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特集／呼吸器感染症研究における新しい展開	Kakeya H, Izumikawa K, Yamada K, Narita Y, Nishino T, Obata Y, Takazono T, Kurihara S, Kosai K, Morinaga Y, Nakamura S, Imamura Y, Miyazaki T, Tsukamoto M, Yanagihara K, Takenaka M, Tashiro T, Kohno S	東京（Intern Med）	2014年7月	国内
Cryptococcus neoformans感染の各種病態：免疫不全において	Nagayoshi Y, Miyazaki T, Minematsu A, Yamauchi S, Takazono T, Nakamura S, Imamura Y, Izumikawa K, Kakeya H, Yanagihara K, Kohno S	U.S.A.（FEMS Yeast Res）	2014年6月	国外

カルバペネム系薬投与患者における耐性菌選択リスク因子の検討	Mihara T, Kakeya H, Izumikawa K, Obata Y, Nishino T, Takazono T, Kosai K, Morinaga Y, Kurihara S, Nakamura S, Imamura Y, Miyazaki T, Tsukamoto M, Yamamoto Y, Yanagihara K, Tashiro T, Kohno S	東京（J Infect Chemother）	2014年6月	国内
Antimicrobial stewardship teamによる 血液培養陽性患者ラウンドのアウトカム評価	金子幸弘	呼吸器内科	2014年7月	国内
③ 薬学生・薬剤師レジデントのための感染症学・抗菌薬治療テキスト	大野秀明，金子幸弘，田辺公一	感染症内科	2014年6月	国内
	前田真之，大戸祐治，村山純一郎，峯村純子，馬場俊之，吉田仁，金子堯一，茅野博行，小林洋一，石野敬子	昭和学士会雑誌	2014	国内

	前田真之，詫間隆博，吉川雅之，内藤結花，土屋亜由美，大戸祐治，峯村純子，宇賀神和久，小司久志，石野敬子，二木芳人	日本化学療法学会雑誌	2015（印刷中）	国内
	石野敬子（分担執筆）	じほう	2015	国内

IV. 研究成果の刊行物・別刷

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Original article

Potent drugs that attenuate anti-*Candida albicans* activity of fluconazole and their possible mechanisms of action

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ABSTRACT

Fluconazole (FLCZ) is a first-line drug for treating *Candida albicans* infections, but clinical failure due to reduced sensitivity is a growing concern. Our previous study suggested that certain drug combinations pose a particular challenge in potently reducing FLCZ's anti-*C. albicans* activity, and cyclooxygenase inhibitors formed the major group of these attenuating drugs in combination with FLCZ. In this study, we examined the effects of diclofenac sodium (DFNa) and related compounds in combination with FLCZ against *C. albicans*, and investigated their possible mechanisms of interaction. DFNa, ibuprofen, and omeprazole elevated the minimum inhibitory concentration (MIC) of FLCZ by 8-, 4-, and 4-fold, respectively; however, loxoprofen sodium and celecoxib did not. An analogue of DFNa, 2,6-dichlorodiphenylamine, also elevated the MIC by 4-fold. Gene expression analysis revealed that diclofenac sodium induced *CDR1* efflux pump activity, but not *CDR2* activity. In addition, an efflux pump *CDR1* mutant, which was manipulated to not be induced by DFNa, showed less elevation of MIC compared to that shown by the wild type. Therefore, DFNa and related compounds are potent factors for reducing the sensitivity of *C. albicans* to FLCZ partly via induction of an efflux pump. Although it is not known whether such antagonism is relevant to the clinical treatment failure observed, further investigation of the molecular mechanisms underlying the reduction of FLCZ's anti-*C. albicans* activity is expected to promote safer and more effective use of the drug.

1. Introduction

Candida albicans is known as the primary cause of systemic candidiasis, which has a high mortality rate [1]. Fluconazole (FLCZ) is a first-line drug for *C. albicans* infections, but clinical failure due to reduced drug sensitivity is a growing concern [2]. In addition, alternative therapeutic options are limited, and the development of new drugs has been slow. Therefore, the identification of effective and safe conventional antifungal agents is necessary. For this purpose, we previously investigated the combinatorial effect of FLCZ with other drugs, and showed that certain stress response inhibitors could enhance the effects of azoles and echinocandins [3,4]. In another study in which we screened the combinatorial effects of FLCZ with 640 drugs approved by the Food and Drug Administration (FDA), we found that some drugs could attenuate the anti-*Candida* activity of FLCZ [5], suggesting that certain combinations have a tendency to potently reduce the antifungal activity. One major group of such attenuating drugs was identified as the cyclooxygenase (COX) inhibitors, which are also known as non-steroidal anti-inflammatory drugs (NSAIDs) and are frequently used as antipyretics and analgesics. In contrast to our findings, other previous reports suggested that COX inhibitors could synergistically or additively enhance the anti-*C. albicans* and anti-*Candida* biofilm activity of FLCZ [6–15]. However, relatively high concentrations of these drugs were used in these studies. In order to estimate the actual interaction of these drugs, we examined their dose- and structure-dependent effects, and investigated the possible mechanisms underlying their combinatorial effects with FLCZ against *C. albicans*.

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2. Materials and methods

2.1. Chemicals

All general chemicals used in this study were purchased from Wako Chemicals (Tokyo, Japan) unless otherwise indicated, and were of the highest purity available. Ultra-pure water dispensed by a Milli-Q water system (Millipore; Bedford, MA, USA) was used for the preparation of buffers and solvents. FLCZ was purchased from Sigma Aldrich (St. Louis, MO, USA). Four COX inhibitors, diclofenac sodium (DFNa), ibuprofen (IBU), loxoprofen sodium (LOX), and celecoxib (CEL), a proton pump inhibitor, omeprazole (OPZ), and an analogue of DFNa, 2,6-dichlorodiphenylamine (2,6-DPA), were used as the combination drugs. Each drug was dissolved in dimethylsulfoxide (DMSO) at 2 mg/mL for stock solution and stored at −20 °C. Standard ergosterol was dissolved in methanol at 1 mg/mL.

2.2. Strains and growth conditions

The standard *C. albicans* strain SC5314 and an efflux pump *CDR1* mutant, TU202, were used in this study [16]. TU202 has an *ACT1* promoter-driven *CDR1* gene instead of the disrupted native *CDR1* gene (Δ*CDR1* with p*ACT1-CDR1*), which results in constant *CDR1* expression independent of exogenous stimulation. The strains used are listed in Supplemental Table S1. We also used two additional *C. albicans* strains ATCC10231 and ATCC10261.

We used yeast nitrogen base medium (YNB; Difco Laboratories; USA) with 2% dextrose (YNB2D) instead of RPMI medium, which is recommended as standard medium by the Clinical and Laboratory Standards Institute (CLSI). YNB2D was used because growth was slow in RPMI, which made it difficult to detect the combinatorial effect. A single colony was inoculated in the medium, and cells were grown in YNB2D at 37 °C with agitation. For microdilution, after the cell density of the overnight culture was measured, the cell suspension was diluted with YNB2D to inoculate approximately 1×10^4 colony-forming units (cfu)/mL of cells for the subsequent experiments. For cellular sterol and gene expression analysis, mid-log phase yeast was incubated with or without DFNa (25 μM) and/or FLCZ (0.5 μg/mL) in 5 mL of medium at 37 °C with agitation, and the cells were pelleted 4 h after treatment.

DFNa is an FDA-approved drug, and we previously reported that it attenuated FLCZ activity against *C. albicans* [5]. We tested the dose-dependent effect of DFNa on the anti-*C. albicans* activity of FLCZ. High concentration of DFNa (>250 μM) alone remarkably inhibited the growth of *C. albicans*; therefore, the combinatorial effect was tested at lower concentrations.

2.3. Microdilution methods for evaluation of combinatorial effects

The cells were seeded in 96-well plates in the presence of the combination drugs and FLCZ and incubated at 37 °C without agitation. FLCZ was serially diluted in the plates and the other drugs were used at the doses indicated in Table 1. After 24 h, cell growth was monitored by measuring the optical density at 630 nm (OD_{630}) by using a microplate reader.

Minimum inhibitory concentration (MIC) was defined as 50% or more growth inhibition compared to the growth without FLCZ treatment, and the growth inhibition was evaluated by the reduction of the OD_{630} as measured above.

2.4. Analysis of cellular sterols

C. albicans is known to have several resistance mechanisms: 1) alternative sterol synthesis, 2) overexpression of ergosterol synthesis, and 3) activated or overexpressed efflux pumps. We first investigated whether DFNa altered ergosterol synthesis by using TLC analysis. Cell pellets were suspended in 4 mL of 1% NaCl solution and lipids were extracted by using Bligh-Dyer methods [17]. The extracted lipids were dried and resolved in chloroform/methanol (2:1). Standard ergosterol (5 μg) and 5 μL of the solution were spotted on a thin layer chromatography (TLC) plate, and lipids were separated in hexan/diethylether/acetate (25:25:1). Spots were visualized after spraying $FeCl_3/CH_3COOH/H_2SO_4$ solution on the plate, and the image was obtained using a scanner.

Table 1
Effects of COX inhibitors and a proton pump inhibitor on the antifungal activity of FLCZ against *C. albicans* SC5314 and TU202.

Strains and drugs		MIC (μg/mL)
SC5314 (wild type)		
FLCZ only		0.5
FLCZ + COX inhibitor	DFNa (25 μM)	4
	IBU (20 μM)	2
	LOX (20 μM)	0.5
	LOX (160 μM)	0.5
	CEL (25 μM)	0.5
	CEL (50 μM)	0.5
FLCZ + analogue of DFNa	2,6-DPA (25 μM)	2
FLCZ + proton pump inhibitor	OPZ (20 μM)	2
TU202 (Δ*CDR1* with p*ACT1-CDR1*)		
FLCZ only		0.5
FLCZ + DFNa (25 μM)		1

2.5. Analysis of gene expression

RNA extraction from the pelleted cells and the subsequent real-time polymerase chain reaction (RT-PCR) were performed as previously reported [3,18]. Briefly, approximately 800 ng of total RNA was used as a template to synthesize the cDNA (final volume, 20 μL) and cDNA equivalent to approximately 40 ng of total RNA was used as a template for RT-PCR. The actin gene, *ACT1*, was used as the internal control, and all expression values were normalized against *ACT1* expression. The sequences of the RT-PCR primers are listed in Supplemental Table S1. The data were analysed using Student's *t*-tests. The data are presented as fold changes in comparison to the control (untreated) and the mean ± standard error (SE) of replicates ($n = 4$). The data are representative of 3 or more individual experiments.

3. Results

3.1. DFNa and related compounds reduce the anti-C. albicans activity of FLCZ

At 2.5 μM and 25 μM, DFNa shifted the dose–response curves, indicating a reduction in the anti-*C. albicans* activity of FLCZ (Fig. 1). DFNa also shifted the dose–response curves in the two additional *C. albicans* strains ATCC10231 and ATCC10261 (Supplemental Fig. S1).

To evaluate whether the COX structure or inhibiting function affect the anti-*C. albicans* activity of FLCZ, the COX-related compounds were tested (Table 1). IBU also elevated the MICs by 4-fold; however, LOX and CEL did not alter the MICs, even at doses of 160 μM and 50 μM, respectively. We also tested an analogue of DFNa, 2,6-DPA, which similarly elevated the MICs by 4-fold. OPZ also elevated the MICs by 4-fold.

3.2. Attenuated anti-C. albicans activity of FLCZ partly depends on induced CDR1 expression

FLCZ quenched ergosterol synthesis (Fig. 2, lane 3), but ergosterol reappeared when DFNa was added (Fig. 2, lane 4), and DFNa alone did not alter the ergosterol spot pattern (Fig. 2, lane 5).

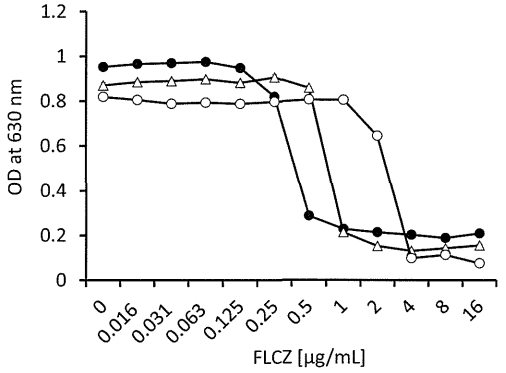

Fig. 1. Effect of DFNa on the antifungal activity of FLCZ against *C. albicans* SC5314. Closed circles, FLCZ alone; open triangles, DFNa (2.5 μM); open circles, DFNa (25 μM). The MIC was 0.5 μg/mL with FLCZ alone, but shifted to 1 μg/mL and 4 μg/mL when 2.5 μM and 25 μM of DFNa were added, respectively.

We next evaluated the expression of genes that are related to ergosterol synthesis and efflux pumps. FLCZ induced *ERG3*, *ERG9*, and *ERG11* gene expressions, which were markedly reduced following the addition of DFNa (Fig. 3a, DFNa + FLCZ), and were not induced by DFNa alone (Fig. 3a, DFNa). These findings suggested that DFNa did not directly affect ergosterol synthesis, and that the reduced anti-*C. albicans* activity is independent of alterations in ergosterol synthesis. *CDR1* was significantly induced by DFNa with or without FLCZ (Fig. 3b, FLCZ + DFNa, DFNa) but not by FLCZ alone (Fig. 3b, FLCZ), whereas *CDR2* was not induced by any drug or drug combination. IBU, 2,6-DPA and OPZ also induced *CDR1* expression, but LOX and CEL did not (Supplemental Fig. S2).

Gene expression analysis suggested that *CDR1* induction was responsible for the reduced anti-*C. albicans* activity of FLCZ. Therefore, we evaluated whether the induction of *CDR1* expression was necessary for the effect. The TU202 mutant has an *ACT1* promoter-driven *CDR1* gene instead of the disrupted native *CDR1* gene (Δ*CDR1* with p*ACT1-CDR1*), which results in the constant expression of *CDR1* independent of exogenous stimulation. The MIC curves of the wild type and TU202 were comparable without DFNa; however, the shift of the curve induced by DFNa in the wild type

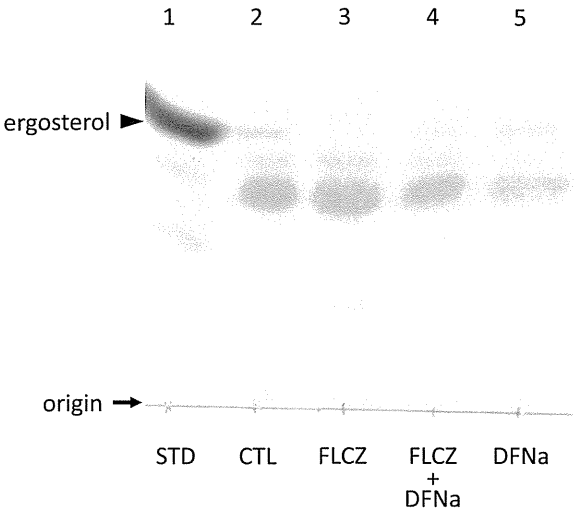

Fig. 2. Profiles of cellular sterols separated by thin layer chromatography. Lane 1, ergosterol standard (STD); lane 2, untreated control (CTL); lane 3, FLCZ-treated; lane 4, FLCZ and DFNa-treated; lane 5, DFNa-treated. The ergosterol spot disappeared when FLCZ was added alone (lane 3), but was restored following the addition of DFNa (lane 4).

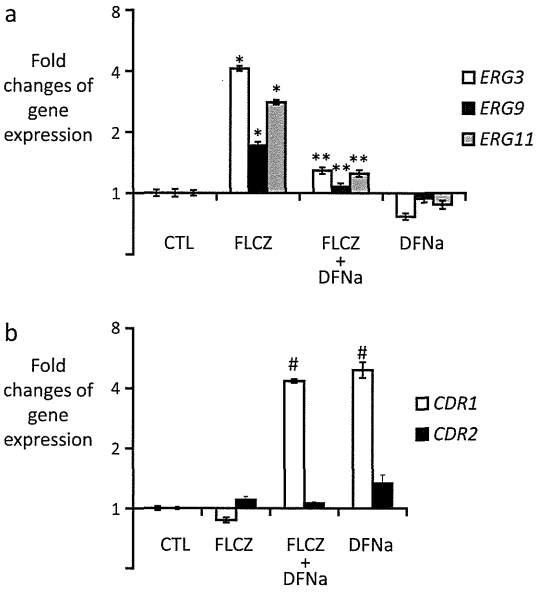

Fig. 3. Expression levels of representative genes related to the FLCZ resistance of *C. albicans*. a, Ergosterol synthesis. White, black, and grey bars indicate the expression of *ERG3*, *ERG9*, and *ERG11*, respectively; FLCZ significantly induced these genes but DFNa did not. b, Efflux pumps. White and black bars indicate *CDR1* and *CDR2* expression, respectively; DFNa significantly induced *CDR1*, with or without FLCZ, but not *CDR2*. CTL, control; FLCZ, fluconazole alone; FLCZ + DFNa, fluconazole with diclofenac sodium; DFNa, diclofenac sodium alone. $^{*}P < 0.001$ compared to CTL; $^{**}P < 0.005$ compared to FLCZ; $\#P < 0.001$ compared to CTL and FLCZ.

was partly diminished in TU202, suggesting that induced *CDR1* partly contributed to the attenuated anti-*C. albicans* activity of FLCZ by DFNa (Fig. 4).

4. Discussion

High doses of COX inhibitors have previously been reported to enhance azoles' anti-*Candida* activity [6–15]. Our study is the first to show that DFNa and IBU are potent factors contributing to the attenuation of this activity at lower doses though Arai R. et al. showed synergism of FLCZ and IBU. When used at therapeutic doses, the maximum blood concentrations of DFNa and IBU were

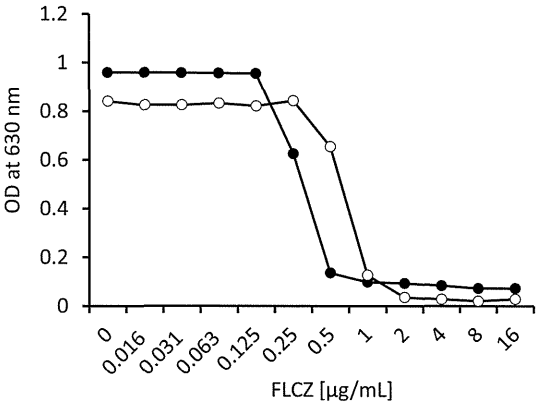

Fig. 4. Effect of DFNa on the antifungal activity of FLCZ in a *CDR1* mutant, TU202. TU202 has an *ACT1* promoter-driven *CDR1* gene instead of the disrupted native *CDR1* gene (Δ*CDR1* with p*ACT1-CDR1*), which results in the constant expression of *CDR1* independent of exogenous stimulation. Closed circles, FLCZ alone; open circles, DFNa (25 μM). The dose–response curves of TU202 were comparable to those of the wild type without the addition of DFNa. DFNa shifted the dose response curve in TU202 less than it did in the wild type.

reported to be 9 μM and 15–50 μM, respectively [19,20]. Therefore, the concentrations we used in this study were closer to the clinically available concentrations than those tested in most previous reports [6–15], with the exception of the study by Arai et al. [14] showing that IBU did not affect FLCZ activity against other strains at a concentration close to that used in the present study. Interestingly, the anti-*Candida* activity of FLCZ was not altered by either LOX or CEL. LOX is frequently used in Japan but not in the US, and CEL is a selective COX2 inhibitor that is dissimilar to traditional COX inhibitors. Therefore, our findings and those of previous reports suggest that the combinatorial effect of COX inhibitors could be dependent on the tested strains and conditions, as well as the specific type of inhibitors used.

We also identified possible mechanisms underlying FLCZ resistance to *C. albicans*. Our data could not completely explain the mechanisms by which DFNa attenuated FLCZ activity, but suggested that induced *CDR1* pumps, which are most frequently related to FLCZ resistance of *C. albicans*, might be at least partially involved in the effect. IBU and OPZ may be also able to disturb the anti-*Candida* activity of FLCZ, possibly via the same mechanism. In contrast to our results, IBU has been reported to reverse the antifungal resistance of *C. albicans* overexpressing *CDR* genes (12). Therefore, the effect of these drugs appears to fluctuate, and it is difficult to accurately predict how the drugs will interact with antifungal agents. We also showed that the mechanism might be independent of COX activity inhibition, as LOX and CEL did not show the reduced anti-*Candida* activity effect. In addition, an analogue of DFNa did show the effect, suggesting that the mechanism might be more related to the structure of the compounds rather than to the inhibition of COX function; however, the structure–activity relationship could not be clearly determined.

In conclusion, several COX inhibitors and proton pump inhibitors show potent effects on reducing the sensitivity of *C. albicans* to FLCZ at clinically realistic concentrations, possibly via inducing an efflux pump. Although it is not known whether this antagonistic effect is the main cause of clinical treatment failure, further investigation of the molecular mechanisms underlying the effect of the reduction on FLCZ's anti-*Candida* activity is expected to promote safer and more effective use of these drugs.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jiac.2014.06.004.

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RESEARCH ARTICLE

Detection and characterization of plasminogen receptors on clinical isolates of *Trichosporon asahii*

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Trichosporon asahii; plasminogen; heparinase; thioredoxin-dependent peroxide reductase.

Abstract

Trichosporon asahii is the major causative agent of deep-seated trichosporonosis. The virulence factors of this yeast pathogen remain uncharacterized. To investigate the pathogenicity of *T. asahii*, we focused on the interactions between surface molecules of the yeast and host biomolecules. We examined the ability of surface molecules to bind human plasminogen using clinical isolates of *T. asahii*. Living *T. asahii* cells accelerated the conversion of plasminogen to plasmin in a dose-dependent manner in the presence of tissue plasminogen activator. Extracts from cells using lithium chloride contained plasminogen-binding molecules based on surface plasmon resonance (SPR) analyses. In all strains tested, several of the fractions obtained using DEAE column chromatography bound and accelerated the conversion of plasminogen to plasmin. Based on far-Western blotting analyses, a common protein was identified within the four strains, which was identified as a hypothetical protein from genome analyses of *T. asahii*. BLAST searches suggested the protein might be heparinase, and heparinase activity was detected in the *T. asahii* extract. Furthermore, affinity chromatography using plasminogen as a ligand detected one protein band by SDS-PAGE, which was identified as thioredoxin-dependent peroxide reductase.SPR analyses suggested the presence of molecules on *T. asahii* cells that could bind plasminogen with differing affinities.

YEAST RESEARCH

Introduction

Trichosporon asahii is a yeast-like fungus distributed in the environment (Sugita, 2011; Sugita *et al.*, 2001) and is the major causative agent of deep-seated trichosporonosis, an opportunistic fungal infection with a poor prognosis and high mortality rate in immunocompromised patients and patients with hematological malignancies (Sugita *et al.*, 1995, 1999; Erer *et al.*, 2000). The low sensitivity of *T. asahii* to antifungal agents may lead to therapeutic problems (Toriumi *et al.*, 2002; Di Bonaventura *et al.*, 2006; Kalkanci *et al.*, 2008; Mekha *et al.*, 2010); however, the virulence factors of this yeast remain uncharacterized. To investigate the pathogenicity, we focused on the interactions between surface molecules of *T. asahii* and host biomolecules. Microbial pathogens express molecules that interact with host proteins and carbohydrates (Henderson & Martin, 2011; Fatoux-Ardore *et al.*, 2014), leading to invasion and colonization. Some pathogenic microorganisms express proteins that bind to and enhance plasminogen activity (Furuya & Ikeda, 2011; Bhattacharya *et al.*, 2012; Sanderson-Smith *et al.*, 2012; Fulde *et al.*, 2013; Godier & Hunt, 2013; Magalhaes *et al.*, 2013). Thus, pathogens utilize the host fibrinolytic system to promote invasion. In *Cryptococcus neoformans*, which is taxonomically related to *T. asahii*, several plasminogen-binding molecules have been reported (Stie *et al.*, 2009; Stie & Fox, 2012; Ikeda & Ichikawa, 2014). In this study, we explored whether *T. asahii* surface polymers interact with plasminogen, and characterized the molecules to analyze *T. asahii* virulence.

Materials and methods

Strains used

Four clinical isolates of *T. asahii* were used. Strains M9434, M9925, and M9928 (Sugita *et al.*, 2002) were isolated from blood, while the fourth strain, M9432 (Sugita *et al.*, 2002), was isolated from the urine of a patient with a pituitary tumor. Strain M9434 was isolated from a patient with aplastic anemia.

Preparation of *T. asahii* surface extracts

Trichosporon asahii cells that had been cultured at 37 °C for 5 days in yeast nitrogen base broth with 2% glucose and 1% casamino acids were suspended in 3 M LiCl to obtain cell surface proteins. After mixing gently on ice for 15 min, the cell extract was dialyzed against water and the surface proteins were collected (Ikeda & Ichikawa, 2014). Crude extracts were fractionated using anion-exchange column chromatography (TOYOPEARL DEAE-650, TOSOH). Unbound molecules were washed with water, and bound molecules were eluted with 0.01 M sodium phosphate buffer (PB), pH 7.4, and eluted via stepwise elution of 0–1.0 M NaCl in PB. The fractions were subjected to further analysis. The proteins were semi-quantified by spotting each fraction on a polyvinylidene difluoride (PVDF) membrane followed by Coomassie Brilliant Blue (CBB) staining.

Effects of intact *T. asahii* cells on plasminogen activation

To examine the effects of intact *T. asahii* on plasminogen activation, cells were added to plasminogen activation assay systems. Reagents were diluted in 50-mM Tris-HCl buffer, pH 7.4. Live cell suspensions (10 μL) of various concentrations (*c.* 10^8, 10^7, 10^6, and 10^5 cells mL^{-1}) were incubated with 40 μL of 500 nM plasminogen for 30 min at 37 °C. After incubation, 10 μL of 10 μg mL^{-1} tissue plasminogen activator (t-PA) (recombinant human; Technoclone GmbH, Vienna, Austria) was added and the mixtures were incubated for 10 min at 37 °C, followed by addition of the chromogenic substrate H-D-valyl-L-leucyl-L-lysyl-*p*-nitroaniline dihydrochloride (S-2251) (40 μL, 0.5 mM solution; Chromogenix, Chapel Hill, NC). The absorbance at 405 nm (which depended on the release of *p*-nitroaniline from the substrate by plasmin activity) was monitored at 10-min intervals.

Interaction between DEAE fractions and plasminogen

To examine the interaction between fractions obtained by DEAE anion-exchange column chromatography and plasminogen, SPR analysis was performed using a Biacore 3000 (GE Healthcare, Milwaukee, WI). Plasminogen (human; Enzyme Research Laboratories, South Bend, IN) was diluted with 10-mM sodium acetate buffer (pH 5.0) to a concentration of 20 μg mL^{-1} and immobilized on a standard sensor chip (CM 5) using an amine-coupling kit, according to the manufacturer's instructions. Running buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 was used. The flow rate was maintained at 10 μL mL^{-1} for immobilization and 20 μL mL^{-1} for analysis.

Effects of DEAE fractions of *T. asahii* on plasminogen activation

The effects of the extracted molecules on plasminogen activation were determined using the methods described above. The experiments were performed using the extracted molecules instead of intact cells.

Binding between surface molecules and plasminogen

Far-Western blotting was performed to identify candidate molecules that interacted with plasminogen. The fractions showing an interaction with plasminogen were separated using SDS-PAGE and blotted onto PVDF membranes. The membranes were treated with plasminogen (100 nM), alkaline phosphatase-conjugated antiplasminogen antibody (goat polyclonal antibody to human plasminogen; EY Laboratories, San Mateo, CA), and alkaline phosphatase chromogenic substrate (15-mL Tris-HCl buffer at 100 mM [pH 9.5] containing 100 mM NaCl and 5 mM $MgCl_2$; 100 μL 0.017% [w/v] nitroblue tetrazolium; 0.23% [v/v] *N*, *N*-dimethyl formamide; and 50 μL 5% 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt in *N*, *N*-dimethyl formamide).

Determination of heparinase activities

Heparin lithium (1 mg, 25 000 units) was dissolved in 325 μL of 50 mM sodium–phosphate buffer, pH 7.4 containing 75 mM NaCl and incubated overnight. The fraction of *T. asahii* M9432 extract eluted with 0.1 M NaCl using DEAE column chromatography (25 μL) was added

to the heparin solution. The mixture was incubated at 32 °C, and absorbance at 232 nm was monitored at 5-min intervals (Hyun *et al.*, 2010).

Affinity chromatography

Affinity chromatography was performed with an Affi-Gel 10 (Bio-Rad) column following the manufacturer's instructions at 4 °C. Briefly, the gel (0.2 mL) was equilibrated with coupling buffer (100 mM, pH 6.6 MOPS buffer). Plasminogen (2 mg 0.4 mL^{-1}) was added to the gel and mixed gently for 4 h. The gel was treated with blocking buffer (200 mM Tris-HCl in 100 mM MOPS buffer, pH 8.0) for 1 h. After adding the sample containing plasminogen-binding proteins, the column was washed with PBS and the bound molecules were eluted with 50 mM EACA, followed by 100 mM EACA.

Results

Effects of *T. asahii* cells on plasminogen activation

To determine whether molecules on the surface of *T. asahii* interacted with plasminogen, live *T. asahii* cells were added to the plasminogen activation system in the presence of tissue plasminogen activator (t-PA). As shown in Fig. 1, cells from all strains facilitated plasminogen activation to plasmin in a dose-dependent manner. The number of live cells varied among the strains because the numbers of colony-forming units were determined 2 days after the experiments. At cell densities of *c.* 10^4 mL^{-1}, reactions proceeded similarly to the control without *T. asahii* for all strains tested. These results suggest that molecules on *T. asahii* interact with plasminogen and

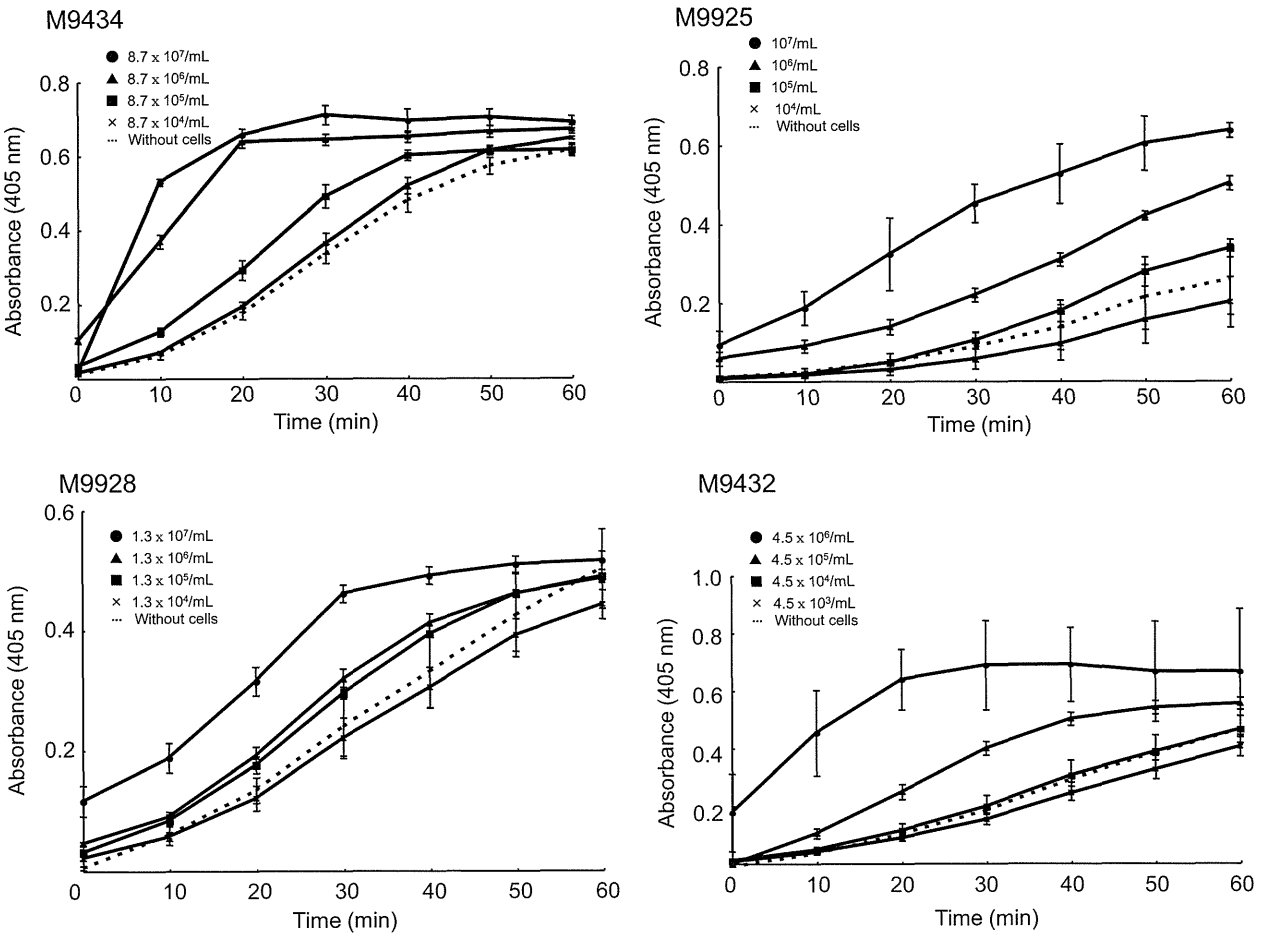

Fig. 1. Effect of live *Trichosporon asahii* cells on plasminogen activation by t-PA. *Trichosporon asahii* M9434, M9925, M9928, and M9432 was used. Plasminogen was preincubated with 10-fold serial dilutions of *T. asahii* cells for 30 min at 37 °C, after which t-PA was added. After incubation for 10 min, S-2251 was added. The absorbance at 405 nm was monitored at 10-min intervals. The number of living cells on Sabouraud dextrose agar plates was counted by incubation of the same cell suspension after dilution.

play a role in plasminogen activation. Strain CBS2479 (type strain) was also used in the experiments. The range of doses of cells used was between 10^2 and 10^5 mL^{-1}. CBS 2479 showed similar results to the four clinical isolates within the range. However, CBS2479 grew extremely poorly at 37 °C. In consideration for virulence research and the amounts of the protein obtained at 37 °C, further experiments were performed using the four clinical isolates.

Interaction between *T. asahii* surface molecules and plasminogen

To explore the role of molecules located on the cell surface in plasminogen activation, cell extracts were fractionated using a DEAE anion-exchange column and binding with plasminogen was analyzed using surface plasmon resonance (SPR). For these experiments, plasminogen was immobilized on the sensor tip as the ligand. As shown in Fig. 2, all strains contained molecules that bound plasminogen. The fractions eluted with 0.1, 0.2, and 0.3 M NaCl reacted at relatively higher affinities. From the shape of the sensorgram, it was speculated that the fractions could contain molecules which interacted to plasminogen with different affinities.

Effects of soluble fractions extracted from *T. asahii* cells on plasminogen activation

To determine whether soluble fractions affect the activation from plasminogen to plasmin, fractions were added to the plasminogen activation system in the presence of t-PA. As shown in Fig. 3, the extracts from all strains accelerated plasminogen activation to plasmin. These results suggest that molecules on *T. asahii* interact with plasminogen and contribute to plasminogen activation.

Identification of plasminogen-binding molecules

To identify molecules that bound plasminogen, far-Western blotting was performed. In all cases, one or two

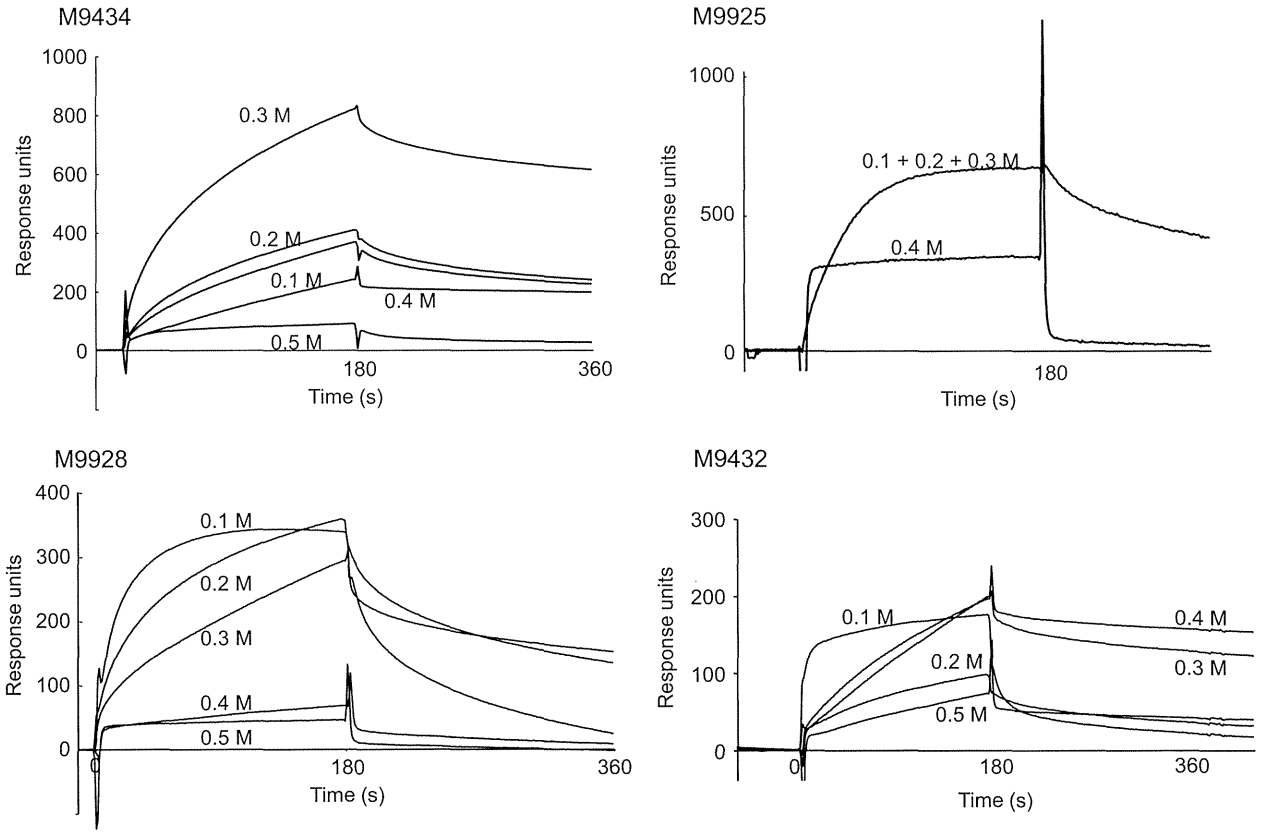

Fig. 2. Interactions between fractions of *Trichosporon asahii* M9434, M9925, M9928, and M9432 extracts using LiCl and plasminogen based on SPR. Human plasminogen was used as the ligand. Analytes were the fractions obtained by DEAE ion-exchange column chromatography eluted with various concentrations of NaCl. In the case of M9925, three fractions were mixed in consideration of the amount of proteins needed for further analyses.

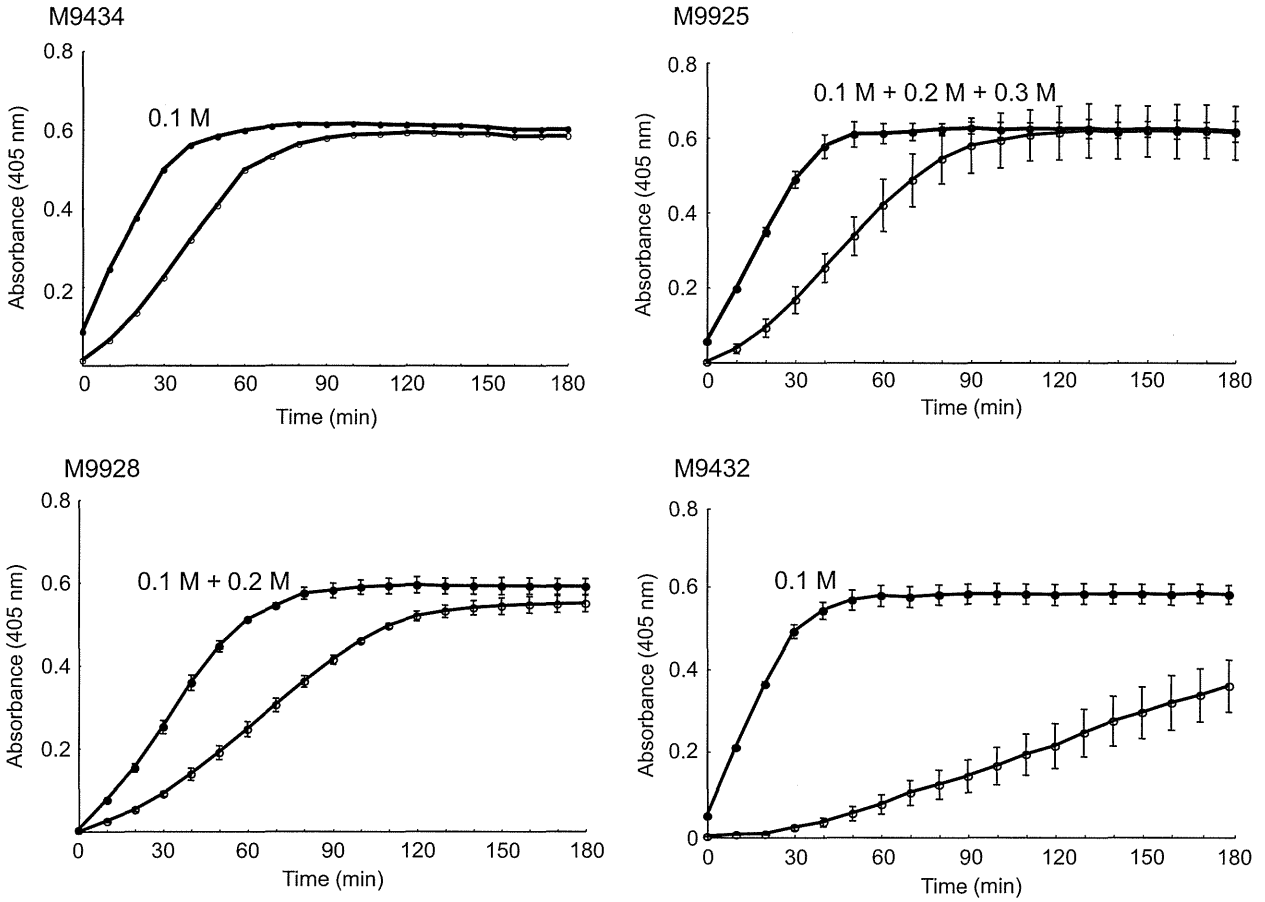

Fig. 3. Effects of DEAE fractions from *Trichosporon asahii* M9434, M9925, M9928, and M9432 on plasminogen activation in the presence of t-PA. Plasminogen was preincubated with the fractions for 30 min at 37 °C, after which t-PA was added. After incubation for 10 min, S-2251 was added. The absorbance at 405 nm was monitored for 3 h at 10-min intervals. Closed circle, with *T. asahii* fractions, open circle, without the fractions. In the case of M9928, two fractions were mixed, and in the case of M9434, 0.1 M fraction was used in consideration of the amount of proteins needed for further analyses.

common protein bands at *c.* 80 and *c.* 35 kDa that bound to plasminogen were identified (Fig. 4) although the figure contained gels of which resolution was less clear in the printed photograph. Amino acids in the N-terminal region corresponding to the bands are shown in Table 1. By comparing these sequences to those from the genome analysis of T. Sugita and M. Takashima (unpublished) using *T. asahii* JCM2466 (CBS2479), the two bands were found to have originated from a single hypothetical protein (coding sequence: 14575559–14578150). Smaller (35 kDa) bands may be derived from the larger band (80 kDa). However, in M9925, larger band was not detected, whereas smaller band was not detected in M9434 and M9432. It is unclear how the smaller bands were derived, although some protease could be speculated. A BLAST analysis of the whole protein sequence showed that it was identical to that of the hypothetical protein A1Q1 00495 (*T. asahii* CBS2479). The amino acid

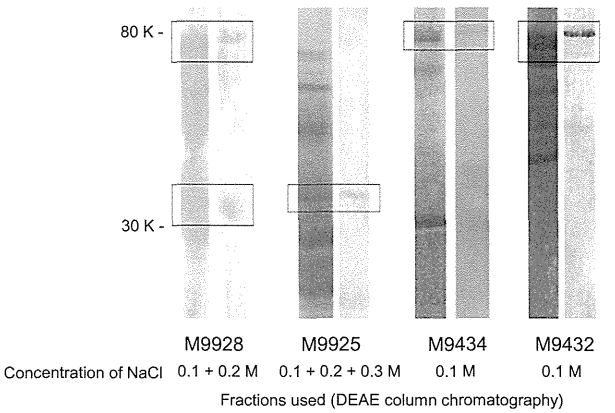

Fig. 4. Detection of plasminogen-binding proteins by far-Western blotting analysis. Left lanes are stained with CBB, and right lanes show the corresponding PVDF membrane for each pair. The bands corresponding to plasminogen binding are indicated by the square.

Table 1. Amino acids at the N-terminal region corresponding to the bands

	Strain			
Band	M9928	M9925	M9434	M9432
35K	W	W		
	Y	Y		
	D	D		
	P	P		
	T	T		
	T	T		
	D	D		
	G	G		
	A	A		
	F	F		
	W	W		
	N	N		
	G	G		
	L	L		
	E	E		
	I	I		
	D	D		
	H	H		
	H	H		
	F	F		
80K	N		N	
	Q		Q	
	G		G	
	G		G	
	D		D	
	K		K	
	G		G	
	Q		Q	Q
	A		A	A
	P		P	P
	A		A	A
	P		P	P
	D		D	D
	A		A	A
	G		G	G
	N		N	N
	T		T	T
	A		A	A
			L	L
				P
				G
				M
				I
				Q
				I
				L
				T
				D

sequences were similar (47%) to that of heparinase II/III family protein of *C. neoformans* (Fig. 5). Thus, the enzymatic activities of heparinase were assayed. Heparin lithium was incubated with the *T. asahii* M9432 fractions eluted by 0.1 M NaCl using DEAE column chromatography, and the increase in absorbance at 232 nm due to degradation of heparin was monitored. As shown in Fig. 6, the absorbance increased sequentially; however, the absorbance of substrate control did not increase, suggestive of the presence of heparinase in the sample.

Fractionation of plasminogen-binding proteins using affinity chromatography

To identify plasminogen-binding proteins, we performed affinity column chromatography using plasminogen as the ligand. The extracts from M9928 and M9432 were fractionated by DEAE column chromatography, and the fractions for affinity chromatography were selected based on the results of SPR analyses. The binding proteins were eluted with EACA. Based on silver staining, one protein band was detected from each strain (Fig. 7). After reductive alkylation, the protein was identified by liquid chromatography tandem mass spectrometry (LC-MS/MS) as a thioredoxin-dependent peroxide reductase in both strains (Fig. 8).

Discussion

Several species of *Trichosporon* are associated with clinical infections; however, in deep-seated trichosporonosis, which occurs in patients with neutropenia and immunodeficiency (Sugita *et al.*, 1995, 1999), *T. asahii* is the species most often isolated. Because of its low sensitivity to antifungal agents, breakthrough infection of trichosporonosis is common (Goodman *et al.*, 2002; Di Bonaventura *et al.*, 2006; Matsue *et al.*, 2006; Kalkanci *et al.*, 2008; Chitasombat *et al.*, 2012). The virulence factors of *T. asahii* remain uncharacterized, although beta-N-acetylhexosaminidase has been proposed as a candidate (Ichikawa *et al.*, 2004). The virulence of microbial pathogens may be attributed to microorganism–host interactions. The binding between human and microbial biomolecules could play an important role in pathogenicity. Several host molecules have been investigated, including fibronectin, plasminogen, and collagen (Singh *et al.*, 2012). Among these, pathogens may utilize the host fibrinolytic system to promote invasion and dissemination. Plasminogen has been identified as being recognized by microorganisms (Furuya & Ikeda, 2011; Bhattacharya *et al.*, 2012; Plow *et al.*, 2012; Sanderson-Smith *et al.*, 2012; Fulde *et al.*, 2013; Godier & Hunt, 2013; Magalhaes *et al.*, 2013). The fungal pathogen *C. neoformans* possesses plasminogen-binding proteins (Stie *et al.*, 2009; Stie & Fox, 2012). Besides proteins, we suspect that carbohydrate-bound plasminogen enhances activation (Ikeda & Ichikawa, 2014).

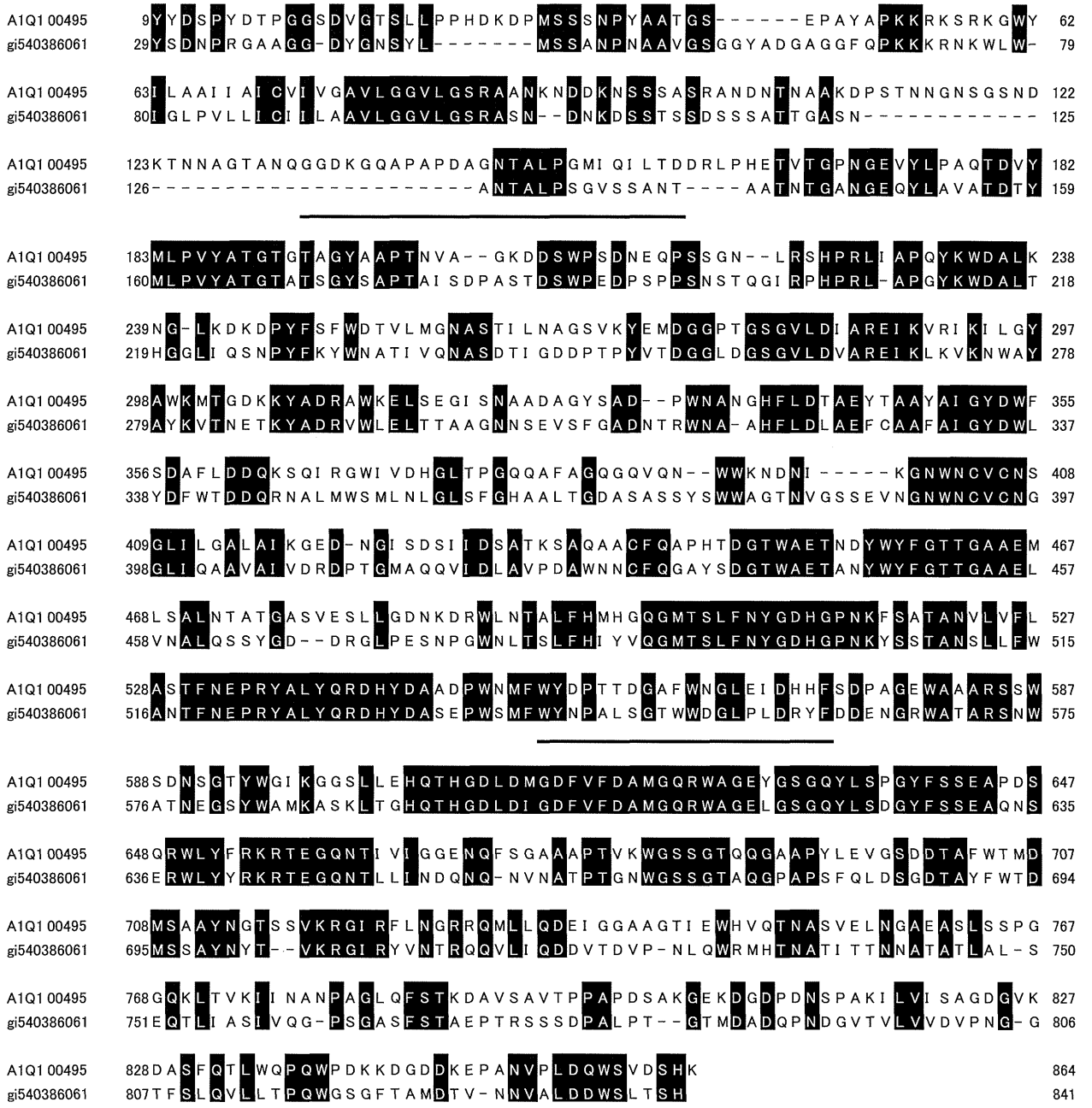

Fig. 5. Alignment of the amino acid sequences of *Trichosporon asahii* A1Q1 00495 and heparinase II/III family protein of *Cryptococcus neoformans*. The residues detected by the N-terminal amino acid sequencing of plasminogen-binding proteins were underlined.

In this study, heparinase was identified as a plasminogen-binding protein on four strains of *T. asahii* clinical isolates (three from blood and one from urine). Heparinase cleaves $\alpha 1 \rightarrow 4$ glycosidic linkages in heparin to oligosaccharides, and some microorganisms have been reported to possess this enzyme, including *Aspergillus* (Tripathi *et al.*, 2012). Heparin has been used as an anticoagulant agent (Liu & Pedersen, 2007) and also regulates cell growth through binding molecules. For example, fibroblast growth factor modulates angiogenesis and metastasis (Conti *et al.*, 2003; Bochenek & Nizankowski, 2012). The potential anticancer activity of low-molecular-weight heparin (LMWH) has been reported, which would be impaired by heparinase (Tang *et al.*, 2014). In tumors, the plasminogen activation system may regulate cell growth (Andreasen *et al.*, 2000). In this study, the activation of

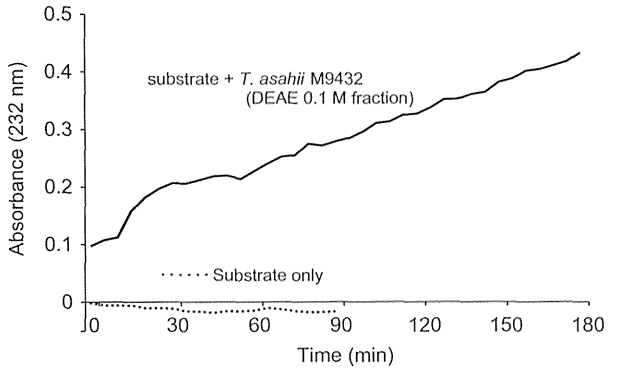

Fig. 6. Confirmation of heparinase activities in *Trichosporon asahii* M9432 extract. The fraction eluted with 0.1 M NaCl was used. The sample and heparin lithium were incubated at 32 °C, and absorbance at 232 nm was monitored at 5-min intervals. An increase in absorbance represents heparin degradation. Solid line, containing *T. asahii* fraction; dotted line, control.

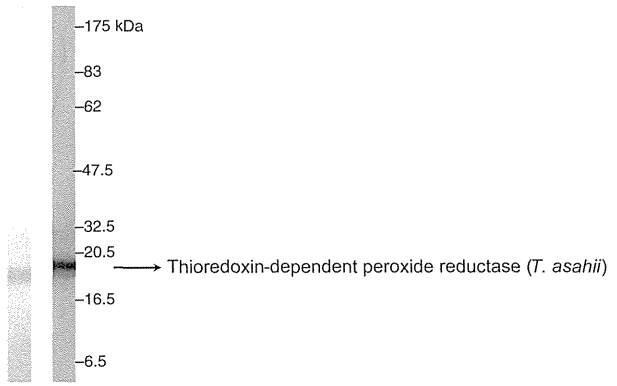

Fig. 7. SDS-PAGE of affinity-purified proteins from *Trichosporon asahii* M9928 (left) and M9432 (right). Protein bands were visualized by silver staining.

1 MSAPSAMIQKPAPAFQGTLVQNGEFKEVKLSDYEGKWVVLFSYPMDYIVC
51 PTEILAFNNALEQFKAINTEVIAFSTDSEFTHLAWSQTPRKEGGLGPNLN
101 LGLLSDRNHSVSKAYGVLLEEEGITLRGTFFIDPKGTLRAMHVHDLPVGR
151 SVEETIRVVKAFQFTDEHGEVCPANWEEGADTIDTADAKKYFSKHGEDAS
201 ASNKREAPGSAEKPAKRARA

Fig. 8. The sequence of thioredoxin-dependent peroxide reductase (*Trichosporon asahii* var. *asahii* CBS 2479). The underlined sequences were obtained by LC-MS/MS.

plasminogen to plasmin was promoted in the presence of *T. asahii* plasminogen-binding molecules. The host coagulation and fibrinolysis systems are commandeered during invasion by, and dissemination of, the fungus.

Recently, *Leishmania* interaction networks with the host extracellular matrix were investigated using SPR imaging. Interestingly, of six *Leishmania* species, 27 new partners (23 proteins, 4 glycosaminoglycans) of procyclic promastigotes and 18 (15 proteins, 3 glycosaminoglycans) of three species of stationary phase promastigotes containing heparin and plasminogen were identified (Fatoux-Ardore *et al.*, 2014). Of the molecules with which *Leishmania* interacted, several regulators of angiogenesis, including antiangiogenic (endostatin and anastellin) and pro-angiogenic factors (ECM-1, VEGF, TEM8, also known as anthrax toxin receptor 1), are regulated by hypoxia.

Additional studies using purified heparinase and mutant strains are required to confirm the identity of the protein as heparinase. Previously, commercially available heparinase II was used. SPR analyses showed that heparinase II from *Bacteroides eggerthii* bound plasminogen in a dose-dependent manner (100, 200, and 400 U mL^{-1}) and that heparinase II activated plasminogen to plasmin (40 and 80 U mL^{-1}) (Supporting Information, Fig. S1).

We also identified thioredoxin-dependent peroxide reductase in the fraction prepared by plasminogen-affinity chromatography, suggesting that multiple *T. asahii* surface molecules interact with plasminogen. We inferred that multiple molecules could bind plasminogen with different affinities. In the experiments using M9432, different batch of the preparation was used (Fig. S2). The preparation in experiment 1 and 2 was applied to far-Western blotting and affinity chromatography, respectively. In Exp. 1, heparinase was identified using 0.1 M fraction, which bound plasminogen rapidly; however, dissociation rate was also quick. While in Exp. 2, 0.3 M fraction was used, dissociation rate of which was slow suggesting higher affinity. These might be the reason that the identification of the proteins which bound plasminogen was different.

In conclusion, *T. asahii* may use human biomolecules, such as plasminogen receptors, for invasion. Although the role of these plasminogen-binding proteins in virulence remains unclear, the findings presented in this report could provide a target gene for investigation of the virulence factors of pathogenic yeast.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (a) Interaction between heparinase II from *Bacteroides eggerthii* and plasminogen as shown by SPR. (b) Effects of heparinase II from *B. eggerthii* on plasminogen activation in the presence of t-PA.

Fig. S2. Comparison of SPR sensorgrams showing the interactions between plasminogen and various DEAE-fractions subjected to far-Western blotting and affinity chromatography.