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Effect of plagiochin E, an antifungal macrocyclic *bis*(bibenzyl), on cell wall chitin synthesis in *Candida albicans*[1]

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Key words

Abstract

Aim: To investigate the effect of plagiochin E (PLE), an antifungal macrocyclic *bis*(bibenzyl) isolated from liverwort *Marchantia polymorpha* L, on cell wall chitin synthesis in *Candida albicans*. **Methods:** The effect of PLE on chitin synthesis in *Candida albicans* was investigated at the cellular and molecular levels. First, the ultrastructural changes were observed under transmission electron microscopy (TEM). Second, the effects of PLE on chitin synthetase (Chs) activities *in vitro* were assayed using 6-*O*-dansyl-*N*-acetylglucosamine as a fluorescent substrate, and its effect on chitin synthesis *in situ* was assayed by spheroplast regeneration. Finally, real-time RT–PCR was performed to assay its effect on the expression of Chs genes (*CHS*). **Results:** Observation under TEM showed that the structure of the cell wall in *Candida albicans* was seriously damaged, which suggested that the antifungal activity of PLE was associated with its effect on the cell wall. Enzymatic assays and spheroplast regeneration showed that PLE inhibited chitin synthesis *in vitro* and *in situ*. The results of the PCR showed that PLE significantly downregulated the expression of *CHS1*, and upregulated the expression of *CHS2* and *CHS3*. Because different Chs is regulated at different stages of transcription and post-translation, the downregulation of *CHS1* would decrease the level of Chs1 and inhibit its activity, and the inhibitory effects of PLE on Chs2 and Chs3 would be at the post-translational level or by the inhibition on the enzyme-active center. **Conclusion:** These results indicate that the antifungal activity of PLE would be attributed to its inhibitory effect on cell wall chitin synthesis in *Candida albicans*.

Introduction

The incidence of fungal infections and associated mortality rates has dramatically increased during the last few decades[1–3]. Systemic mycoses and some forms of dermatomycosis are the cause of mortality in many patients who underwent anticancer chemotherapy, radiation therapy, parenteral nutrition, or organ transplants[4]. This has resulted in the increased use of broad-spectrum antifungal agents and the initiation of protocols for antifungal prophylaxis in patients at risk. The long-term use of antifungal agents, such as fluconazole, has led to drug resistance in some cases, possibly due to alteration of the drug target, changes at the cellular level of the demethylase enzyme, or changes in drug permeability[5–7]. Antifungal drug resistance has become an important problem in a variety of infectious diseases today. For instance, 33% of late-stage AIDS patients had drug-resistant strains of *Candida albicans* in their oral cavities[8]. Antifungal drug resistance has exacerbated the need for the next generation of new safe antifungal agents.

The macrocyclic *bis*(bibenzyls) isolated from liverworts[9–11] has demonstrated a wide range of biological activities, including antifungal, antibacterial, cytotoxic (antitumoral and antileukemia), and 5-1ipoxygenase- and calmodulin-inhibitory activities[12–14]. In our ongoing program for the discovery of new antifungal ingredients from liverworts, a macrocyclic *bis*(bibenzyl), plagiochin
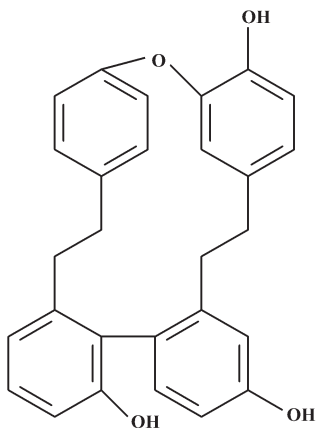
Figure 1. Chemical structure of PLE.

E (PLE; Figure 1), was isolated from *Marchantia polymorpha* L (Marchantiaceae) by bioassay-guided separation and was found to have antifungal activity against *Candida albican*s[15]. PLE had identical antifungal activity against both fluconazole-susceptible and fluconazole-resistant isolates of *Candida albicans* assayed by broth microdilution[16], which suggested that the antifungal mechanism of PLE is possibly distinct from that of fluconazole, one of the inhibitors of ergosterol biosynthesis[17,18].

As we know, the fungal cell wall plays an important role in the growth and viability of fungi; the 3 major structural elements, glucan, chitin, and mannan[19,20], are generally considered therapeutic targets[21,22]. Chitin, a long linear homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc), is synthesized by the incorporation of GlcNAc units from the precursor uridine 5'-diphospho-*N*-acetyl-glucosamine (UDP-GlcNAc) in a reaction catalyzed by chitin synthetase (Chs)[23,24]. Three different Chs (Chs1, Chs2, and Chs3) and their associated genes (*CHS1, CHS2,* and *CHS3*) have been identified in *Candida albicans*[25]. Chitin is indispensable for the construction of the cell wall, and therefore, for fungal survival. The inhibition of chitin polymerization may affect cell wall maturation, septum formation, and bud ring formation, damaging cell division and cell growth in the process. Several compounds that affect chitin synthesis have been identified, such as nikkomycin Z and polyoxin D, which are well-known competitive Chs inhibitors, presumably due to structural similarity with the Chs substrate[26–28].

To understand the biochemical basis of the antifungal activity of PLE, the changes of ultrastructure in *Candida albicans* was observed under transmission electron microscopy (TEM) after incubation with PLE for 24 h. The results showed that the cell wall of *Candida albicans* was seriously damaged and cell division was inhibited, which suggested that the antifungal effect of PLE was associated with its effect on the fungal cell wall. Therefore, a further study on the effect of PLE on chitin synthesis in *Candida albicans* was carried out at the cellular and molecular levels for identifying a promising cellular target of PLE.

Materials and methods

Chemicals PLE was isolated from liverwort *Marchantia polymorpha* L (Marchantiaceae) in our laboratory, and its structure had been identified by nuclear magnetic resonance (NMR) and mass spectrometry (MS). A stock solution of PLE at a concentration of 20 480 mg/L was prepared in DMSO and frozen at –20 °C. In each assay, DMSO comprised <1% of the total test volume. *N*-acetyl glucosamine, dansyl chloride, osmium tetroxide, EPON-812, ethidium bromide, bovine serum albumin, lyticase, and chitinase were purchased from Sigma (St Louis, MO, USA). Trypsin, soybean trypsin inhibitor, and MOPS (morpholinepropanesulfonic acid) were purchased from Amresco (Solon, OH, USA). Tryptone, yeast extract, glucose, and agar were purchased from Oxo (London, UK). RPMI-1640 and agarose were purchased from Gibco (Grand Island, NY, USA). The advantage RT-for-PCR kit and SYBR green real-time PCR master mix were purchased from TOYOBO (Osaka, Japan).

Microorganism and media The isolate of *Candida albicans* [CA2, minimal inhibitory concentration (MIC)=16 mg/L] used in this study was kindly donated by Shandong Provincial Qianfoshan Hospital (Ji-nan, China), and stored at –80 °C in medium containing 20% glycerol. Microbiological media were either YPD medium (2% tryptone, 1% yeast extract, and 2% glucose) or YPD solid medium with 2% agar or RPMI-1640 buffered with 0.165 mol/L MOPS to pH 7.0. Prior to testing, the isolate was passaged on YPD solid medium to ensure purity and viability.

Effect of PLE on ultrastructure of *Candida albicans* TEM was performed to observe the effect of PLE on the cell ultrastructure of *Candida albicans*. The cells were collected after being treated with 16 mg/L PLE at 35 ºC for 24 h, washed twice with phosphate-buffered solution (PBS), and centrifuged for 5 min at 500×*g*. The pellet was fixed in 2.5% glutaraldehyde at 4 ºC for 24 h and then placed in 1% osmium tetroxide in 0.1 mol/L sodium cacodylate (pH 7.4) for 1 h. The cells were desiccated in graded series of acetone and embedded with EPON-812. Ultra-thin sections were prepared and observed

under TEM (JEM-200EX; JEOL, Tokyo, Japan) with 5000 magnification after double staining with uranium and plumbum. At the same time, the untreated cells were used as the control.

Effect of PLE on Chs activity *in vitro* The substrate 6-*O*-dansyl-*N*-acetylglucosamine (DNAG) was synthesized in 3 steps from *N*-acetyl glucosamine according to a previously-described procedure[29,30].

Chs was extracted according to a previously-described procedure with some modifications[31]. Logarithmic phase cells grown in 100 mL YPD medium were collected, washed twice with sterile water, and homogenized. Broken material was collected and cell debris was removed by centrifugation at 5000×*g* for 5 min at 4 ºC. The supernatant was collected and stored at –80 ºC. The protein concentration was measured by the Lowry's method, using bovine serum albumin as the standard.

Chs activity assay was performed, as previously described, with slight modifications[32]. For Chs1 activity, the standard assay mixture containing 4.3 mmol/L magnesium sulfate, 32 mmol/L Tris-HCl at pH 6.5, 5 µL trypsin (2 g/L), 1.1 mmol/L DNAG, and 17 µL membrane suspension in a total volume of 46 µL was used. For Chs2, the standard assay mixture containing 3.2 mmol/L cobaltous nitrate and 32 mmol/L Tris-HCl (pH 8.0) was used. For Chs3, it containing 3.2 mmol/L cobaltous nitrate, 10 mmol/L nickelous nitrate, and 32 mmol/L Tris-HCl (pH 8.0), but did not need trypsin activation. Mixtures were incubated for 15 min at 30 ºC. Proteolysis was stopped by adding 5 µL soybean trypsin inhibitor (3 g/L) on ice. Different concentrations of PLE were added. Each reaction was incubated for 2 h at 30 ºC and stopped by the addition of 1 mL of 10% trichloroacetic acid. The pellet was then treated for 10 min with hot 5% KOH. The incorporated fluorescence was determined by diluting the hot alkali-insoluble material in water and measuring the light emitted at 501 nm with an excitation wavelength of 321 nm in a fluorospectrophotometer. All reactions were carried out in triplicate.

Effect of PLE on chitin synthesis *in situ* Fluorescent microscopy was used to assay the effect of PLE on chitin biosynthesis of *Candida albicans in situ*[29,30]. Logarithmic phase cells grown in 100 mL YPD medium were collected, washed once with 50 mmol/L Tris-HCl (pH 7.4), and resuspended in 20 mL of 1 mol/L sorbitol, 100 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, and 15 mmol/L β-mercaptoethanol at 35 ºC for 30 min. In total, 50 000 U lyticase and 5000 U chitinase were added and the suspension was incubated at 35 ºC for 1 h. The spheroplasts were collected by centrifugation for 5 min at 200×*g* and resuspended in regeneration medium RPMI-1640 supplemented with 1 mol/L sorbitol and 1 g/L DNAG to $1×10^6$ to $5×10^6$ CFU/mL. PLE was added from stock solution to a final concentration of 16 mg/L. After incubation at 35 ºC for 24 h, the cells were collected by centrifugation for 5 min at 200×*g*, washed twice, resuspended in PBS buffer, then observed with an Olympus fluorescent microscope (Olympus 1×81; Olympus, Tokyo, Japan) equipped with a 60× oil immersion lens.

Effect of PLE on *CHS* expression Real-time RT-PCR was performed to assay the effect of PLE on *CHS* expression. RNA was isolated by the hot phenol method. *Candida albicans* was initially grown for 24 h at 35 °C in liquid YPD medium. The cultures were then diluted to $1×10^5$ CFU/mL in 40 mL fresh YPD medium and allowed to grow for an additional 8 h at 35 °C. The cells were collected by centrifugation at 1500×*g* for 3 min, washed once with distilled water, and then centrifuged at 5000×*g* for 10 s at 4 °C. The pellet was resuspended in 400 µL *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) buffer and 400 µL acid phenol was then added. The pellet was vortexed for 10 s, incubated for 30–60 min at 65 °C, and then incubated for 5 min on ice. After centrifugation at 5000×*g* for 5 min at 4 °C, the upper aqueous phase was transferred to a new microcentrifuge tube. Then 400 µL of chloroform was added. The mixture was vortexed for 10 s, centrifuged at 5000×*g* for 5 min at 4 °C, and the upper aqueous phase was transferred to a new microcentrifuge tube. Isopropanol was added to precipitate RNA. Samples were incubated at 4 °C for at least 2 h, and centrifuged at 2000×*g* for 8 min. The pellet was washed with 75% ethanol and resuspended in DEPC-treated water in order to protect the RNA from degradation by RNases. RNA was quantitated by diluting 1 µL into 100 µL with water, and the A_{260} and A_{280} were determined. Single-stranded cDNA was synthesized with oligo(dT) primers from 5 µg total RNA using the Advantage RT-for-PCR kit accordingly to the manufacturer's instruction. The accumulation of PCR products was measured in real-time PCR by using the SYBR green real-time PCR master mix. The sequences of the primers are listed in Table 1. The reaction was performed in a MiniOpticon real-time PCR system (Bio-Rad, Hercules, CA, USA). The basic protocol for real-time PCR was an initial incubation at 50 °C for 2 min, 95 °C for 10 min, followed by 44 cycles of 95 °C for 15 s, and 60 °C for 1 min. After that, a melting curve was constructed for verification of the specificity of PCR products by increasing the temperature from 60 to 94 °C in

Table 1. Sequences and melting temperatures (Tm) of primers used in the real-time RT-PCR.

Primer	Sequence	TM (ºC)
CHS1–5	5'-TCGATGAAGATTACCTTGAGA-3'	54.11
CHS1–3	5'-GCATAATCATCACCATCAAGA-3'	54.11
CHS2–5	5'-GTATGGACAAGTTCTCATTCA-3'	54.11
CHS2–3	5'-GAAGTTGTCAATATCCTAAACA-3'	52.62
CHS3–5	5'-AACTGTTTGTTCGAGTCAAGT-3'	54.11
CHS3–3	5'-GCTTTAGGATGTTGTGACGA-3'	55.75
18SrRNA-5	5'-GCCAGCGAGTATAAGCCTTG-3'	59.85
18SrRNA-3	5'-AGGCCTCACTAAGCCATTCA-3'	57.80

sequential steps of 0.5 °C for 10 s. All samples were run in triplicate, and 18S rRNA was used as the housekeeping gene for arbitrary unit calculation for every tested gene.

Statistical analysis Values are presented as mean±SD. Statistical significance was determined by Student's *t*-test, and *P*<0.05 was considered statistically significant.

Results

Effect of PLE on cell ultrastructure of *Candida albicans* analyzed by TEM The *Candida albicans* images under TEM after incubation with PLE for 24 h are shown in Figure 2. In the cells treated with PLE, the most obvious morphological change appeared in the cell wall, which was much thicker and had a significantly lower electron density than that of the untreated cells whose cell wall was intact. Many cells were also aggregated after treatment with PLE, in which the mother and daughter cells were connected by immature septa.

Effect of PLE on Chs activity *in vitro* Chs activity is often assayed by radioactivity using UDP-[14C] GlcNAc. In this study, a fluorescent method was selected for assessing Chs activity using DNAG as the substrate. Three different Chs can be catalyzed by different divalent metal ions at different pH optimums as described by Chio *et al* who have formulated conditions for the specific determination of each synthetase in the presence of the others[32].

In this assay Chs activity was measured in the *Candida albicans* at the logarithmic growth phase. The results revealed that residual activity of the 3 enzymes (percentage of incorporation compared to the incorporation in the absence of PLE) decreased obviously in relation to the doses of PLE, as shown in Figure 3. When the concentration of PLE was 16 mg/L, the residual activities of Chs1, Chs2, and Chs3 were 77.41%±2.39%, 68.35%±1.96%, and 50.71%±1.51%, respectively.

Effect of PLE on chitin synthesis *in situ* The fungal
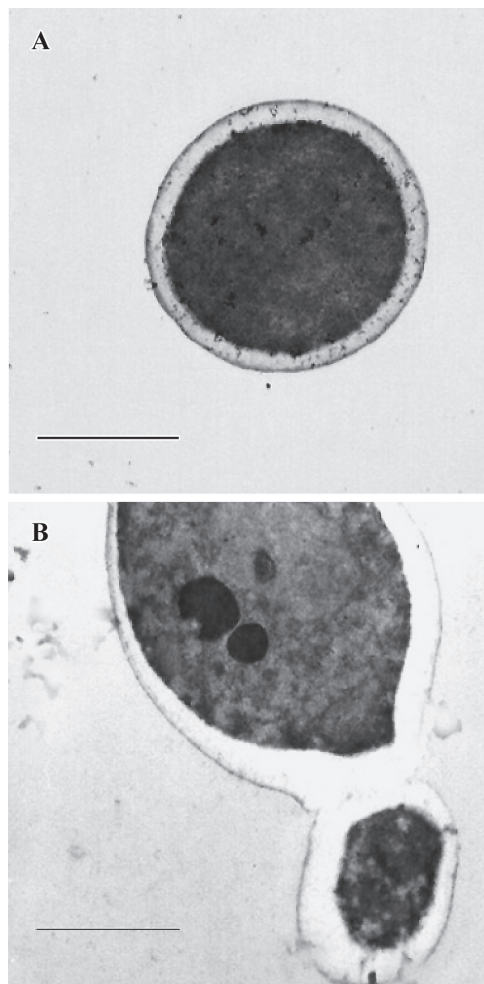
Figure 2. Effects of PLE on the ultrastructure of *Candida albicans* demonstrated by TEM of ultra-thin sections. Scale bars: 1 μm. ×5000. (A) cells from control sample with intact cell wall; (B) cells from samples treated with 16 mg/L PLE at 35 °C for 24 h, demonstrating disorganization of the cell wall and immature septum.

cell wall is a complex matrix of polysaccharides (glucans, mannans, and chitins), proteins, and minor amounts of lipids. The removal of this essential structure for cell integrity and viability by controlled enzymatic digestion in an isoosmotic medium brings about spheroplast formation. When spheroplasts are incubated in an osmotically-stabilized liquid nutrient medium, cell wall precursors are secreted into the culture medium to *de novo* synthesize the cell wall[33].

To assay the effect of PLE on the chitin synthesis of *Candida albicans in situ*, spheroplast regeneration in the presence of DNAG was followed. First, the cells were converted to spheroplasts by enzymatic digestion of chitinase and lyticase, as described earlier, and then
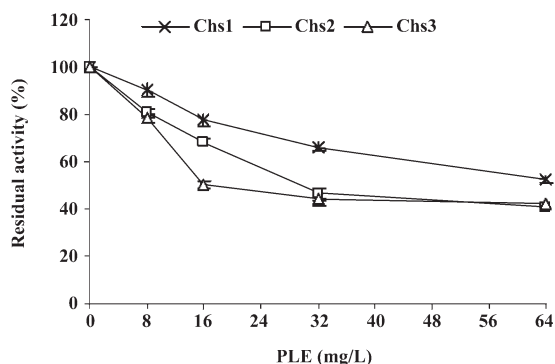
Figure 3. Effects of PLE on the 3 different Chs activities *in vitro* using DNAG as a fluorescent substrate. Activities were expressed as residual activity of the enzyme (percentage of incorporation compared to the incorporation in the absence of PLE). Results are presented as mean±SEM for triplicate experiments.

suspended in the regeneration medium supplemented with 1 mol/L sorbitol as the osmotic stabilizer in the presence of DNAG. The cells were regenerated for 24 h. Spheroplasts regenerated in the presence of DNAG were fluorescent, but samples treated with PLE at concentration of 16 mg/L were less fluorescent and aggregated, as shown in Figure 4. The results indicated that the incorporation of DNAG into chitin in cells was moderately inhibited by PLE. Because it is a selective incorporation of DNAG into the chitin biopolymer rather than a non-specific absorption in the cell wall matrix[30], the results suggested that PLE could moderately inhibit the synthesis of chitin *in situ*.

Effect of PLE on the *CHS* expression Real-time RT-PCR was performed to determine the effect of PLE on the relative expression levels of 3 different *CHS* genes, which were shown as ratios to the mean levels of the control. The results showed that the relative expression level of *CHS1* decreased significantly to 0.2137±0.0207 after treatment with 16 mg/L PLE at 35 °C for 8 h, while the relative expressions of *CHS2* and *CHS3* increased slightly to 1.0325±0.2708 and 1.0257±0.06570, respectively.

Discussion

Fungal cells are coated with a carbohydrate-containing wall, which is necessary for cell integrity[34,35]. As a consequence, the division of fungal cells is different from that of mammalian cells, whose cytokinesis is affected by the contraction of an actomyosin ring that invaginates the plasma membrane until it pinches off, splitting the dividing cell into 2 daughter cells[36]. This implies the formation of the septum between the mother and daughter cells to
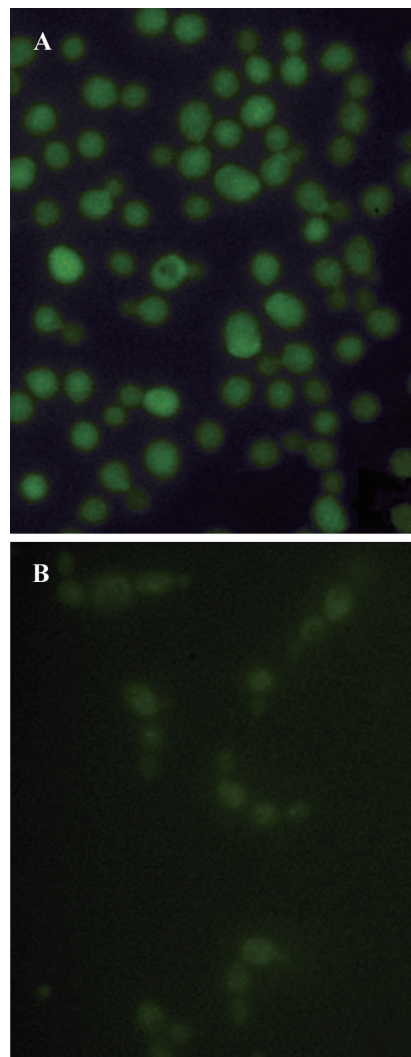
Figure 4. Effects of PLE on chitin synthesis in *Candida albicans in situ* observed under an Olympus fluorescence microscope. ×600. (A) untreated cells; (B) cells treated with 16 mg/L PLE for 24 h.

permit their separation without lysis. The yeast septum is made in 3 different stages, and Chs plays a central role in this process. First, a chitin ring is formed at the basis of an emerging bud. Second, the membrane invaginates, and chitin is secreted from it to form the primary septum. Next, secondary septa are built up from both the mother and daughter cell sides. Finally, the 2 cells are separated by the action of a chitinase that partially hydrolyzes the primary septum[37–39].

Three Chs isoforms have been identified in *Saccharomyces cerevisiae* and their functions are known[40,41]: ScChs1 repairs damaged chitins during cell separation, ScChs2 is involved in the formation of the primary septum disk, and ScChs3 is responsible for the synthesis of the
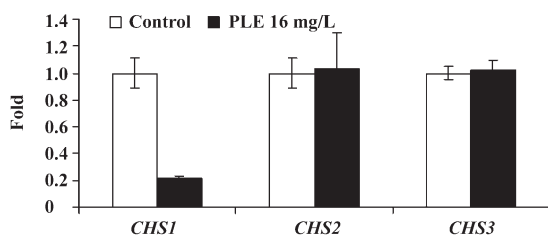
Figure 5. Effects of PLE on the expression of *CHS* genes by real-time RT-PCR. Real-time RT-PCR of *Candida albicans* 18S rRNA was performed as a control.

chitin ring at the base of an emerging bud and cell wall chitin synthesis. *ScCHS2* mutants are unable to construct a primary septum. Although they usually succeed in completing cell division, their septa are thick, amorphous, and often show lacunae. Chitinase cannot act on these septa, and as a result, cell separation is defective and the cells form large clumps[42]. *ScCHS3* mutants do not lay down the chitin ring at the base of emerging buds, but they have a tri-layered septum. They also lack the chitin dispersed in the cell wall and involved in cross-links[43]. Three isoforms of Chs in *Candida albicans* have also been identified. Sequence homology in amino acids matched Chs1 with ScChs2, Chs2 with ScChs1, and Chs3 with ScChs3, and their functions[44–46]. Polyoxins are a competitive inhibitor of Chs of *Candida albicans*. It has been reported that after treatment with polyoxin D, some pairs of abnormal cells consisted of 2 lysed cells from which the cytoplasm had been extruded at the cell junction or 2 highly-refractile cells were joined by a thin bridge[47].

In our experiments, we observed the ultrastructural changes of *Candida albicans* after treatment with PLE under TEM. The first change was that the cell wall became thick and had a low electron density, which suggested that the PLE changed the structure of the cell wall in *Candida albicans*. The second change was that many cells aggregated with immature septa, which suggested the septum formation and cell division were inhibited by PLE. As we know, Chs plays a central role in cell division of budding yeast, so the effect of PLE on chitin synthesis was assayed. The results of the enzymatic activities showed that PLE had a moderate inhibitory effect against all 3 different Chs *in vitro*, which is in accordance with the predicted results. The effect of PLE on chitin synthesis *in situ* was also determined by assaying the incorporation of DNAG into chitin during the spheroplast regeneration of *Candida albicans* in presence of PLE, and the results showed that PLE inhibited chitin synthesis *in situ*, which

verified the above results. Chitin is a major structural element of the fungal cell wall, thus, the inhibition of chitin synthesis by PLE would decrease the proportion of chitins in the cell wall, which leads to a damaged cell wall with lower electron density, as observed under TEM. The partial inhibition of Chs by PLE would affect the formation of primary septa between mother and daughter cells and inhibit cell division, which induces many aggregated cells observed under TEM and fluorescent microscopy.

The different roles of Chs require that each be regulated independently, so that it will execute its function at the required time and location in the cell. The present work indicated that Chs were regulated at both the transcriptional and post-translational stages. ScChs2 is largely regulated by a process of synthesis and degradation. The shut-off transcription of *ScCHS2* resulted in the rapid disappearance of ScChs2. However, the regulation of ScChs1 and ScChs3 occurs at the post-translational level, which appears to be quite stable in the absence of transcription[48,49]. The effect of PLE on chitin synthesis *in situ* would relate to the decreased expression of *CHS*. The results of the real-time RT-PCR in our experiment showed that compared with the control, the expression of *CHS1* in *Candida albicans* was significantly downregulated after treatment with PLE, and the expressions of *CHS2* and *CHS3* were slightly upregulated. Because Chs1, whose sequence homology in amino acids matched that of ScChs2, is regulated at the transcriptional and post-translational stages, the downregulation of the expression of *CHS1* by PLE would decrease the level of Chs1 and inhibit its activity. The regulation of Chs2 and Chs3 occurs at the post-translational level, and their activities have little correlation with the levels of transcription. Thus, the upregulation of the expressions of *CHS2* and *CHS3* by PLE would not affect the activities of Chs2 and Chs3. The inhibitory effects of PLE on Chs2 and Chs3 would be at the post-translational level or by the inhibition on the enzyme-active center of PLE. Overall, the effect of PLE on chitin synthesis would be caused by the decreased expression of *CHS* or the inhibition on the enzyme-active center.

The fungal cell wall plays a very important role in maintaining the structural integrity of fungal cells. Once the continuity of the cell wall is damaged, cellular morphology can not be maintained any longer, and the cells are rendered osmotically fragile[50]. A very small lesion in the cell wall can cause swelling in the area of the lesion, rupture of the cell membrane, and growth inhibition. The inhibition of chitin biosynthesis by PLE would affect the continuity of the fungal cell wall and damage cell growth.

The results reported in the present study allow us to infer that PLE acts by inhibiting the biosynthesis of chitin as one of its possible modes of action. Because mammalian cells do not have a cell wall, these kinds of antifungal agents should be safe for humans[51]. Thus, PLE would be a potential candidate for the development of safer antifungal drugs.

Acknowledgements

Author contribution

Hong-xiang LOU designed research; Xiu-zhen WU performed research; Ling-mei SUN contributed new analytical tools and reagents; Ai-xia CHENG analyzed data; Xiu-zhen WU wrote the paper.

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