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Phellinsin A, a Novel Chitin Synthases Inhibitor Produced by *Phellinus* sp. PL3

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Phellinsin A, a novel chitin synthases inhibitor was isolated from the cultured broth of fungus PL3, which was identified as *Phellinus* sp. PL3. Phellinsin A was purified by solvent partition, silica gel, ODS column chromatographies, and preparative HPLC, consecutively. The structure of phellinsin A was assigned as a phenolic compound on the basis of various spectroscopic analyses including UV, IR, Mass, and NMR. Its molecular weight and formula were found to be 358 and $C_{18}H_{14}O_8$, respectively. Phellinsin A selectively inhibited chitin synthase I and II of *Saccharomyces cerevisiae* with an IC_{50} value of 76 and 28 μg/ml, respectively, in our cell free assay system. This compound showed antifungal activity against *Colletotrichum lagenarium*, *Pyricularia oryzae*, *Rhizoctonia solani*, *Aspergillus fumigatus*, and *Trichophyton mentagrophytes*.

Chitin is a structural polymer present in most fungal cell walls. Although its amount varies from 1~30% of the cell wall, it have been shown to be indispensable for the maintenance of the fungal cell[1]. Mutations that affect chitin synthesis cause osmotic sensitivity[2,3], abnormal morphology, aggregation, and growth arrest with elongated buds[4,5].

There are three different chitin synthases in *Saccharomyces cerevisiae*: chitin synthase I is nonessential repair enzyme of damaged chitin[6,7]; chitin synthase II is an essential enzyme for primary septum formation between mother and daughter cells[5,8~10]; chitin synthase III makes 90~95% of the cellular chitin, including the chitin synthesized during mating and spoluration[5]. Therefore, specific inhibitors of chitin synthase II and III might be interesting lead compounds to develop effective antifungal agents.

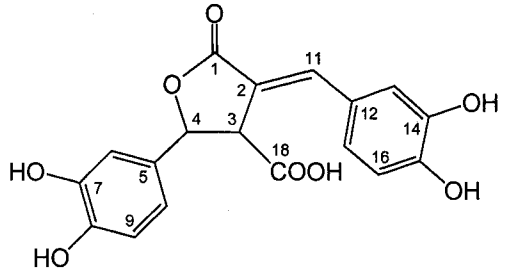
As a part of our continuing efforts to discover naturally occurring new antifungal agents, we have screened for chitin synthase II inhibitors of microbial origin. We report herein on the taxonomy and fermentation of the phellinsin A-producing strain and structural elucidation, physicochemical properties and biological activity of a novel antifungal metabolite, phellinsin A (Fig. 1).

Materials and Methods

Phellinsin A-producing Strain

The phellinsin A-producing mushroom, strain PL3, was collected from the trunk of *Morus alba* at Mountain

Fig. 1. Structure of phellinsin A.



Kyeryong, Chungcheongnam province, Korea. Chemical reaction of basidiospores was tested according to GILBERTSON and RYVARDEN[11]. Microscopical structures of hypae and basidiospores were observed using 4% KOH solution, Cotton-blue solution or Melzer's reagent as mouting solutions. For the evaluation of cultural characteristics, the strain was grown on CYA (yeast extract 0.5%, $NaNO_3$ 0.3%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $ZnCl_2 \cdot 7H_2O$ 0.001%, sucrose 3.0%, agar 1.5%), CYA20S (CYA medium with sucrose increased to 20%), Czapek dox agar ($NaNO_3$ 0.3%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, sucrose 3.0%, agar 1.5%), MEA (malt extract 2.5%, agar 1.5%), PDA (Difco Co.), YMA (yeast extract 0.3%, malt extract 0.3%, tryptone 0.5%, agar 1.5%) media, for 14 days at 25°C to 42°C. Morphological observation was carried out using a light microscope (Nikon, EFD3). The color names used were based on the ISCC-NBC Color-Name Chart Illustrated with Centroid Colors.

Fermentation

One frozen stock vial (1 ml of spore suspension in 10% glycerol, −80°C) of strain PL3 was inoculated into a 500 ml baffled-flask containing seed medium(glucose 0.5%, soluble starch 1.5%, yeast extract 0.2%, polypeptone 0.5%, KH_2PO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 5.8 prior to autoclaving). Distilled water was used in preparation of seed and production media. The seed culture was incubated for 4 days at 26°C on a rotary shaker at 150 rpm (radius 7 cm). Twenty ml of the seed culture was transferred into a 5-liter baffled-flask containing 1 liter of production medium (Potato dextrose broth; Difco Co.). The fermentation was carried out for 5 days at 26°C on a rotary shaker at 150 rpm. To determine the cell mass, mycelia were collected by filtration using Whatman No. 2 filter paper, then washed twice with distilled water and oven dried at 105°C to a constant weight.

Spectral Analysis

The UV spectrum was recorded on a Shimadzu UV265 UV-Visible spectrophotometer. The pH was measured with an Orion research digital pH/millivolt meter 611 and Ross electrode. ESI mass spectrum was recorded on a Hewlett Packard 5989A. ^1H- and ^{13}C-NMR spectra were obtained on a Varian UNITY 500 spectrometer using CD_3OD. Chemical shifts are given in ppm using TMS as internal standard.

Chitin Synthases Assay

Saccharomyces cerevisiae YPH499 (*ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*), *S. cerevisiae* ECY38-38A (*MAT*a *chs1-23 chs2::LEU2 cal1/csd2 ura3-52 trp1-1 leu2-2* pAS6), and *S. cerevisiae* ECY38-38A (*MAT*a *chs1-23 chs2::LEU2 cal1/csd2 ura3-52 trp1-1 leu2-2* pWJC6) were used as sources of chitin synthase I, II, and III activity, respectively. *S. cerevisiae* YPH499 is wild type for all three synthases and grown in YEPD (yeast extract 1%, peptone 2%, glucose 2%). *S. cerevisiae* ECY38-38A (pAS6) and *S. cerevisiae* ECY38-38A (pWJC6), which can only overexpress the chitin synthase II and III, respectively, when grown in YPG (yeast extract 1%, peptone 2%, galactose 2%).

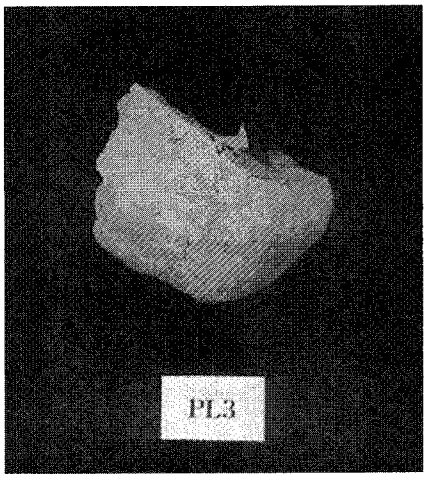
Cells suspended in 50 mM Tris-HCl (pH 7.5) containing 5 mM magnesium acetate were broken by vortex mixing with glass beads[12]. Cell walls were sedimented at 4,000×*g* for 5 minutes and the supernatant fluid was centrifuged at 130,000×*g* for 45 minutes. The membrane pellet was suspended in the 50 mM Tris-HCl (pH 7.5) containing 33% glycerol used in the breakage, to a final volume of 1.6 ml/g (wet weight) of cells. Chitin synthase II activity was measured by the described procedure[12]. For the proteolytic activation step, reaction mixtures contained 32 mM Tris-HCl (pH 7.5), 1.6 mM cobalt acetate, 1.0 mM UDP-[^{14}C]-*N*-acetylglucosamine (400,000 cpm/μmol, NEN), 2 μl of trypsin at the optimal concentration for activation (2.0 mg/ml), 20 μl of membranes, and 14 μl of the test sample in a total volume of 46 μl. The mixtures were preincubated for 15 minutes at 30°C. Proteolysis was stopped by adding 2 μl of a soybean trypsin inhibitor solution (4.0 mg/ml) at a concentration 2 times that of trypsin solution used, and tubes were placed on ice for 10 minutes. GlcNAc was added to a final concentration of 32 mM and incubation at 30°C was carried out for 90 minutes. For chitin synthase III activity[12], the assay was performed the same as that for chitin synthase II except that 32 mM Tris-HCl (pH 7.5) and 4.3 mM magnesium acetate were used. For chitin synthase I activity[12], reaction mixtures contained 37 mM Tris-HCl (pH 7.5), 0.12% digitonin, 4.8 mM magnesium acetate, 2 μl of trypsin (1.0 mg/ml), 6 μl of membrane suspension, and 14 μl of the test sample in a total volume of 41 μl. After 15 minutes of incubation at 30°C, 2 μl of trypsin inhibitor (2.0 mg/ml) was added, and the tubes were placed on ice. 32 mM GlcNAc and 1.0 mM UDP-[^{14}C]-GlcNAc were added as for the chitin synthase II and III assay, and the mixtures were incubated for 30 minutes at 30°C. In all cases, the reaction was stopped by the addition of 10% trichloroacetic acid and the radioactivity of the insoluble chitin formed was counted

after filtration through glass fiber filters (GF/C, Whatman). The concentration of protein was measured by the method of LOWRY[13]. Blank values were measured with addition of 25% aqueous MeOH instead of both enzyme and test sample. Percent inhibition of chitin synthase activity was calculated by subtracting the blank values from both control and test sample values.

$$\% \text{ Inhibition} = \left[1 - \frac{\text{Sample (cpm)} - \text{Blank (cpm)}}{\text{Control (cpm)} - \text{Blank (cpm)}}\right] \times 100$$

The chitin synthase II and III activities of the enzyme were confirmed using positive controls polyoxin D and nikkomycin Z (Calbiochem. Co.), respectively.

Fig. 2. The fruiting body of *Phellinus* sp. PL3.



Antifungal Activity

Minimum inhibitory concentrations (MIC) were determined by the two-fold serial agar dilution method. Human pathogenic fungi were grown on Sabouraud's agar medium, and plant pathogenic fungi were grown on potato dextrose agar medium. Antifungal activity was observed after 24-hour incubation at 30°C for yeasts and 48-hour incubation for fungi at 25°C.

Results

Taxonomic Studies of the Producing Organism

The fruiting body of strain PL3 was corky, yellowish brown, and sessile (Fig. 2). Basidiospores were subglobose to ellipsoidal in shape. They were hyaline in young ones, became yellowish-brown with age, and 4.0~5.7×3.0~4.7 μm in size. They showed weakly cyanophilic and non-dextrinoid reaction in Cotton-blue solution and Melzer's reagent, respectively. Hyphal structures of trama were dimitic with generative and skeletal hyphae. Hymenial setae were abundantly found and 27~57×5.7~9.3 μm in size. Pores were formed in 7~9 per mm on hymenial layer. Setal hyphae were not found.

The cultural characteristics of strain PL3 after incubation on six agar media at 25°C to 42°C for 14 days are summarized in Table 1. The optimal temperature for growth of the fungus PL3 was at 30°C with little growth observed at 42°C. The mycelium was pale orange to yellow brown and the reverse side was pale orange yellow to dark brown in color. The simple septa without clamp-connection was also observed in hyphae like other *Phellinus* sp[14].

Table 1. Cultural characteristics of fungus PL3 on several media.

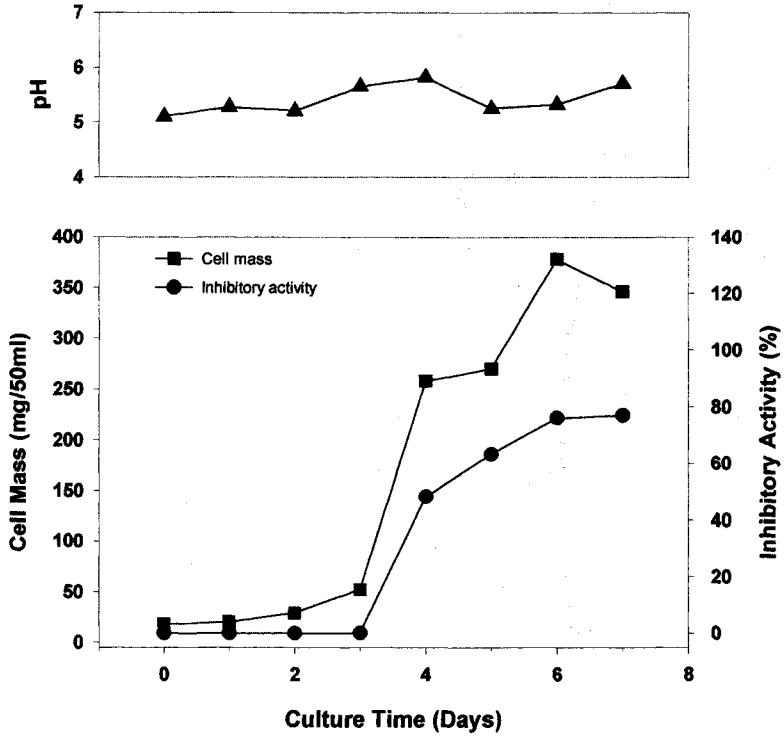
Media	Colony size(mm)[a]/°C				Color of mycelium[b]	Color of reverse side[b]
	/25	/30	/37	/42		
CYA	45	52	33	7	Pale orange yellow	Pale orange yellow
CYA20S	47	53	42	8	Pale yellow	Pale orange yellow
Czapek	48	58	34	7	Pale orange yellow	Pale orange yellow
MEA	85	85	40	7	Vivid yellow	Dark yellow
PDA	85	85	45	7	Vivid yellow	Dark orange yellow
YMA	71	85	37	7	Strong yellowish brown	Dark brown

[a]Colony size measured after 14 days.

[b]ISCC-NBS color-name chart illustrated with centroid colors, U. S. Dept. of Comm. Suppl. to NBS Circular 533, Washington, D. C., 1985

Fig. 3. Time course of phellinsin A production by *Phellinus* sp. PL3.

Phellinus sp. PL3 was cultivated at 26°C in 50 ml potato dextrose broth medium.

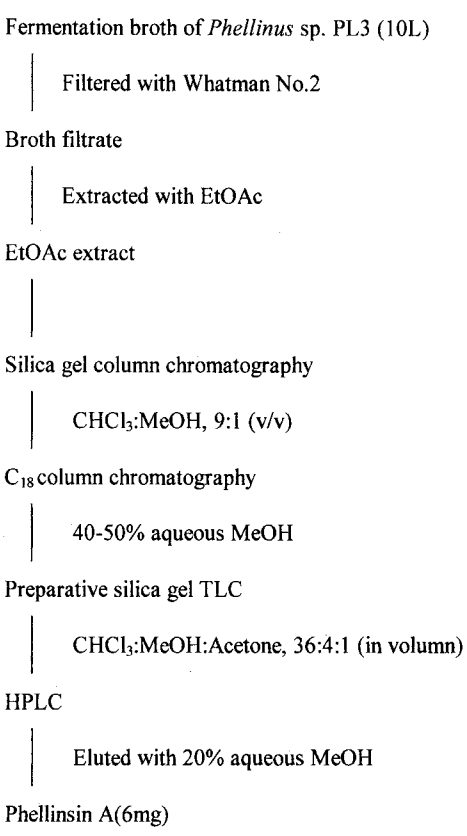


Based on the morphological and biochemical characteristics, strain PL3 was classified into *Phellinus* species owing to abundant hymenial setae, blackening of their basidiocarps to KOH treatment, and dimitic hyphal structures[15]. The combination of morphological and biochemical characteristics of basidiospores, setae, basidiocarps and host specificity led to the conclusion that strain PL3 is closely related to *P. linteus*, *P. johnsonianus* or *P. baumii*, which belong to LARSEN and COBB's Group III[16]. However, strain PL3 is slightly different from the above three species. *P. johnsonianus* has been reported to be resupinate to reflexed in the morphology of basidiocarps, while strain PL3 is distinctly sessile[17]. Strain PL3 was consistent with description on *P. baumii* according to DAI and XU[18], concerning the thickness in KOH solution of tramal skeletal hyphae. Tramal skeletal hyphae were 2.3~4.8 μm thick in strain PL3, 2.5~3.5 μm in *P. baumii* and 3.9~6.0 μm in *P. linteus*. However, this strain had a little larger basidiospores than of *P. baumii* (3.3~4.5× 2.4~3.5 μm) according to DAI and XU descriptions[18]. Size of basidiospores in strain PL3 (4.3~5.5×3.8~4.8 μm) was similar to that of *P. linteus*. In addition, the contextural skeletal hyphae were thinner than those of *P. linteus* and *P. baumii*. The contextural skeletal hyphae in KOH solution were 2.4~4.1 μm thick in strain PL3, while 4.5~6.1 μm and 4.8~7.0 μm in *P. linteus* and *P. baumii*, respectively. In conclusion, strain PL3 seems to be a relative but a distinctly different species of the three *Phellinus* species. Therefore, this fungus was designated as *Phellinus* sp. PL3. It was deposited in the Korean Collection for Type Culture (KCTC) as KCTC 0613BP.

Fermentation

The fermentation was followed by monitoring for chitin synthase II inhibitory activity. A typical time course production of phellinsin A in a 500 ml baffled Erlenmeyer flask fermentation is shown in Fig. 3. The inhibitory activity for chitin synthase II started after 3 days and reached a maximum after 6 days of cultivation. Cell mass also reached a maximum after 6 days of fermentation.

Fig. 4. Purification steps for phellinsin A.



Isolation

The isolation procedure for phellinsin A is shown in Fig 4. A five-day old fermentation broth of *Phellinus* sp. PL3 (10 liters) was filtered with Whatman No. 2 filter paper. The broth was extracted twice with 10 liters of ethyl acetate and the ethyl acetate layers were evaporated *in vacuo*. The residue was applied to a column of silica gel (Merck, Kieselgel 60, 230~400 mesh) and the column was eluted with chloroform - methanol, 9 : 1 (v/v), to give a yellow brown residue, which inhibited chitin synthase II by 60~70% at 280 μg/ml. The residue was purified with an ODS column (Merck, Lichroprep RP-18, 40~63 μm) eluted with a gradient of water - methanol (4 : 1 to 100% methanol, v/v) and then the yellow residue was found in 40~50% aqueous methanol fractions, which inhibited chitin synthase II by 70% at 280 μg/ml. The residue was further purified on preparative silica gel TLC developed with chloroform - methanol - isopropyl alcohol, 36 : 4 : 1 (in volume). The active zone (Rf 0.15) was extracted with methanol and the resulting solution was concentrated *in vacuo* to yield a yellow material. Finally, the material was dissolved in methanol and subjected to preparative HPLC using an ODS column (YMC-Park, C18 SH-343-5, No 204788(W)). The column was eluted with a mixture of methanol - water, 2 : 8 (v/v) at a flow rate of 2 ml/minute. An active substance was eluted as a peak with retention time of 30 minutes and detected by a UV spectrophotometric detecter (213 nm). The substance was collected and concentrated *in vacuo* to give a yellow powder of pure phellinsin A (6 mg).

Physico-chemical Properties and Structure

The physico-chemical properties of phellinsin A are summarized in Table 2. Phellinsin A was obtained as yellow power and showed a Rf value of 0.5 in MeOH - H_2O, 2 : 8 (v/v), on a Merck RP-18 TLC plate. Phellinsin A was soluble in MeOH, DMSO and distilled water but insoluble in $CHCl_3$ and *n*-hexane. The molecular formula was determined to be $C_{18}H_{14}O_8$ on the basis of the ESI-MS spectrum in combination with ^{1}H- and ^{13}C-NMR spectral data (Table 3). The IR spectrum suggested the presence of hydroxyl groups (3400 cm^{-1}) and carbonyl groups (1718~1737 cm^{-1}).

The structure determination of phellinsin A was carried out by NMR spectroscopic analyses. The ^{1}H-NMR spectrum of phellinsin A showed two *AMX* spin systems due to two 1,3,4-trisubstituted benzenes (6.65, 6.71, 6.74 ppm and 6.80, 7.03, 7.13 ppm) in the aromatic region, and three methine proton peaks at 4.01, 5.59 and 7.51 ppm.

In the ^{13}C-NMR spectrum, all eighteen carbons were observed. The DEPT experiment revealed the presence of nine quaternary and nine methine carbons, and a HMQC experiment established all direct ^{1}H-^{13}C connectivities. The ^{1}H- and ^{13}C-NMR spectral data of phellinsin A are shown in Table 3. The ^{1}H-^{1}H COSY spectrum revealed the three partial structures indicated in Fig. 5 including allylic coupling between H-3 (4.01 ppm) and H-11 (7.51 ppm). These partial structures were further connected with HMBC data, which showed long-range correlations from the methine proton at 7.51 ppm to two aromatic carbons at 118.1 and 125.5 ppm, and from an oxygenated methine proton at 5.59 ppm to two aromatic carbons at 113.4 and 118.3 ppm, assigning the attached positions of two benzene rings. The γ-lactone moiety was unambiguously assigned by the long-range couplings from the methine proton at 5.59 ppm to sp^2 quaternary carbons at 174.8 and 121.6 ppm. In addition, correlations between the proton at 7.51 ppm and carbons at 174.8 and 56.7 ppm completed the structure of phellinsin A as shown in Fig 5. The regio-stereochemistry of olefin carbon (C-11) was determined to be *trans* by the NOE effect observed between the methine

Table 2. Physico-chemical properties of phellinsin A.

Properties	Phellinsin A
Appearance	Yellow powder
ESI-MS (*m/z*)	
positive mode	358.9 $[M+H]^+$
negative mode	356.9 $[M-H]^+$
Molecular formula	$C_{18}H_{14}O_8$
$[\alpha]_D^{25}$	0 (c 0.03 MeOH)
UV λ_{max}^{MeOH} nm(log ε)	336 (3.79), 205 (4.20)
IR νcm^{-1} (KBr, disk)	3400, 2922, 2852, 1737, 1718
Rf value[a]	0.5
Soluble	MeOH, DMSO, H_2O
Insoluble	$CHCl_3$, *n*-hexane, CH_2Cl_2, EtOH, EtOAc, CH_3CN, *i*-PrOH, Acetone

[a]On the TLC plate (Merck, Lichroprep® RP-18, 40-63μm), MeOH:H_2O (2:8, v/v).

Table 3. ^1H- and ^{13}C-NMR spectral data of phellinsin A.

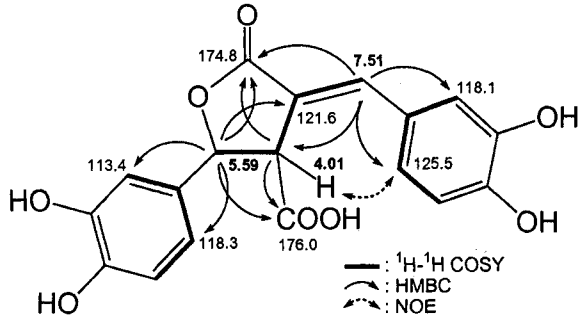
No	^1H-NMR (δ_H, *J*=Hz)	^{13}C-NMR (δ_C, ppm)
1		174.8
2		121.6
3	4.01 (1H, t, 2.2)	56.7
4	5.59 (1H, d, 2.5)	83.7
5		133.3
6	6.74 (1H, d, 8.3)	113.4
7		146.8
8		146.9
9	6.71 (1H, d, 2.0)	116.5
10	6.65 (1H, dd, 8.3, 2.0)	118.3
11	7.51 (1H, d, 2.0)	140.9
12		127.2
13	7.13 (1H, d, 2.2)	118.1
14		146.6
15		149.6
16	6.80 (1H, d, 8.3)	116.6
17	7.03 (1H, dd, 8.3, 2.2)	125.5
18		176.0

• Measured in CD_3OD. s; singlet, d; doublet, t; triplet, dd; doublet of doublets.

proton at 4.01 ppm and two aromatic protons at 7.03 and 7.13 ppm. Stereochemistries of chiral carbons at 83.7 (C-4) and 56.7 ppm (C-3) remain to be established.

Fig. 5. ^1H-^1H COSY, HMBC, and NOE experiments of phellinsin A (CD_3OD).

Arrows are directing H to C.



Biological Activity

Inhibition of Chitin Synthases by Phellinsin A

Phellinsin A inhibited chitin synthase I and II in a dose-dependent manner at concentrations up to 280 μg/ml (Fig. 6). The IC_{50} value of phellinsin A for chitin synthase I and II were 76 and 28 μg/ml, respectively. These results suggest that phellinsin A is 2.5 times stronger inhibitory activity than that of the previously identified chitin synthases inhibitor, polyoxin D (IC_{50}, 70 μg/ml)[19,29], whereas it exhibited very weak inhibitory activity against chitin synthase I as compared with polyoxin D (IC_{50},

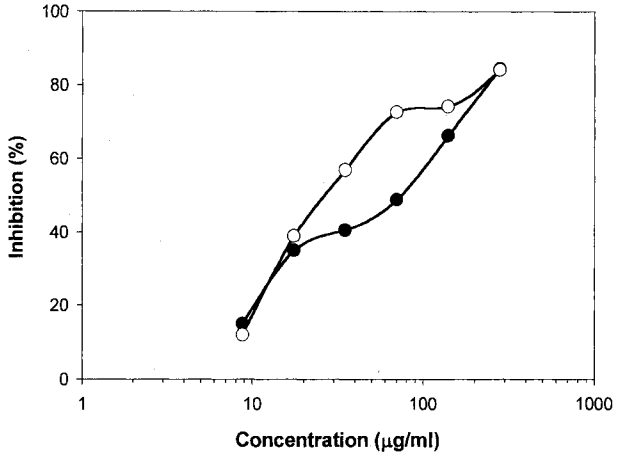
$\leq$5.2 μg/ml)[19]. Phellinsin A did not exhibit inhibitory activity against chitin synthase III from *S. cerevisiae* ECY38-38A (pWJC6) (Data not shown).

Antifungal Activity

Phellinsin A exhibited antifungal activity against human fungal pathogens such as *Trichophyton mentagrophytes* and *Aspergillus fumigatus* with MIC value of 50 μg/ml, but very weak activity against other human fungal pathogens, such as *Cryptococcus neoformans* and *Coccidioides immitis* (MIC, 100 μg/ml) (Table 4). This compound also exhibited strong antifungal activity against the plant pathogens *Colletotrichum lagenarium*, *Pyricularia oryzae*, and *Rhizoctonia solani* with MICs of 12.5 and 50 μg/ml, respectively. It showed no activity against *Candida albicans*, *C. lusitaniae*, *C. krusei*, *C. tropicalis* and *Fusarium oxysporum* at a concentrations up to 100 μg/ml.

Fig. 6. Inhibition of chitin synthase II by phellinsin A.

●, Chitin synthase I; ○, Chitin synthase II.



Discussion

The fungal cell wall has been considered as an ideal target for the development of novel antifungal agents. Several enzymes in the biosynthesis of the cell wall such as chitin and glucan synthases are unique to fungi, and thus serve as suitable targets[20~23]. Various compounds affecting the chitin synthesis have been discovered[19,24,25]. Among them, nikkomycins exhibit synergic effects with azole and glucan synthase inhibitors[25]. In *S. cerevisiae*, chitin synthase I and III are more sensitive to nikkomycin derivatives than is chitin synthase II[19]. Nikkomycin Z is inhibitory specifically for chitin synthase III in *S. cerevisiae*[27]. *C. albicans* and other medically important fungi are resistant to polyoxins because of their poor transport across the cell membrane[28]. Thus, the potency of

Table 4. The antifungal activities of phellinsin A against various human and plant pathogens.

(MIC : μg/ml)

Pathogens		Phellinsin A	Polyoxin D
Human	*Candida albicans* ATCC 10231	>100	>100
	C. lusitaniae ATCC 42720	>100	>100
	C. krusei ATCC 6258	>100	>100
	C. tropicalis ATCC 13803	>100	>100
	Coccidioides immitis ATCC 34020	100	100
	Cryptococcus neoformans ATCC 36556	100	>100
	Aspergillus fumigatus ATCC 16424	50	>100
	Trichophyton mentagrophytes ATCC 9533	50	>100
Plant	*Alternaria kikuchiana*	100	>100
	Botrytis cinerea	100	>100
	Colletotrichum lagenarium	12.5	>100
	Fusarium oxysporum	>100	>100
	Pyricularia oryzae	50	100
	Rhizoctonia solani	50	50

an inhibitor may depend on the cell wall as well as its affinity to a given enzyme.

In the course of our continuous screening to find new antifungal agents from microorganisms, we isolated a novel compound, phellinsin A, possessing a phenol and γ-lactone from *Phellinus* sp. PL3. Phellinsin A was shown to be as an inhibitor of chitin synthase I and II , which is responsible for the synthesis of fungal cell wall. The IC_{50} value of phellinsin A against chitin synthase I and II were 76 and 28 μg/ml, respectively, which is more inhibitory than polyoxin D and chaetoatrosin A (Fig. 6). Phellinsin A did not exhibit inhibitory activity against chitin synthase III at concentrations up to 280 μg/ml. The structure of phellinsin A is different from other chitin synthase II inhibitors previous reported such as polyoxin D from *Streptomyces* sp.[19], chaetoatrosin A from *Chaetomium* sp.[29], catechin from *Taxus cuspidata*[30], and ursolic acid from *Crataegus pinnatifida*[31]. Phellinsin A was active against various pathogenic fungi but it exhibited weak activity against pathogenic yeasts and *Fusarium oxysporum* (MIC, $\geq$100 μg/ml) (Table 4). Overall, the antifungal activity of phellinsin A is more potent than that of polyoxin D. Thus, phellinsin A is unique in that it is a potent chitin synthase I and II inhibitor with antifungal activity *in vitro*. Therefore, we consider that phellinsin A may be a useful lead compound for development of new antifungal agents through the regulation of chitin biosynthesis. Structural modifications of phellinsin A to improve its antifungal activity are now in progress.

Acknowledgments

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