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Evaluation of Fatty Acid Synthase as a Molecular Target for Stress-Dependent Fungicidal Activity of 1-Geranylgeranylpyridinium

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Abstract

Among various isoprenoid compounds, 1-geranylgeranylpyridinium (GGPy) showed remarkable lethal effects on Saccharomyces cerevisiae cells similarly under hypo- and hyperosmotic conditions at 30°C. In addition to such osmotic stress, GGPy exhibited temperature-dependent lethal effects against S. cerevisiae and the pathogenic yeast Candida albicans at the human body temperature of 37°C. Fatty acid synthase (FAS) was identified as one of the GGPy-binding proteins and was considered a molecular target of GGPy in its inhibitory effect on the fungal stress adaptation. GGPy was not inhibitory to the activity of FAS assayed upon NADPH oxidation involved in acyl chain elongation by this multi-functional enzyme complex. Nevertheless, the lethality of GGPy was repressed in the medium where polyoxyethylene sorbitan monopalmitate (Tween 40) supplemented as the water-soluble and esterase-dependent source of palmitic acid. These findings may suggest that GGPy is permissive for acetyl unit incorporation into the growing chain of fatty acyl-CoA by FAS butis restrictive to its ultimate elongation to palmitoyl-CoA as a donor of the long-chain saturated fatty acid for the synthesis of stress-tolerant glycerophospholipids.

Index terms— Saccharomyces cerevisiae; Candida albicans; fungal stress adaptation; geranylgeraniol derivative; fungicidal activity.

1 Introduction

ntifungal chemotherapy is currently very limited and dominated mainly by the classical antifungal agents such as azole class of ergosterol biosynthesis inhibitors and polyene macrolides that can bind with this neutral lipid (Pianalto and Alspaugh, 2016). New semisynthetic lipopeptide antifungal agents which is commonly known as micafungin and pneumocandin are recently used in the treatment of systemic fungal infectious diseases (Morrison, 2006). This class of agents inhibits fungal cell wall biosynthesis by targeting ?-1, 3-glucan synthase. Rho1p is one of the fungal GTP-binding proteins in which C-terminal enables its plasma membrane association and the resulting activation of ?-1, 3-glucan synthase (Inoue et al., 1999;Levin, 2005). Therefore, GGTase is considered an alternative target for the development of antifungal agents with an increased selectivity toward fungal cell wall biosynthesis (Murthi et al., 2003;Nishimura et al., 2009;Sunami et al., 2002).

Farnesol (FOH) is naturally-occurring isoprenoid alcohol with a shorter isoprenyl chain than geranylgeraniol (GGOH), which is also involved in protein prenylation as a result of pyrophosphorylation of the terminal hydroxyl group. We had found a marked antifungal activity of FOH in addition to the predominant apoptosis-inducing activity against various tissuecultured mammalian cells (Machida et al., 1998;Machida and Tanaka, 1999;Voziyan et al., 2005). 1-Farnesylpyridinium (FPy, Fig. 1) is a newly synthesized derivative of FOH, is characterized by

an extreme increase in the apoptosis-inducing activity especially against human leukemiacells (HL-60) (Hamada et al., 2002;Hamada et al., 2006). Such a hybrid structure of FPy generated a unique antifungal activity as judged by the transformation of the rod-shaped cell wall to the swollen spherical architecture in the fission yeast Schizosaccharomyces pombe. Isoprenoid may give us a novel type of antifungal agent as a result of the structural modification of its terminal hydroxyl group.

In this study, 1-geranylgeranylpyridinium (GGPy, Fig. 1) was newly synthesized as a possible inhibitor of GGTase, and its antifungal activity was evaluated based on the inhibitory activity on ?-1, 3-glucan synthetic reaction. Unlike the cell wallassociated toxic event, however, GGPy exhibited a marked fungicidal activity similarly under low and high osmotic environments. We additionally found the thermal stress-dependent lethality of GGPy against the human pathogenic fungus Candida albicans at the human body temperature of 37°C. Fatty acid synthase (FAS) was identified as one of the GGPy-binding proteins, being considered a target for the newly detected stress-dependent fungicidal activity of GGPy.

A Candida albicans at the human body temperature of 37ºC.

2 materials and methods

3 a) Syntheses of isoprenoid-derivatives

FPy, geranylgeranylchloride, and geranylgeranylamine (GGNH 2) were synthesized as previously described (Hamada et al., 2002;Tanaka et al., 2004). GGPy was synthesized by following the procedures for FPy synthesis, as described below. A mixture of pyridine (0.6 mmol, 48.5 µl) and geranylgeranylchloride (0.6 mmol, 180.6 µl) was heated at 80°C for 1 h. After cooling to room temperature, the reaction mixture was diluted with 1.0 ml of dichloromethane and applied onto a silica gel column (ø 3.0 × 5.0 cm), which had been equilibrated with this solvent. After washing the column with dichloromethane, stepwise elution was done by increasing methanol concentration in dichloromethane up to 7% (v/v). GGPy was eluted in the fraction consisting of 97% (v/v) dichloromethane and 3% (v/v) methanol and was finally isolated as a faintly reddish slimy liquid (113.9 mg) after evaporation of the organic solvent. The spectral data of GGPy were taken on a JEOL JNM-LA400 spectrometer in chloroform- 3-amino-GGPy was synthesized by following the method of GGPy synthesis except for the use of a mixture of 3-amino pyridine (0.6 mmol) and geranylgeranylchloride (0.6 mmol). 3-amino-GGPy was obtained as a faintly red-orange oily liquid, and its FAB (+)-MS m/z spectrum value of 367.5 coincided with m/z 367.5905 calculated for C 25 H 39 N 2 .

4 b) Measurement of yeast cell growth and viability

Unless otherwise stated, S. cerevisiae W303-1A was used in the following experiments to examine the effects of FPy, GGOH, and its derivatives on cell growth and cell viability (Ogita et al., 2010). C. albicans NBRC 1061 was also used in addition to S. cerevisiae BY4741 and its glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene deletion mutant ? tdh1 (Yutani et al., 2011). Cells of these yeast strains were grown overnight in YPD medium, which contained (per liter) 10 g of yeast extract, 20 g of peptone, and 20 g of glucose, with vigorous shaking at 30°C. After dilution of the overnightgrown culture with distilled water (DW) to 10 7 cells/ml, cells were incubated without or with each compound in DW, YPD medium, and YPD medium containing 1.2 M D-sorbitol as an osmotic stabilizer. Viable cell number was determined by the methylene blue method or by counting colony-forming units after a 48-h incubation at 30°C in YPD medium containing 1.8% (w/v) agar (Iida et al., 1990). Minimum growth inhibitory concentration (MIC) of GGPy was determined by the serial broth dilution method using a 96-well microplate, in which S. cerevisiae W303-1A cells were suspended at 10 6 cells/ml in YPD medium and incubated for 48 h at 30°C and 37°C.

5 c) Purification of GGPy-binding proteins

To bind GGPy residue covalently to Sepharose 4B gel as a molecular ligand, ECH Sepharose TM 4B (2 g) was subjected to carbodiimide-mediated coupling with amino-GGPy (20 mg) in DW according to the direction of the supplier's manual (GM Healthcare). ECH Sepharose TM 4B was also treated with carbodiimide in the absence of amino-GGPy, and such chemically treated Sepharose 4B gel itself was used as a control for the detection of proteins, which are bound directly to Sepharose 4B column without GGPy as a ligand.

Overnight-grown cells of S. cerevisiae were inoculated into 200 ml of YPD medium and incubated with vigorous shaking at 30°C for 8 h. Without or with the additional incubation at 37°C for 1 h, the cells were collected by centrifugation and washed twice with phosphate-buffered saline (PBS) consisting of 137 mM NaCl, 8.10 mM Na 2 HPO 4 ?12H 2 O, 2.68 mM KCl, and 1.47 mM KH 2 PO 4 . The finally obtained cell pellets were suspended in 5 ml of PBS. The yeast cells were then disrupted by repeated vortexing with glass beads, and the supernatant (200 µl) obtained by centrifugation was applied onto the column of 3-amino-GGPy-Sepharose 4B (1.5 × 20 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was extensively washed with the same buffer and then with 10 ml of the buffer containing 100 µM GGPy. The fractions (10 ml) finally eluted with the buffer alone, and the following fraction eluted with the buffer containing GGPy were concentrated to 35 µl in Amicon Ultra Centrifugal Filters ULTRA CELL-10 K by centrifugation at 7,000 rpm. Protein contents in these fractions were measured by the Bradford method using bovine serum albumin as a standard. These concentrated samples were

subjected to SDS-PAGE using a 15% (w/v) polyacrylamide gel and an ATTO Mini-Slab Electrophoresis System (Tokyo, Japan) at a constant current of 10 mA. The gels were stained with 0.25% (w/v) coomassie brilliant blue for 30 min and de-stained with a mixture of 5% (v/v) acetic acid and 5% (v/v) methanol in DW.

6 d) Identification of GGPy-binding proteins

GGPy-binding proteins were identified by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) **??**Iida et al., 2017). The major protein bands were excised from the gel and digested with trypsin. Mass spectra were collected by MALDI-TOF-MS using a Brucker Daltonics auto flex speed TOF/TOF system (Billerica, MA, USA) for protein identification. Peptide mass fingerprinting was carried out using the MASCOT program for protein identification (www.matrixscience.com/).

7 e) Assays of enzyme activities

The activity of GAPDH was determined using the reaction mixture containing 1.0 mM NAD + , 10 mM EDTA, 0.1 mM dithiothreitol, 4.0 mM glyceraldehyde-3phosphate, and the enzyme in 100 mM potassium phosphate buffer (pH 7.4) without or with 100 µM GGPy (McAlister and Holland, 1985). After the addition of the purified preparation of GAPDH from baker's yeast (Sigma-Aldrich), the increase in the absorption was monitored at the wavelength of 340 nm in a spectrophotometer at 37°C as the corresponding production of NADH. Glyceraldehyde 3-phosphate dehydrogenase activity is defined as the enzyme amount that can produce 1 µmol of NADH per min under the above conditions.

The activity of FAS was determined by NADPH oxidation coupled with both 3-ketoacyl reductase and enoyl reductase activities involved in each cycle of acetyl unit elongation by FAS (Hori et al., 1987). The reaction mixture consists of 200 µM NADPH, 50 µM acetyl-CoA, 50 µM malonyl-CoA, and the enzyme in 100 mM potassium phosphate buffer (pH 7.0) without or with 100 µM GGPy. FAS was partially purified from 100 µl of the cell-free extract, which was used for the detection of GGPy-binding proteins, with the aid of the ultrafiltration device VIVA SPIN 1000 k (Zartrius Co.) depending on its extremely enormous molecular weight (2,600 k). The time-dependent decrease in the absorption at 340 nm due to oxidation of NADH to NAD + was monitored in a spectrophotometer at 37°C. The enzyme activity is defined as the enzyme amount that can oxidize 1 µmol of NADPH to NADP + per min under the above conditions. The specific activity of the partially purified preparation was 1.09 units per mg protein.

8 f) Chemicals

Farnesol (FOH) and geranylgeraniol (GGOH) were products of Sigma & Aldrich (St. Louis, MO, USA). Glyceraldehyde-3-phosphate, NAD, NADPH, acetyl-CoA, malonyl-CoA, and cerulenin were also purchased from Sigma & Aldrich. Polyoxyethylene sorbitan monolaurate (Tween 40) was the product of Wako (Osaka, Japan). Other chemicals are of analytical reagent grade.

9 III.

10 Results

11 a) Effects of GGPy on the cell viability of S. cerevisiae under various osmotic conditions

We first examined the lethal effects of GGPy in DW, YPD medium, and YPD medium with D-sorbitol added as an osmotic stabilizer to estimate its relation to the loss of cell wall integrity. As shown in Fig. 2A, GGPy lethality was markedly enhanced when cells were incubated in DW, suggesting the possibility of its dependence on inhibition of the yeast cell wall biosynthetic reaction. In contrast to our expectation, however, GGPy lethality was similarly enhanced under the hyperosmotic conditions with D-sorbitol, which was thought to protect GGPy-treated cells against the plasma membrane disruptive damage. These findings supported the idea of attributing GGPy lethality to its interference with a mechanism of cellular adaptation to the osmotic imbalance. It was therefore required to confirm the loss of cell viability directly in the GGPycontaining medium. Methylene blue staining was thus employed for the real-time evaluation of cell viability, giving rise to the results comparable to those obtained by the CFU-dependent analysis (Fig. 2B). This method indicated that GGPy lethality is enhanced in response to the osmotic imbalance or the environmental osmotic stress.

Figure 2C shows the structure-activity relationship in the osmotic stress-dependent fungicidal activity, indicating the loss of the corresponding lethality in the molecular structure of FPy with a shorter isoprenyl chain. GGOH is a naturally-occurring source of geranylgeranyl chain, being ineffective in causing the osmotic stress-dependent lethality. GGNH 2 was able to enhance the lethality only in YPD medium with D-sorbitol, but not in DW, suggesting the dependence of its fungicidal activity on a different type of toxicity. These results may indicate that both the geranylgeranyl chain and the structural modification of the terminal hydroxyl group are the minimum required for the generation of osmotic stress-dependent fungicidal activity. We next examined whether or not GGPy can enhance the loss of fungal cell viability under the condition with environmental stress

other than the osmotic imbalance. As shown in Fig. 3, GGPy lethality was markedly enhanced even in the YPD medium alone by merely increasing the incubation temperature up to 37°C. As shown in Fig. 4, cells of C. albicans were more sensitive to GGPy than S. cerevisiae cells as judged from the partial loss of cell viability during incubation in YPD medium alone at 30°C. In spite of such an increased sensitivity to GGPy, GGPy lethality could be more increased when cells were placed under low and high osmotic environments. This human pathogenic fungal strain was also made sensitive to GGPy at the human body temperature of 37°C.

12 c) Identification of GGPy-binding proteins

It is highly probable that GGPy causes a stressdependent lethality using the molecular interaction with a protein functional for fungal stress adaptation. We, therefore, attempted to purify and identify GGPy-binding protein as a possible molecular target of GGPy. As shown in Fig. 5, only two proteins bands were detected upon SDS-PAGE for the protein sample purified by the affinity chromatography using GGPy as a molecular ligand. No protein band was found when the affinity chromatography was done using the Sepharose 4B column alone prepared without GGPy, indicating that these two proteins should have predominant binding affinities with GGPy. As summarized in Figs. 6A and B, the mass spectral data revealed that the proteins 1 and 2 are a mixture of ?-and ?-subunits of FAS and GAPDH isozyme 3, respectively (Fig. 6C).

13 d) Effects of GGPy on the activities of GAPDH and FAS

GAPDH isozyme 3 (Tdh3p) is involved in the oxidation of D-glyceraldehyde-3-phosphate to 1, 3bisphosphoglycerate in the yeast glycolytic pathway, in cooperation with the other two isozymes Tdh1p and Tdh2p (McAlister and Holland, 1985). As expected from the existence of these isozymes, cells of ?tdh3 are viable, being characterized by the same MIC value of GGPy (6.25 µM) at 37°C as those found with ?tdh1, ?tdh2, and even with the parent strain. Indeed, GGPy was not inhibitory to the activity of GAPDH, as shown in Fig. 7A. These results should support the idea of excluding GAPDH from the molecular target in the stress-dependent fungicidal activity of GGPy.

FAS catalyzes the synthesis of palmitoyl-CoA, which is a donor of palmitic acid as a constituent of stress-tolerant cellular glycerophospholipids (Tehlivets et al., 2007). However, GGPy was not inhibitory to the enzymatic oxidation of NADPH, which is coupled with the incorporation of the acetyl unit into the growing chain of acyl moiety by FAS (Fig. 7B). This means that FAS can also be excluded from the molecular target of GGPy on the assumption that the repeated incorporation of acetyl unit results in its ultimate elongation to yield palmitoyl-CoA.

14 e) Protective effects of palmitic acid on GGPy-induced growth inhibition

We finally confirmed whether or not the exogenous supplementation of palmitic acid can protect the yeast cells against GGPy lethality by using Tween 40 as the water-soluble and esterase-sensitive source of palmitic acid (Ohba et al., 1979; **??**aleng and Lands, 1975). As shown in Table **??**, cerulenin-mediated growth inhibition could be fully protected in medium with Tween 40, as judged from the highly increased MIC values, agreeing with its selective inhibitory effect on 3-oxoacyl-ACP synthase activity of FAS (Funabashi et al., 1989). Such an inhibitory effect of cerulenin was not affected by the reaction temperature, as seen from the mostly identical MIC values (100 µM) found at 30 and 37°C. The MIC value of GGPy could be similarly increased with the addition of Tween 40 at 30°C, though its MIC value was kept at the lower level at 37°C, agreeing with the temperature-dependent increase in the toxicity of GGPy. These findings supported the idea that GGPy is permissive for the reaction of acetyl unit incorporation into the growing chain of fatty acid by FAS as judged by the successful oxidation of NADPH. Thus, the palmitic acid-dependent growth recovery of GGPy-treated cells likely indicates the failure in the enzymatic elongation of the acyl chain to yield palmitoyl-CoA as a donor of the long-chain saturated fatty acid for the synthesis of stress-tolerant glycerophospholipids (Klose et al., 2012;Leach and Cowen, 2014). cellular resistance to cell wall stress caused by ?-1, 3-glucan synthase inhibitor caspofungin (Brown et al., 2014). However, Hog1 cannot be considered a primary target for the osmotic stress-dependent fungicidal activity of GGPy since the cell survival under low and high osmolarity conditions should require a more complicated responsive mechanism (Saxena and Sitaraman, 2016).

The osmotic stress-dependent fungicidal activity was generated only in the hybrid structure consisting of the geranylgeranyl chain and the nitrogencontaining attached group but not detected with GGOH itself (Figs. 1 and 2). Unlike GGPy, however, the lethality of GGNH 2 cannot be simply elucidated by the stressdependent fungicidal activity, since GGNH 2 -induced cell death could be highly enhanced in medium with D-sorbitol, but not in DW. Our previous study demonstrated that GGNH 2 could exhibit an antifungal activity depending on its molecular structure with a terminal amino group, which can serve as a substrate for fungal mitochondrial amine oxidase, producing H 2 O 2 as a toxic by-product (Tanaka et al., 2004). Thus, D-sorbitol-dependent toxicity of GGNH 2 may be elucidated by the enhancement of its incorporation across the fungal plasma membrane under high osmolarity conditions, resulting in the acceleration of its oxidation by the mitochondrial amine oxidase (see

Fig. 2). GGPy is only one with a clinical interest if its stress-dependent lethality can be applied against the temperature of 37°C (Figs. 3 and 4).

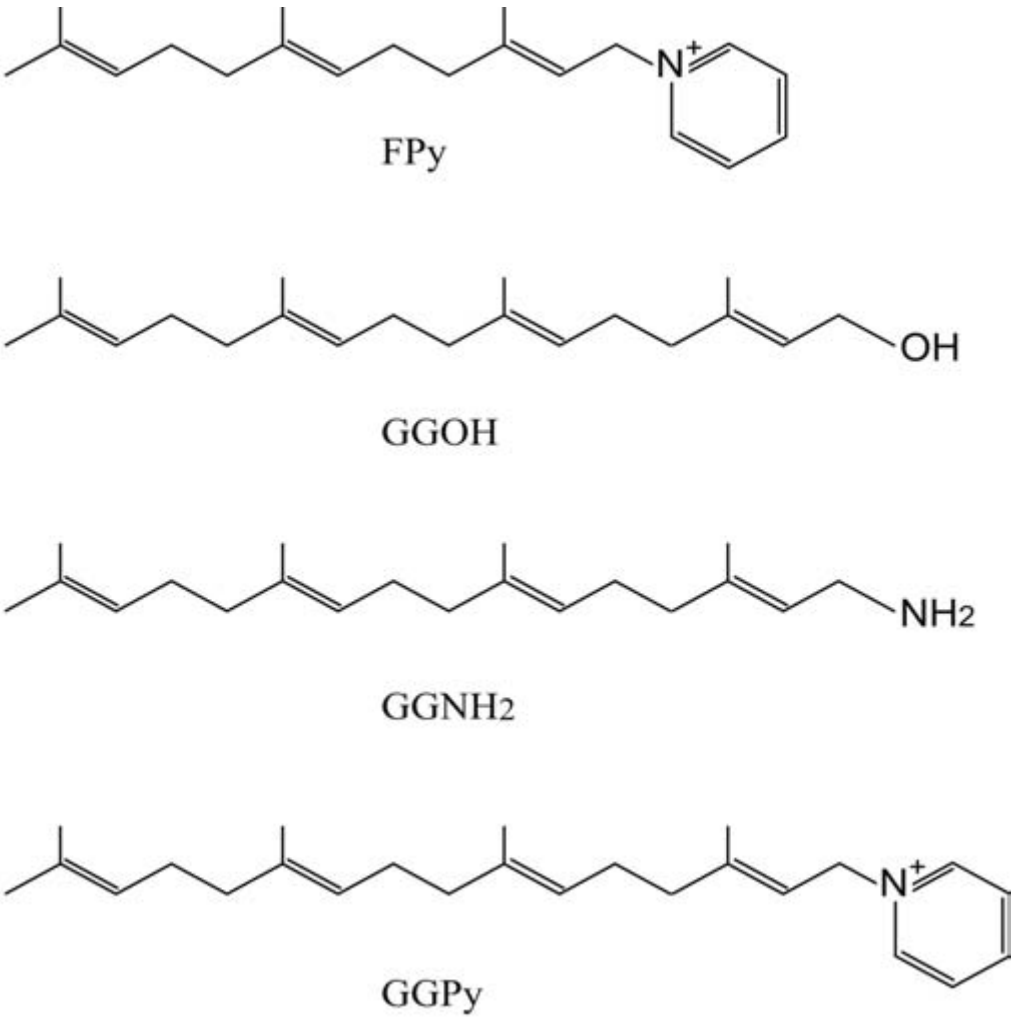
It is noteworthy that only two protein bands were detected on SDS-PAGE of GGPy-binding proteins, which are identified to be GAPDH isozyme 3 and each of ?-and ?-subunits of FAS, respectively (Figs. 5 and 6). This result supported the existence of a selective molecular interaction between GGPy and GAPDH, though such an intensive binding ability of GGPy could not be a cause of inhibition of the enzymatic activity. Another GGPy-binding protein FAS catalyzes the longchain fatty acyl-CoA synthetic reaction as a form of ? 6 ? 6 heteromultimeric complex, being responsible for the ultimate synthesis of palmitoyl-CoA as a donor of the long-chain saturated fatty acid. Under various stress conditions, the length of the fatty acyl chain must be fully elongated to yield palmitic acid, which is evaluated as a constituent of stress-tolerant glycerophospholipids (Tehlivets et al., 2007). Cerulenin is a typical inhibitor of 3-oxoacyl-ACP synthase activity involved in the overall palmitoyl-CoA synthetic reaction catalyzed by the fungal FAS complex (Funabashi et al., 1989).Indeed, ceruleninmediated inhibition of fatty acyl-CoA synthesis results in cell death in fungi regardless of the environmental stress condition (Nguyen and Nosanchuk, 2011). Unlike the case with cerulenin, however, GGPy has none of the structural relatednesses to acetyl-ACP or malonyl-ACP pathogenic fungi like C. albicans at the human body **Palmitic acid was provided as its water-soluble derivative (Tween 40) at the final concentration of 0.1% (w/v).

IV.

15 Discussion

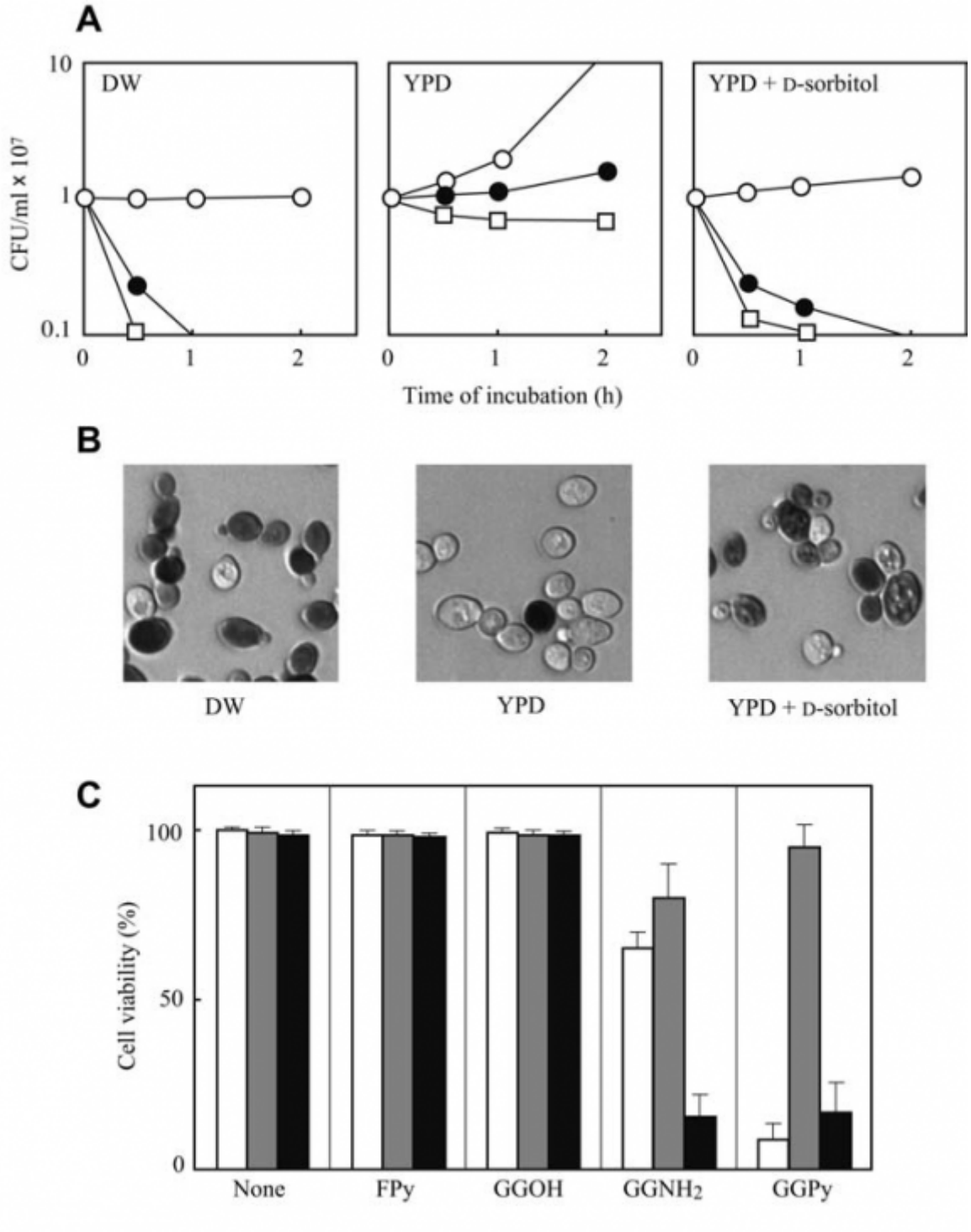
Fungal cells can survive under the conditions with various stresses like thermal stress, ionic stress, oxidative stress, and osmotic stress by provoking the corresponding response for stress adaptation (Brown et al., 2014;Hallsworth, 2018). Among these cellular responses, the Hog1 (high-osmolarity glycerol response) mitogen-activated protein kinase pathway is known for a pivotal role in the adaptation of S. cerevisiae to the stress from high external osmolarity. In C. albicans, the Hog1 pathway is also involved in the added as the substrates for fatty acyl chain elongation by FAS, agreeing with its inability of inhibiting the apparent activity of FAS.

In the cellular membrane of S. cerevisiae, the degree of phospholipid saturation is lowest at 15°C and highest at 37°C with the length of fatty acyl chain becoming longer with increasing environmental temperature (Klose et al., 2012;Leach and Cowen, 2014). This means that palmitic acid synthesis is essential for the yeast cell survival under the thermal stress condition at 37°C, agreeing with our finding that the exogenous supplementation of this long-chain fatty acid can protect the yeast cells against the toxicity of GGPy. Thus, it may be postulated that GGPy is permissive for the fatty acyl chain elongation by FAS to yield short-or medium-chain fatty acid, as represented by the successful oxidation of NADPH. Even in the case, GGPy may be inhibitory to the ultimate elongation of fatty acyl chain to yield palmitic acid. It is also possible to elucidate the growth inhibitory effect of GGPy at 30°C by the accumulation of short-or medium-chain fatty acids in GGPy-treated cells, as is the case with the growth inhibition by the production of octanoic and decanoic acids during the process of yeast alcohol fermentation (Viegas et al., 1989). Further investigation is needed to solve how GGPy can interact with FAS for modification of the enzymatic fatty acyl chain elongation.



1

Figure 1: Fig. 1 :


A
DW
YPD
YPD + D-sorbitol
CFU/ml x 10^7
10
1
0.1
0
1
2
Time of incubation (h)
B
DW
YPD
YPD + D-sorbitol
C
Cell viability (%)
100
50
0
None
FPy
GGOH
GGNH2
GGPy

1

Figure 2: d 1 .

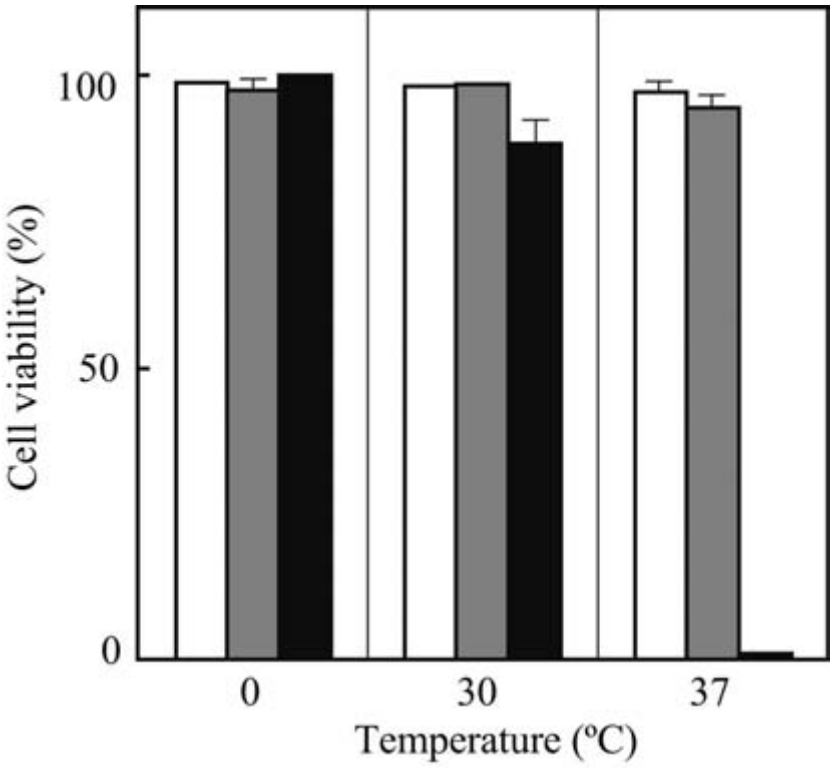


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Figure 3: Fig. 2 :

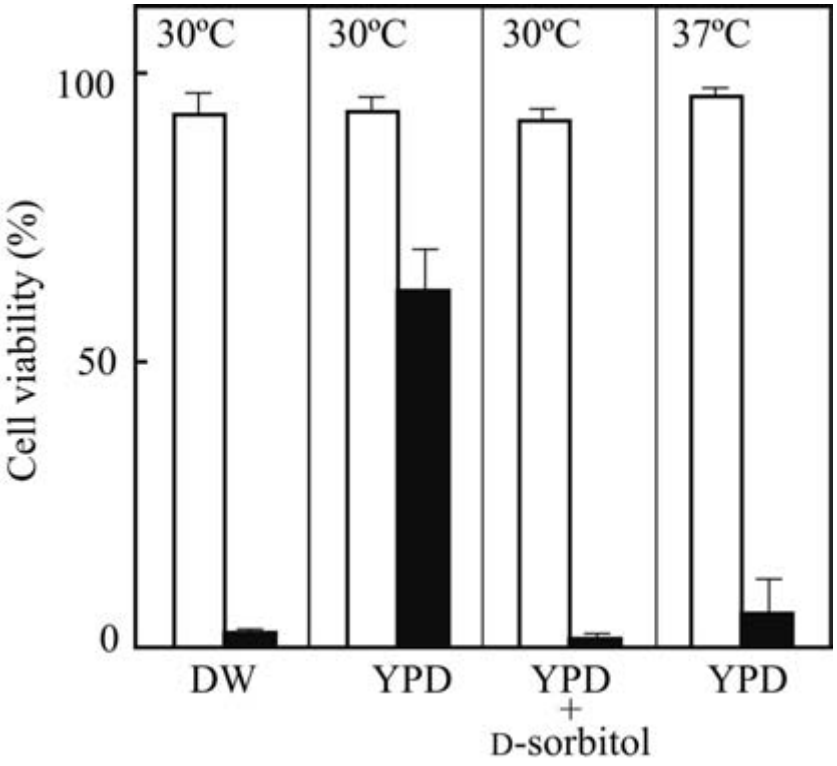

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Figure 4: Fig. 3 :

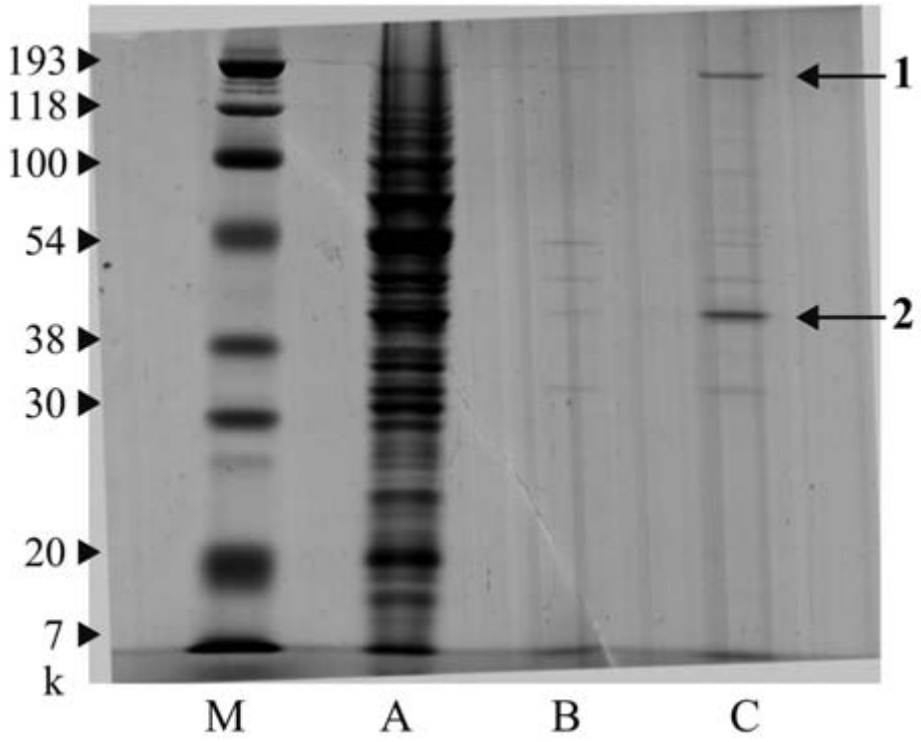

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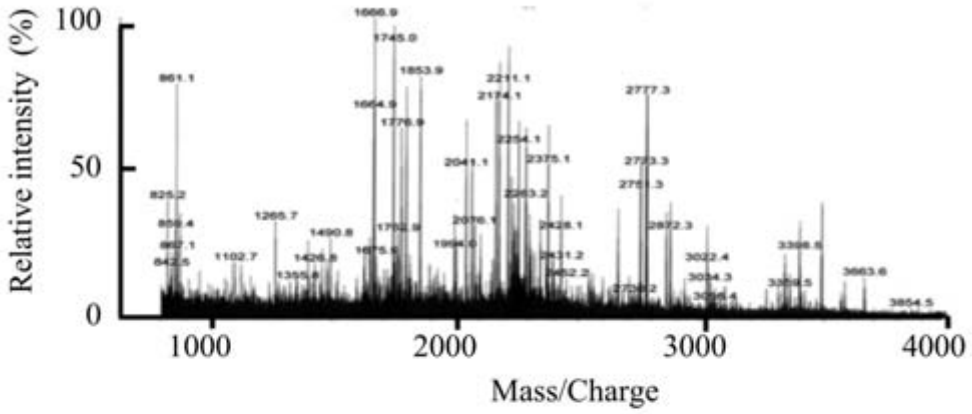
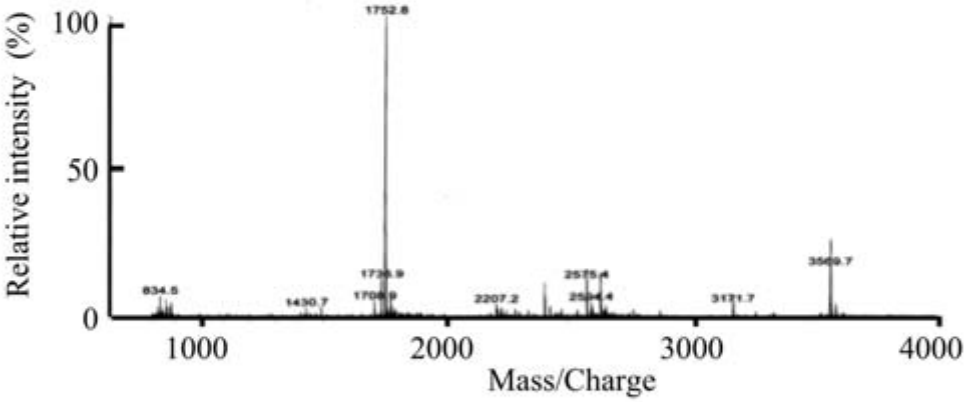
Figure 5: Fig. 4 :

A



B

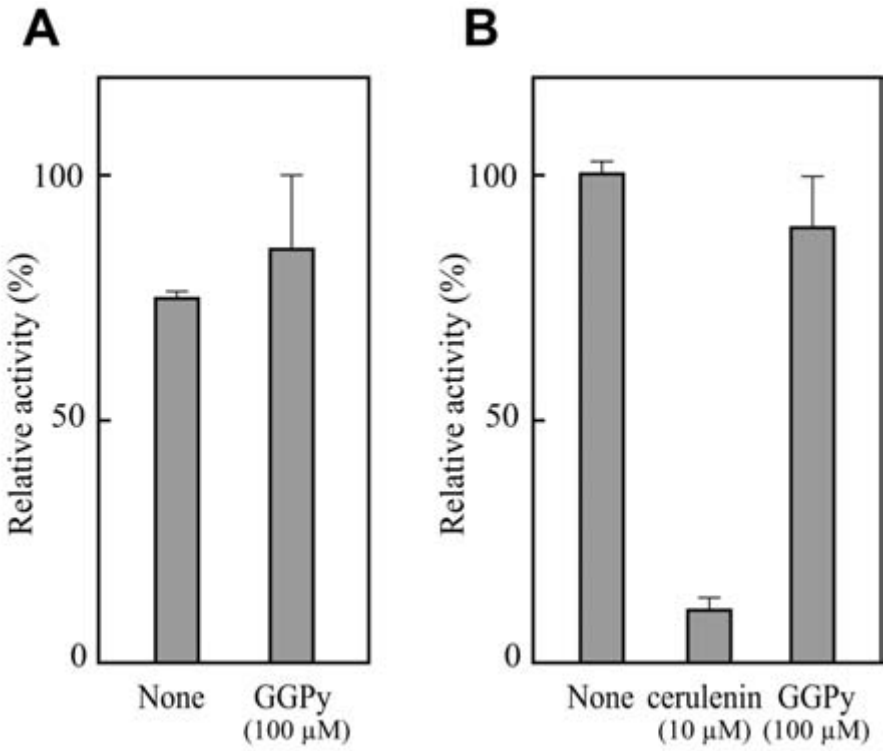


C

Band	Identified Protein		MOUSE score	Function	MOUSE score	Sequence coverage (%)
1	fatty acid synthetase	α-subunit	82	fatty acid synthesis	14/38	12
		β-subunit	71	fatty acid synthesis	13/38	11
2	glyceraldehyde-3-phospate dehydrogenase isozyme 3		98	glycolysis	8/37	52

5

Figure 6: Fig. 5 :



6

Figure 7: Fig. 6 :

Addition	MIC (μM)*			
	Cerulenin		GGPy	
	30°C	37°C	30°C	37°C
None	3.13	3.13	12.5	6.25
Palmitic acid** (Tween 40)	>100	100	100	25

7

Figure 8: Fig. 7 :

[Note:). 13C-NMR (150 MHz, CDCl 3)]

Figure 9:

.1 Acknowledgements

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