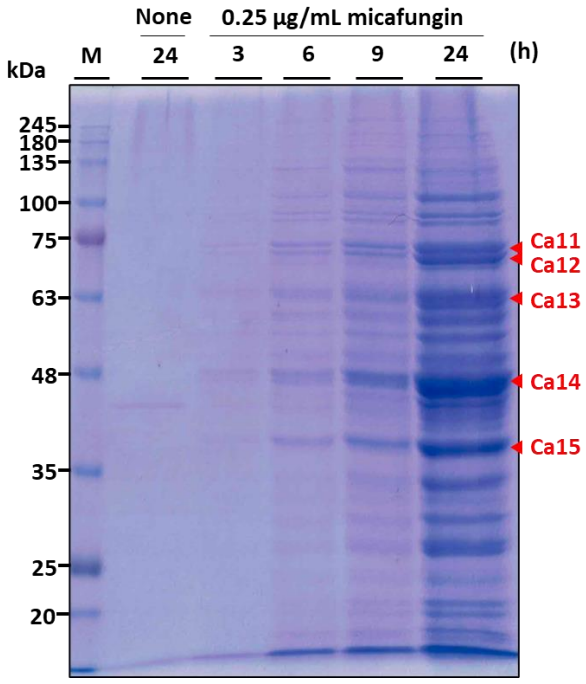
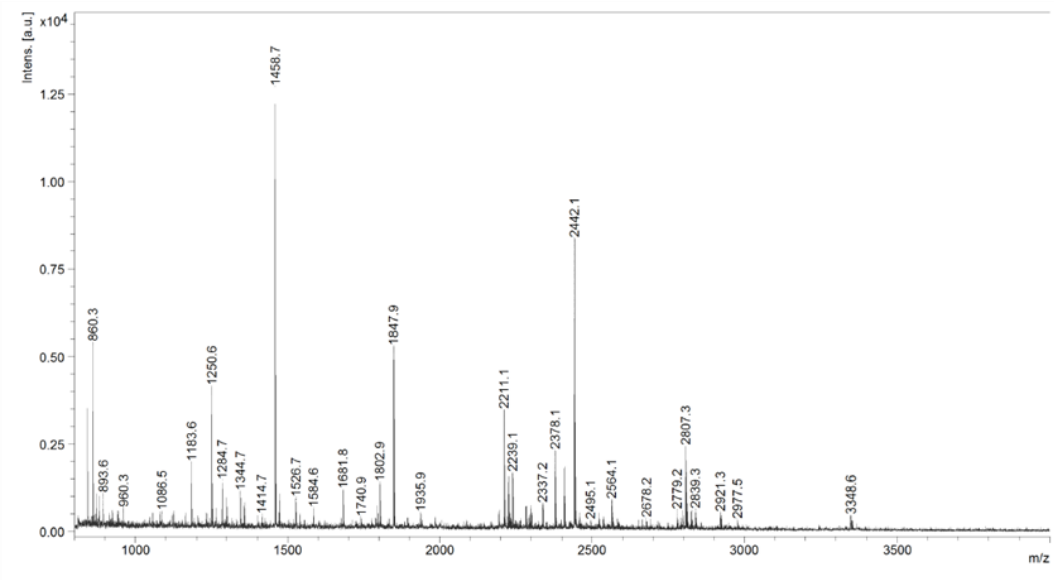


Fig. 4-8 The analysis over time of supernatants after culturing the *C. albicans* IFO1061 strain in YNBPS with 0.25 µg/mL micafungin (Continued). (C) SDS-PAGE of culture supernatants. M: WIDE-VIEW Prestained Protein Size Marker III (Fujifilm Wako Pure Chemical) was used as a molecular weight marker. Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

Table 4-4 The characteristics of proteins identified by mass spectrometry after micafungin treatment. Reprinted with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

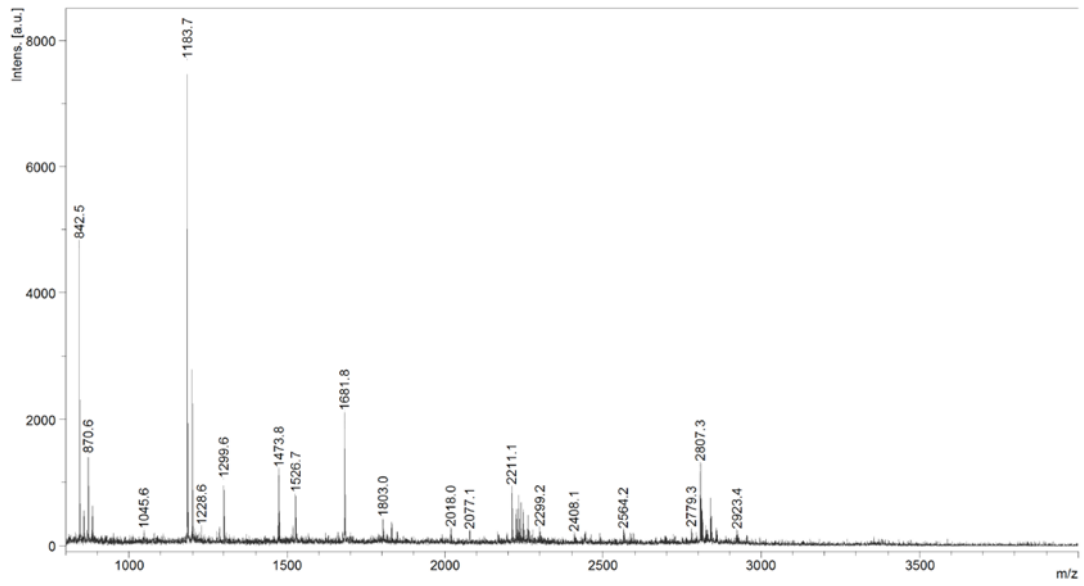
	Protein	MW [kDa]	Score	Localization (referred to CGD)	Function
Ca11	Tkl1	73.8	82	Cell wall and cell membrane	Transketolase
Ca12	Hsp71	70.5	73	Cell wall, cell membrane, cytoplasm, and nucleus	Heat shock protein
Ca13	Kpyk	55.8	101	Cell wall, cell membrane, and cytoplasm	Pyruvate kinase
Ca14	Eno1	47.2	87	Cell wall, cell membrane, cytoplasm, and nucleus	Enolase 1
Ca15	Fba1	39.4	116	Cell wall, cell membrane, and cytoplasm	Fructose-bisphosphate aldolase

MW is the molecular weight estimated from the amino acid sequence. The scores >70 were considered significant ($p<0.05$).

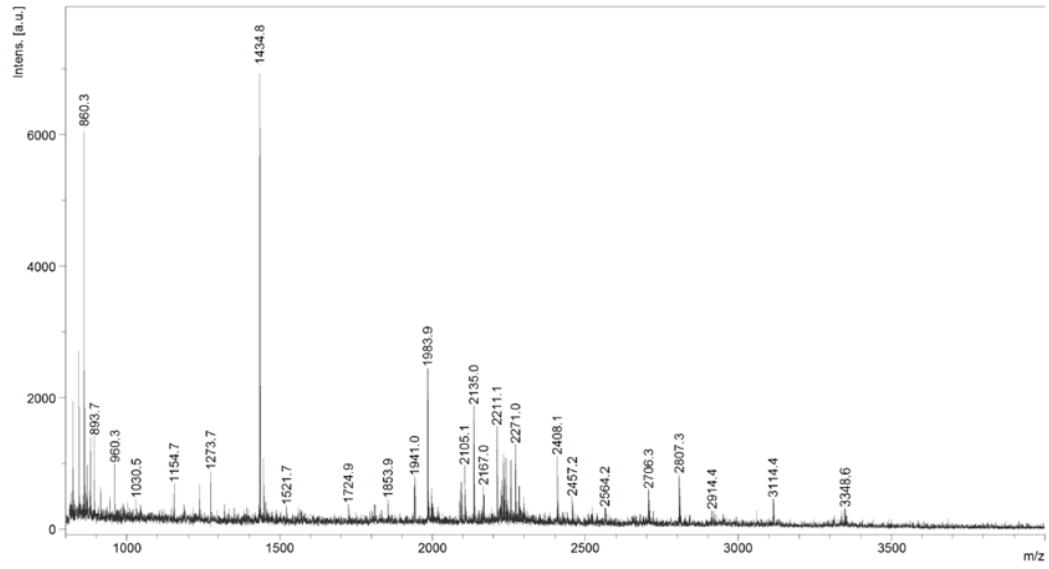


Mass spectrum of protein band Ca11

Fig. 4-9 The mass spectrum of protein bands detected by SDS-PAGE (Fig. 4-8C). The search parameters were as follows: type of search, peptide mass fingerprint; database, SwissProt 2021_03; taxonomy, Fungi; enzyme, trypsin; fixed modification, carbamidomethyl (C); variable modifications, oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ±50 ppm; peptide charge state, 1+; maximum missed cleavages, 2. The protein score was calculated as –10*Log(P), where P is the probability that an observed match is a random event. The protein scores above 70 were assumed to be significant ($p<0.05$). Reprinted with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

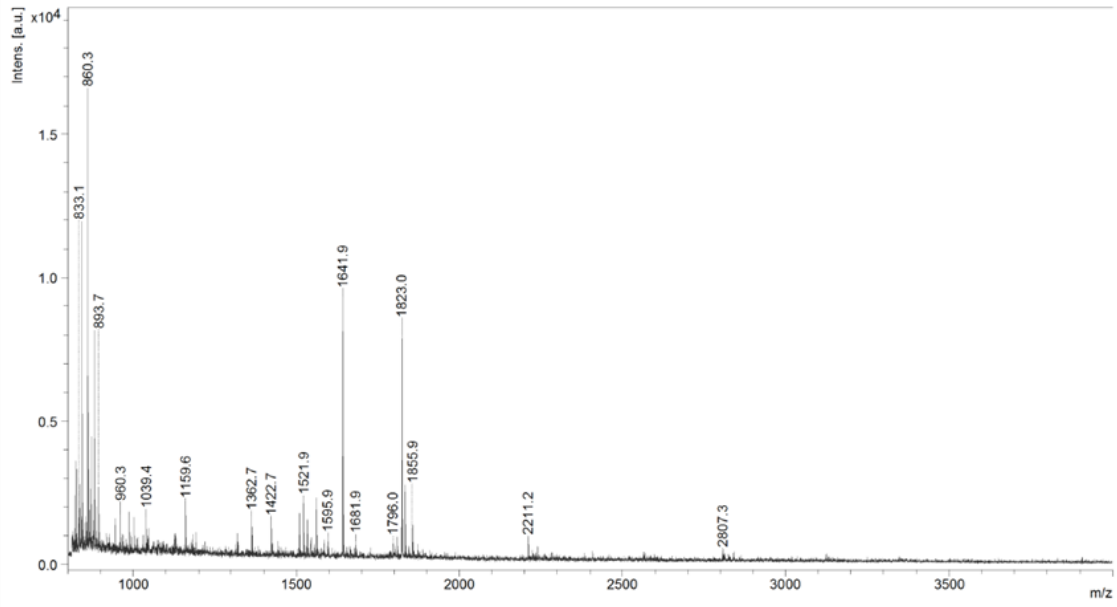


Mass spectrum of protein bands Ca12

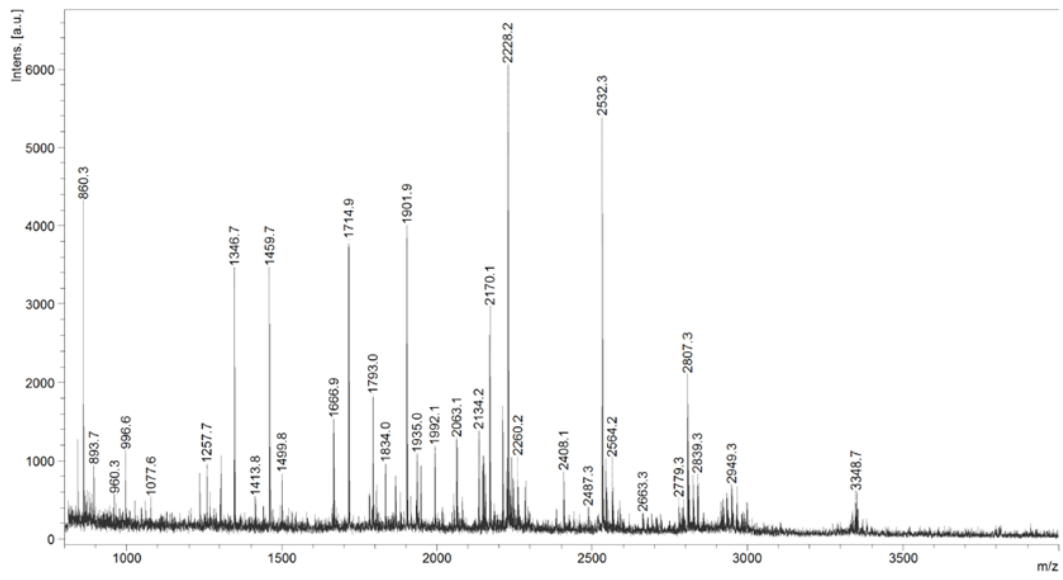


Mass spectrum of protein bands Ca13

Fig. 4-9 The mass spectrum of protein bands detected by SDS-PAGE (Fig. 4-8C). (Continued)



Mass spectrum of protein bands Ca14



Mass spectrum of protein bands Ca15

Fig. 4-9 The mass spectrum of protein bands detected by SDS-PAGE (Fig. 4-8C). (Continued)

4-3-6 Emulsification activity of recombinant Tkl1, Kpyk, Eno1, and Fba1

In order to evaluate the emulsification activities of Tkl1, Kpyk, Eno1, and Fba1, recombinant proteins tagged with 6 × histidines at their C-terminal positions were purified. First, the plasmids expressing recombinant TKL1, HSP71, KPYK, ENO1, and FBA1 were constructed under the PGK1 promoter in *S. cerevisiae*. In this section, the *S. cerevisiae anp1*Δ*gup1*Δ strain was transformed with the constructed plasmid. As shown in Fig. 2-2, the mannan of the outer-most layer is remarkably decreased in each *anp1*Δ and *gup1*Δ strain. In chapter 2, it was confirmed that the emulsifying proteins released by washing the cell wall mutants, in which mannan remarkably decrease, with PBS. Additionally, it is known that Tkl1, Kpyk, Eno1, and Fba1 localize to the cell wall despite having no secretion signal [17, 18]. Therefore, we attempted to release the recombinant proteins by washing an *anp1*Δ*gup1*Δ cells with PBS and purifying the protein.

Western blotting and CBB staining after SDS-PAGE confirmed that the recombinant proteins, Tkl1, Kpyk, Eno1, and Fba1, except for Hsp71, were released from the cells when the cells were suspended in PBS. The recombinant Hsp71 was detected in disrupted cell debris (data not shown), and Hsp71 may not be secreted in this system. Then recombinant Tkl1, Kpyk, Eno1, and Fba1 were purified from a PBS solution by a Ni-NTA affinity column. As shown in Fig. 4-10, SDS-PAGE and western blotting confirmed the high purity of each protein. Subsequently, the purified Tkl1, Kpyk, and Eno1 were dialyzed with pure water and freeze-dried, and the purified proteins were used to evaluate the emulsification activity. Meanwhile, the emulsification activity of recombinant Fba1 was evaluated without dialysis and freeze-dried because recombinant

Fba1 was aggregated by dialysis with pure water. After the protein concentrations were adjusted to 10, 20, 30, 40, and 50 μg/mL in pure water, the emulsification activities were evaluated (Fig. 4-11). As a result, Fba1 showed a strong emulsification activity, and Tkl1 and Eno1 showed a weaker emulsification activity than Fba1. Meanwhile, Kpyk showed no emulsification activity. These results indicated that Fba1, Tkl1, and Eno1 contributed to emulsification by culture supernatant obtained when *C. albicans* β-1,3-glucan synthesis was inhibited under osmotic protection.

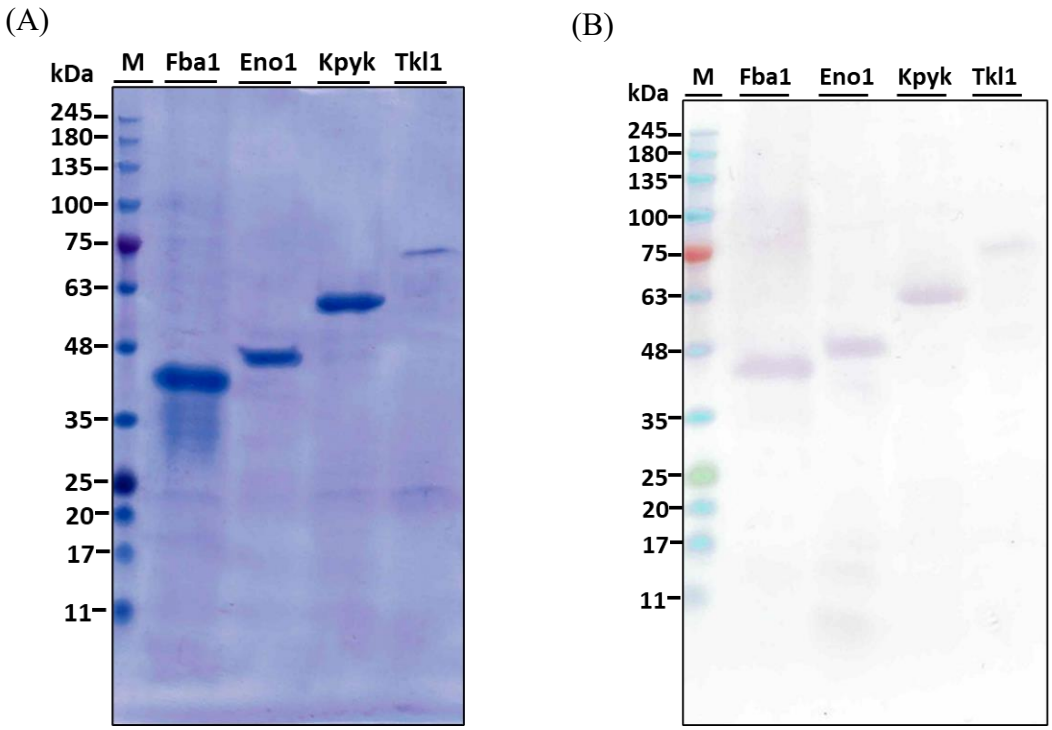


Fig. 4-10 SDS-PAGE and western blotting of recombinant proteins. SDS-PAGE (A) and western blotting (B) of Tkl1, Kpyk, Eno1, and Fba1 purified using Ni-NTA affinity column. Purified recombinant Kpyk, Eno1, and Fba1 (2 μg) were used for electrophoresis. In recombinant Tkl1, 0.1 μg of protein was used. Recombinant proteins were detected with anti-His-tag mAb. M: WIDE-VIEW Prestained Protein Size Marker III (Fujifilm Wako Pure Chemical) was used as a molecular weight marker. Reprinted with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

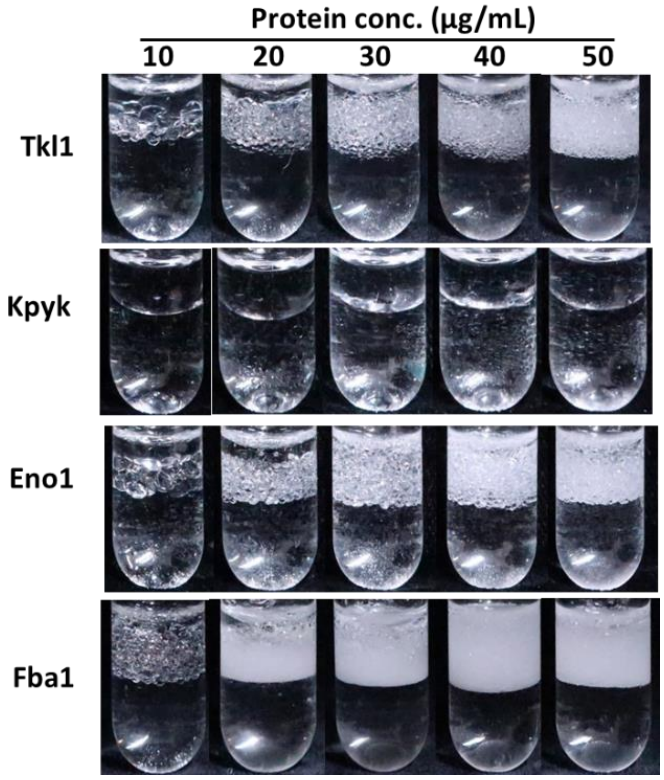


Fig. 4-11 Emulsification by recombinant Tkl1, Kpyk, Eno1, and Fba1, which were expressed in the *S. cerevisiae anp1*Δ*gup1*Δ strain. The Tkl1, Kpyk, and Eno1 solutions were prepared with pure water. The Fba1 solution was prepared with pure water containing 10–50 mM imidazole (due to dilution without dialysis), and 50 mM imidazole solution showed no emulsification activity (data not shown). Reproduced with permission from Nerome *et al.*, J Microbiol Methods. Copyright 2023 Elsevier.

4-4 Conclusion

In this chapter, it was confirmed that emulsification was also detected in the culture medium when *C. albicans* was cultured with an inhibitor of GPI-anchor synthesis. This result strongly supports that a novel screening method using emulsion formation as an indicator can be applied to detect inhibition of β-1,3-glucan and GPI-anchor synthesis. However, inhibition of β-1,3-glucan and GPI-anchor synthesis can be distinguished by evaluating the cell growth in an osmotically stabilized medium because cell growth after inhibition of GPI-anchor synthesis did not recover by osmotic protection unlike after inhibition of β-1,3-glucan synthesis, or the inhibitions can be distinguished by the strength of emulsification activity because emulsification observed in GPI-anchor synthesis inhibition was weak compared with β-1,3-glucan synthesis inhibition.

Furthermore, it was confirmed that Phr2, Tkl1, Eno1, and Fba1 have emulsification activity, especially Phr2 and Fba1 have strong activity. Phr2, which is GPI anchored cell wall protein and homolog of *S. cerevisiae* Gas1, released from cell surface and showed emulsification activity when GPI-anchor synthesis or β-1,3-glucan synthesis was inhibited under osmotic protection. Meanwhile, Tkl1, Eno1, and Fba1, which is also localized to cell surface, released from cell surface and showed emulsification activity when β-1,3-glucan synthesis was inhibited under osmotic protection.

I anticipate that additional research into the emulsifying capabilities of mannoproteins such as Phr2, Pga4, Tkl1, Eno1, and Fba1 may lead to practical applications in the future. The examination by emulsion is conducted visually, which is straightforward and adequate. If emulsification activity could be evaluated quantitatively, the proposed screening method would be more trustworthy.

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Chapter 5

Summary

For the first time, I examined the relationship between changes in yeast cell surface and emulsification activity in detail, and I also proposed a novel screening method using yeast-derived emulsification as an indicator for inhibition of fungal cell wall synthesis, which will undoubtedly lead to the development of new antifungal drugs.

In chapter 1, general introductions about emulsifiers, yeast cell walls, antifungal drugs, and the outline of this study were described.

Chapter 2 of the study focused on examining gene mutations that enhance the emulsification ability of *S. cerevisiae*. The findings revealed that certain mutant strains (*mcd4*Δ, *anp1*Δ, *och1*Δ, *mnn10*Δ, *mnn11*Δ, and *hoc1*Δ) with decreased cell wall mannan content exhibited strong emulsification activity. Additionally, emulsifying substances were observed to be released from the cells when they were washed with PBS, and these substances had a high molecular weight ranging in the tens of thousands. Through screening strains with emulsifying activity, two strains (*asc1*Δ and *scp160*Δ) were identified as having a strong emulsification ability despite not exhibiting a significant decrease in mannan content. While the direct impact of these defective genes on cell wall synthesis remains unknown, they were found to affect the structure of the cell wall. There have been no reports indicating such mutations impair yeast cell emulsion formation, but the accumulation of such discoveries is required for the creation of a yeast-derived emulsifier.

In chapter 3, it was examined whether the emulsion phenomenon was detected by

inhibition of fungal cell wall synthesis. As a result, strong emulsification was detected when pathogen *C. albicans* cells were cultured with a β-1,3-glucan synthesis inhibitor under osmotic protection. The emulsion phenomenon was not obtained by treatment with the antifungal compounds, which act on other cell components, including inhibition of *N*-linked protein glycosylation, chitin synthesis, cell membrane, and nucleic acid synthesis. Furthermore, it was found that the emulsion form is caused by the released proteins upon inhibition of β-1,3-glucan synthesis. In the osmotically stabilized medium containing β-1,3-glucan synthesis inhibitor, *C. albicans* cells with an incomplete cell wall can grow, and the mannoproteins release from the cell surface, resulting in strong emulsifying ability. The above results strongly support the idea that we can use emulsification as an indicator for inhibition of β-1,3-glucan synthesis.

In chapter 4, we focused on the cell wall structure, specifically the linkage of many cell wall proteins to β-1,3-glucan via the carbohydrate moiety of the GPI-anchor. The objective was to examine whether emulsification could be achieved by inhibiting GPI-anchor synthesis. The results confirmed that emulsification was detected in the culture supernatant following GPI-anchor synthesis inhibition, although the emulsifying activity was weaker compared to inhibition of β-1,3-glucan synthesis. This suggests that emulsion formation can be used as a novel screening method to detect inhibition of both β-1,3-glucan and GPI-anchor synthesis. However, it was possible to distinguish between inhibition of β-1,3-glucan and GPI-anchor synthesis based on the strength of emulsification activity or by evaluating cell growth in an osmotically stabilized medium. Unlike after β-1,3-glucan synthesis inhibition, cell growth did not recover with osmotic protection after GPI-anchor synthesis inhibition.

Furthermore, in order to understand the mechanism of the emulsion phenomenon

after inhibition of β-1,3-glucan or GPI-anchor synthesis, the proteins included in the culture supernatant after treatment with manogepix and micafungin were analyzed. In conclusion, it was found that Phr2, Tkl1, Eno1, and Fba1, which are known to localize on the cell wall, have emulsification activity. Especially, Phr2 releases and contributes to the emulsification when GPI-anchor synthesis or β-1,3-glucan synthesis is inhibited. Meanwhile, Tkl1, Eno1, and Fba1 release and contribute to emulsification only when β-1,3-glucan synthesis is inhibited.

The findings from this study about cell wall mutation and release of emulsifying protein could be useful to improve the productivity of yeast derived emulsifiers for food and cosmetical industries. Furthermore, the findings of emulsion phenomenon obtained by inhibiting yeast cell wall synthesis could be useful for the first screening of antifungal agents inhibiting β-1,3-glucan synthesis.

List of publications

Shinsuke Nerome, Masaya Onishi, Daiki Saito, Ayano Mizobuchi, Tatsuya Ando, Yui Daira, Azusa Matsumoto, Yoshihiro Ojima, and Masayuki Azuma: Cell surface changes that advance the application of using yeast as a food emulsifier. Food Chem. 315: 126264 (2020).

(Chapter 2)

Shinsuke Nerome, Naoki Yokota, Yoshihiro Ojima and Masayuki Azuma: Evaluating β-1,3-glucan synthesis inhibition using emulsion formation as an indicator. J Microbiol Methods. 190:106327 (2021).

(Chapter 3)

Shinsuke Nerome, Mai Tsudzuki, Minori Nizuka, Mao Takata, Yoshihiro Ojima, and Masayuki Azuma: Identification of emulsification proteins released from the cells by inhibiting the synthesis of GPI-anchor or β-1,3-glucan in *Candida albicans*. J Microbiol Methods. 209:106728 (2023).

(Chapter 4)

Acknowledgements

I am grateful to Prof. Masayuki Azuma, Osaka Metropolitan University, for his valuable advice, inspiration, and all research facilities to purpose and complete this research. I am also grateful to Prof. Masaya Kitamura and Prof. Akira Tachibana for valuable discussions and suggestions.

I thank Associate Prof. Yoshihiro Ojima and Associate Prof. Koichi Igarashi for valuable helps and inspirations, and Tomomi Shimonaka of Osaka Metropolitan University for mass spectrometry.

I thank members of Cell Engineering Lab., especially, Masaya Onishi, Daiki Saito, Ayano Mizobuchi, Tatsuya Ando, Yui Daira, Azusa Matsumoto, Naoki Yokota, Mai Tsudzuki, Minori Nizuka, and Mao Takata, for their kind cooperations.

I also thanks Akio Koda Research Institute director and Takayuki Bogaki Senior Adviser, Ozeki Corporation, for giving me a chance to pursue this study.

Finally, I am grateful to my family for all kind of support.