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Characterization of the interactions between human high-molecular-mass kininogen and cell wall proteins of pathogenic yeasts *Candida tropicalis**

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***Candida tropicalis* is one of the most frequent causes of serious disseminated candidiasis in human patients infected by non-albicans *Candida* species, but still relatively little is known about its virulence mechanisms. In our current study, the interactions between the cell surface of this species and a multifunctional human protein – high-molecular-mass kininogen (HK), an important component of the plasma contact system involved in the development of the inflammatory state – were characterized at the molecular level. The quick release of biologically active kinins from candidal cell wall-adsorbed HK was presented and the HK-binding ability was assigned to several cell wall-associated proteins. The predicted hyphally regulated cell wall protein (Hyr) and some housekeeping enzymes exposed at the cell surface (known as "moonlighting proteins") were found to be the major HK binders. Accordingly, after purification of selected proteins, the dissociation constants of the complexes of HK with Hyr, enolase, and phosphoglycerate mutase were determined using surface plasmon resonance measurements, yielding the values of 2.20×10^{-7} M, 1.42×10^{-7} M, and 5.81×10^{-7} M, respectively. Therefore, in this work, for the first time, the interactions between *C. tropicalis* cell wall proteins and HK were characterized in molecular terms. Our findings may be useful for designing more effective prevention and treatment approaches against infections caused by this dangerous fungal pathogen.**

Key words: contact system, kinins, inflammation, candidiasis, cell wall proteins, adhesion

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INTRODUCTION

Although *C. albicans* is still the most prevalent species responsible for life-threatening systemic and deep-seated candidal infections, changes in epidemiology and an alarming increase in the incidence of candidiases caused by non-albicans *Candida* species – *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* – have been observed in the last decade (Pfaller & Diekema, 2007; Chaudhary *et al*., 2015). The frequency of invasive infections caused by particular *Candida* species varies in terms of geographical distribution and prevalence among different groups of patients. However, the predominance of *C. tropicalis* infections in the particular regions of the world – especially in South America and South Asia – and the fact that this species is considered to be the second, after *C. albicans*, most commonly isolated species from the bloodstream in many locations, suggest that special attention should be paid to *C. tropicalis* (Chai *et al*., 2010; Ma *et al*., 2013; Magalhães *et al*., 2015).

C. tropicalis is an opportunistic human pathogen that colonizes the skin as well as gastrointestinal and urogenital tracts, and it is often isolated from the surfaces of medical devices (Negri *et al*., 2012). The main risk factors predisposing for disseminated nosocomial infections include surgical procedures and bone marrow transplantation or severe immunosuppression associated with neutropenia and hematological malignancy (Horn *et al*., 2009; Chai *et al*., 2010; Fernández-Ruiz *et al*., 2015). Relevant results suggest higher pathogenicity of this species than that of the other non-albicans *Candida* species; however, the pathogenetic mechanisms of infections caused by *C. tropicalis* are still insufficiently described (Kothavade *et al*., 2010; Arendrup, 2013; Priest & Lorenz, 2015). *C. tropicalis* displays several virulence factors, including the ability to adhere to host cells and to form drug-resistant biofilms on tissues and medical devices, and to secrete hydrolytic enzymes that can destroy host defense mechanisms and barriers and deregulate systems responsible for the homeostasis in the human organism (Rapala-Kozik *et al*., 2010; Silva *et al*., 2012; Sardi *et al*., 2013; Negri *et al*., 2015). Likewise, the ability to bind host's proteins – e.g., extracellular matrix proteins and the soluble components of important plasma proteolytic cascades – at the fungal cell surface contributes to *C. tropicalis* pathogenicity and might be assigned to a fraction of surface-exposed proteins (Karkowska-Kuleta *et al*., 2010; Kozik *et al*., 2015; Dühring *et al*., 2015). Numerous putative cell wall-as-

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Abbreviations: Als, agglutinin-like sequence; BSA, bovine serum albumin; Eno1, enolase 1; FXII, coagulation factor XII; FXIIa, active coagulation factor XII; GPI, glycosylphosphatidylinositol; Gpm1, phosphoglycerate mutase 1; HK, high-molecular-mass kininogen; Hyr, hyphally regulated cell wall protein; LC-MS/MS, liquid chromatography-coupled tandem mass spectrometry; PBS, phosphate buffered saline; pHPK, human plasma prekallikrein; RU, resonance units; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid

sociated proteins and adhesins are encoded in the *C. tropicalis* genome, including large families of Pga30-like (predicted glycosylphosphatidylinositol (GPI)-anchored) proteins, Iff/Hyr-like (hyphally regulated) and Als-like (agglutinin-like sequence) proteins, and others (Butler *et al*., 2009). The presence of three potentially adhesive Als-like proteins at the *C. tropicalis* cell surface was confirmed using an immunoassay (Hoyer *et al*., 2001) and several *C. tropicalis* surface-exposed proteins were identified with the "cell surface shaving" method and shotgun proteomic approach, including a few predictably covalently bound typical cell wall proteins and a number of atypical ("moonlighting") proteins, more loosely associated with the fungal cell surface (Karkowska-Kuleta *et al*., 2015).

The quite high prevalence reported for *C. tropicalis* bloodstream infections in comparison with other non-albicans *Candida* species, high mortality rates in the range of 40–60% related with candidemia caused by these fungi (Leung *et al*., 2009; Chai *et al*., 2010), and their remarkable potential for dissemination within the host organism (Okawa *et al*., 2008; Negri *et al*., 2012; Arendrup, 2013) prompted us to pay attention to putative interactions between the cell surface of *C. tropicalis* and the proteinaceous components of the kinin-generating system (the contact system). This important proteolytic cascade activated in human plasma consists of high-molecular-mass kininogen (HK) – a precursor of bradykinin-related peptides, called kinins – and two serine proteinases, coagulation factor XII (FXII) and plasma kallikrein (HPK), and is involved in inflammatory responses during infection (Long *et al*., 2015). Kinins act as potent vasodilatory and proinflammatory mediators responsible for increased blood flow and vascular permeability (Colman & Schmaier, 1997). Therefore, these peptides are engaged in the host defense against infection by facilitating the influx of immune cells to the site of infection; however, on the other hand, this process is also related with the spread of pathogens within the host organism (Potempa & Pike, 2009). Moreover, systemic activation of the contact system and massive kinin production can lead to hypotension, vascular leakage, and subsequently systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) (Oehmcke & Herwald, 2010).

Recently, it has been shown that several proteins isolated from the cell wall of *C. albicans* hyphal forms strongly bind HK (Karkowska-Kuleta *et al*., 2011). The candidal hyphae or pseudohyphae are generally considered to be the most invasive forms of these fungal pathogens, capable of tight binding to host cells and proteins and involved in the damage of epithelial cells, constituting the first defense barrier of the host against the infection (Chai *et al*., 2010; Silva *et al*., 2012). In the light of evidence that *C. tropicalis* filamentous forms bound kininogen molecules to a much higher capacity than the yeast forms (Rapala-Kozik *et al*., 2008) and that proteins belonging to the contact system might gather at the cell surface of *C. tropicalis* pseudohyphae (Karkowska-Kuleta *et al*., 2010), our present study aimed to present a missing molecular characteristic of the binding of HK to the cell surface of *C. tropicalis* pseudohyphae. The specific aims included identification of the particular cell wall proteins that are involved in HK binding and kinetic and thermodynamic characterization of the interactions between this host's protein and the fungal proteins isolated from the fungal cell wall and purified.

MATERIALS AND METHODS

***C. tropicalis* strain and culturing conditions**. The *C. tropicalis* strain T1 (ATCC®MYA-3404™) was obtained from American Type Culture Collection (Manassas, VA, USA). Unicellular yeast forms were cultured to the stationary phase by incubation at 30°C for 16 hours in YPD medium (1% yeast extract, 2% soybean peptone, and 2% glucose) (Sigma, St. Louis, MO, USA). Then, the pseudohyphal forms were obtained by inoculation of yeasts into RPMI 1640 medium (PAA Laboratories GmbH, Pasching, Austria) and growth at 37°C. Filamentous forms were cultured for 24 hours for the further experiments on detection of kinin production, or for 72 hours in order to isolate proteins from the cell wall with β-1,3-glucanase.

Proteins. Human plasma HK, human plasma prekallikrein (pHPK), and active human coagulation factor XII (FXIIa) were purchased from Enzyme Research Laboratories (South Bend, IN, USA). β-1,3-glucanase was purchased from Sigma and β-1,6-glucanase from Takara Bio Inc. (Otsu, Shiga, Japan), trypsin was obtained from Sigma, Promega (Madison, WI, USA) and Biocentrum (Krakow, Poland), the horseradish peroxidase-conjugated streptavidin solution (SA-HRP) from MP Biomedicals (Solon, OH, USA), and bovine serum albumin (BSA) from BioShop Canada Inc (Burlington, Ontario, Canada). Labeling of HK with biotin was performed as described previously (Barbasz *et al*., 2008).

Kinin release from HK bound to the surface of *C. tropicalis* pseudohyphae. *C. tropicalis* pseudohyphae (obtained from 5×10^8 cells) were washed twice with phosphate buffered saline (PBS) (pH 7.4) and transferred to reaction tubes treated overnight with 1% BSA, resuspended in 100 μl of a HK solution (300 nM), incubated with gentle shaking for 2 hours at 37°C, washed twice with PBS, and transferred to new BSA-treated tubes. These HK-loaded cells were incubated with a mixture of FXIIa and pHPK (60 nM each) at 37°C for various periods, which was followed by stopping the reaction on ice for 15 minutes and collecting the supernatant by centrifugation at 4°C. The supernatant from above the HK-loaded fungal cells, which were only incubated with PBS, served as a control. Another approach involved incubating 5×10^9 fungal cells that were resuspended in 500 μl of 15 mM HEPES buffer (pH 7.4) with 135 mM NaCl, 50 μM $ZnCl_2$, and 500 μl human plasma (r2 Diagnostics, South Bend, IN, USA) at 37°C, which was followed by removing the fungal cells and protein precipitation with 1% trifluoroacetic acid (TFA; Sigma). This was followed by centrifuging and collecting the peptide-containing supernatant. The peptides were extracted using C-18 Sep Columns (Bachem, Torrance, CA, USA). This procedure consisted of the following steps: (i) washing the column with 1 ml of 60% acetonitrile (Merck, Darmstadt, Germany) with 1% TFA (buffer B) and 9 ml of 1% TFA (buffer A); (ii) injecting the sample with buffer A as the mobile phase; (iii) elution with 3 ml buffer B; and (iv) washing the column with 6 ml buffer B and additional 6 ml of buffer A. The collected fractions were evaporated in a vacuum concentrator (Martin Christ, Osterode am Harz, Germany). The amount of released kinin-like peptides was determined using an enzyme-linked immunosorbent assay (ELISA) and a dedicated kit (Bachem); the manufacturer's instructions were strictly followed.

Binding of biotinylated HK to the *C. tropicalis* pseudohyphal forms that were pretreated with β-1,3-glucanase, β-1,6-glucanase, heat, and trypsin. *C. tropicalis* pseudohyphal forms obtained from 5×10^8 yeast-like

cells were placed in Eppendorf tubes and incubated for 1 hour at 37°C with: (i) 100 U of β-1,3-glucanase and 40 mM β-mercapthoethanol in 100 µl of 50 mM Tris buffer (pH 7.5); (ii) 0.2 U of β-1,6-glucanase in 100 µl of McIlvaine buffer (pH 6.0), or (iii) 10 U of trypsin (Sigma) in 100 µl of 50 mM potassium phosphate buffer with 5 mM EDTA (pH 6.1). Additionally, a separate portion of cells (100 µl) was heated at 80°C for 20 minutes in PBS buffer and rapidly cooled according to the method of Bouchara and coworkers (1990). Then, the fungal cells were washed with 1 ml of PBS, centrifuged, mixed with 100 µl of a 50 nM solution of biotinylated HK (HK-Bt) in PBS, and incubated for 1.5 hours at 37°C. The amount of bound HK-Bt was determined as described previously (Rapala-Kozik *et al*., 2008) with the SA-HRP/TMB detection system.

Biotinylation and isolation of proteins associated with the *C. tropicalis* cell wall. Prelabeling of *C. tropicalis* cell wall-associated proteins with biotin was performed as previously described (Kozik *et al*., 2015) by incubation of 0.4 g (wet weight) of the *C. tropicalis* pseudohyphal forms in 100 mM bicarbonate buffer (pH 8.5) with biotin N-hydroxysuccinimide ester (NHS-D-biotin; Sigma) (1 mg in 50 µl dimethylformamide). The mixture of fungal cell wall-isolated proteins was obtained similarly as reported earlier (Karkowska-Kuleta *et al*., 2011; Kozik *et al*., 2015) with some minor modifications. Briefly, the pseudohyphal forms of *C. tropicalis* cells were incubated in 2 ml of 50 mM Tris-HCl buffer (pH 7.5) with the addition of 40 mM β-mercaptoethanol and 500 U of β-1,3-glucanase for 24 hours at 37°C. After centrifuging the remaining cells, the supernatant was dialyzed against PBS for 48 hours at 4°C. SYTOX® Green (Invitrogen Life Technologies, Carlsbad, CA) and Trypan Blue (Sigma) staining were used to verify that > 95% of cells remained viable. Unlabeled cell wall-associated proteins were isolated as described above, omitting incubation with NHS-biotin. The protein concentration was measured using the Bradford method (Bradford, 1976). These isolation procedures yielded about 2 mg of total protein from 1 g of fungal cell wet weight.

Identification of putative HK-binding proteins present in the mixture of *C. tropicalis* cell wall-extracted proteins. The procedure for affinity chromatography described previously, based on the use of HK that was covalently attached to Affi-Gel 10 (Bio-Rad, Hercules, CA, USA), was applied here (Karkowska-Kuleta *et al*., 2011). Briefly, a 50 µl portion of the affinity gel was incubated in PBS buffer for 4 hours at 37°C with gentle stirring along with 300 µg of proteins that were released from the cell wall of *C. tropicalis* using β-1,3-glucanase. After extensive washing out the unbound proteins with 1 ml of PBS, elution of HK-bound fungal proteins was performed by boiling the gel in 2% SDS for 20 minutes. The extracted proteins were separated using sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis and the Laemmli system with 10% separating gel and visualized with Coomassie Brilliant Blue R-250 staining. Then, particular protein bands (if, however, were not detected in the control samples eluted from agarose gel with blocked reactive ester groups but without immobilized HK) were identified by mass spectrometry with Method 1 (see below).

Purification of HK-binding proteins – enolase 1 (Eno1), phosphoglycerate mutase 1 (Gpm1), and predicted hyphally regulated cell wall protein (Hyr) – from the whole mixture of *C. tropicalis* cell wall-isolated proteins using ion-exchange chromatography. The mixture of proteins extracted from the cell wall of *C. tropicalis* pseudohyphae with β-1,3-glucanase was dialyzed against 20 mM Tris-HCl buffer pH 8.0, centrifuged for 15 minutes at 15 000 × *g*, and applied to a pre-equilibrated Resource ™ Q 1 ml column (GE Healthcare, Uppsala, Sweden). Protein separation was carried out with a Knauer (Berlin, Germany) chromatograph (equipped with an HPLC Pump K-1001 with a solvent organizer/proportioning valve K-1500, a UV detector 2600 with a control unit IF2, and EuroChrom software for pump control, data acquisition, and analysis); the elution was performed with a 20 ml linear gradient from 20 mM Tris-HCl buffer pH 8.0 without NaCl to the same buffer with 0.5 M NaCl, at a flow rate of 1 ml/min, with detection based on absorbance measurements at 280 nm, in accordance with the protocols described previously (Bras *et al*., 2012; Seweryn *et al*., 2015). The homogeneity of eluted proteins was confirmed with SDS-PAGE electrophoresis and identification thereof was carried out with mass spectrometry (Method 2) as described below.

Protein identification with mass spectrometry. Particular fungal proteins were identified after electrophoretic separation by SDS-PAGE in the Laemmli system and manual excision of selected bands from gel as described in detail elsewhere (Seweryn *et al*., 2015). Two methods based on liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) were used for the analysis of peptides obtained after protein digestion with trypsin. Method 1 was based on the use of an UltiMate 3000RSLCnano System (Dionex, Carlsbad, CA, USA), coupled to a microTOF-QII mass spectrometer (Bruker, Bremen, Germany), containing an Apollo Source ESI nano-sprayer equipped with a low-flow nebulizer, and was applied to identify fungal proteins obtained by affinity chromatography. Method 2 was carried out with an HCT Ultra ion-trap mass spectrometer equipped with an electrospray ionization ion source and an electron-transfer dissociation II fragmentation module (Bruker,Bremen, Germany) and coupled to an ultrahigh-performance liquid chromatography Dionex Ultimate3000 system. The conditions of chromatographic separation and parameters applied in order to search the obtained lists of peaks against the NCBI protein database with an in-house Mascot server (v.3.0, Matrix Science, London, UK) were described in detail previously (Kozik *et al*., 2015).

Binding of biotinylated HK to *C. tropicalis* pseudohyphal forms in the presence of purified Hyr, Eno1, and Gpm1. The binding assay was performed on *C. tropicalis* pseudohyphal forms (1×10^6) grown in RPMI 1640 medium for 3 hours at 37°C in the wells of MaxiSorp microtiter plates (Nunc, Roskilde, Denmark). The unoccupied well surface was blocked with 3% BSA in PBS and after each stage the cells were washed three times with 300 µl of PBS. Then, the solution of HK-Bt (2 pmoles per 40 µl) and solutions of unlabeled purified fungal protein, Hyr, Eno1, or Gpm1 (24 pmoles per 10 µl), all prepared in PBS, were added to the wells, gently mixed, and incubated for 1.5 hours at 37°C. The amount of bound HK-Bt was determined with the SA-HRP/TMB detection system as described previously (Karkowska-Kuleta *et al*., 2010).

Semi-quantitative analysis of binding of fluorescein-labeled fungal proteins to microplate-immobilized HK. Purified fluorescein-labeled *C. tropicalis* proteins – Eno1, Gpm1, and Hyr – were prepared by addition of 1 mg of NHS-fluorescein (Thermo Fisher Scientific, Waltham, MA, USA) dissolved in 100 µl of dimethyl sulfoxide to 100 µg of particular fungal protein dissolved in 200 µl of 0.1 M bicarbonate buffer, pH 8.3. Then the

mixture was incubated at 4°C for 4 hours and the excess reagent was removed by extensive dialysis against PBS at 4°C. HK (3 pmoles/50 µl of PBS per well) was immobilized in the wells of a MaxiSorp 96-well microplate (Sarstedt, Nümbrecht, Germany) by overnight incubation at 4°C. After each step, the wells were washed three times with 250 µl of PBS. The unoccupied surface in each well was blocked with 300 µl of 0.5% BSA in PBS for 3 hours at 37°C; then the solutions of the particular prelabeled fungal protein prepared in PBS (50 µl) at increasing concentrations were incubated in the wells for 1.5 hours at 37°C. After that, the fluorescence intensity was measured with the multi-mode microplate reader Synergy™ H1 (BioTek, Winooski, VT, USA). The signal for non-specific binding, obtained after addition of the mixture containing fluorescein-labeled protein with ten-fold molar excess of the particular corresponding unlabeled protein to microplate-immobilized HK, was subtracted from the total binding signal. A one-site binding model was fitted to the experimental data with the use of GraphPad Prism software.

Kinetic and thermodynamic analysis of binding of fungal proteins to HK using surface plasmon resonance (SPR) measurements. The characterization of interactions between HK and Eno1, Gpm1, or Hyr in kinetic and thermodynamic terms was performed with a BIACORE 3000 system (GE Healthcare). After dialysis against 10 mM HEPES buffer with 150 mM NaCl, Eno1, Gpm1, and Hyr were immobilized onto a CM5 sensor chip. The immobilization was carried out at 25ºC in 10 mM sodium acetate buffer, pH 4.5, at a flow rate of 10 µl/min for 7 minutes and was preceded by activation of the surface of the sensor chip by injecting 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 200 mM N-hydroxysuccinimide (NHS). The level of immobilization was *ca.* 600 resonance units (RU) for Hyr and Eno1, and 280 RU for Gpm1. 10 mM HEPES with 150 mM NaCl and 0.005% (w/v) surfactant P20, pH 7.4, was used as a running buffer. HK solutions at variable concentrations in the range of 40–1500 nM were pumped through the chip cell with a flow rate of 30 µl/min at 25°C and the association and dissociation time was 2 minutes. After that, the surface of the chip was regenerated by injecting 1 M NaCl for 30 seconds. BIAevaluation 4.1 software (GE Healthcare) was used to analyze the sensograms and calculate binding parameters – the dissociation and association rate constants (k_d and k_a) and the equilibrium dissociation constants (K_D) – on the basis of a global fit of the entire set of sensograms with a simple Langmuir model (1:1) with a drifting baseline.

Statistical analysis. The results presented in the bar graphs are reported as the mean ± standard deviation (SD) and the binding plots represent data fits with the nonlinear-regression one-site binding model (hyperbola), obtained with the use of GraphPad Prism software for data points reported as the mean ± standard deviation (SD). Data were analyzed using the Student's *t*-test. Statistical comparisons were performed using non-parametric ANOVA. Results were considered statistically significant at $p < 0.05$.

RESULTS

Biologically active kinin peptides were released from *C. tropicalis* cell wall-adsorbed HK in the presence of other proteins of the human plasma contact system

Kinin production from HK, which was pre-bound to the cell surface of the pseudohyphal forms of *C. tropicalis*, could be detected after 5 minutes of incubation with a mixture of pHPK and FXIIa (Fig. 1A). The time-dependent profiles of kinin release demonstrate a progressive increase over 1 hour. The maximal yield of kinin production corresponded to about 1% of the kininogen molecules added to the fungal cells (i.e., approximately 10% of kininogen that seems to be actually adsorbed to the fungal surface) (Rapala-Kozik *et al.*, 2008). Quick kinin release was also observed after contact of the fungal cells with human plasma (Fig. 1B). The peptides formed were progressively degraded, presumably by numerous peptidases (kininases) present in the plasma.

Major contribution of β-1,3-glucanase-extractable cell-wall proteins to HK binding by *C. tropicalis* pseudohyphae

C. tropicalis pseudohyphae were extracted with β-1,3-glucanase to obtain a cell wall fraction that was assumed to be a good source of native proteins for further identification and purification of HK-binding factors (see the following paragraphs). However, we first determined the suitability of this material for those purposes, in terms

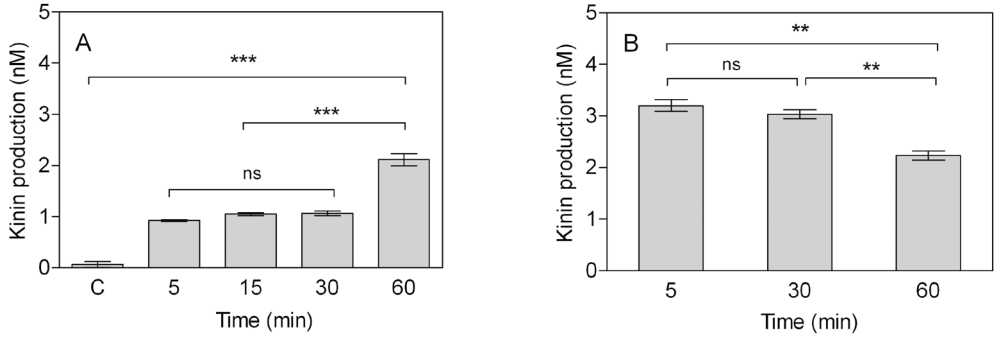

Figure 1. Release of kinin peptides from (A) HK bound at the *C. tropicalis* cell surface and (B) human plasma after contact with *C. tropicalis* cells.
Pseudohyphal forms of *C. tropicalis* were obtained after propagation in RPMI 1640 medium for 24 hours at 37°C and incubated at 37°C with (**A**) 300 nM HK for 2 hours, followed by incubation for the specified time with a mixture of pHPK and FXIIa (60 nM each) or (**B**) human plasma for the specified time. The amounts of generated kinins were determined using ELISA. The control ("C") sample in (A) represents HK-loaded cells that were incubated without any proteins for 60 minutes at 37°C. The amount of kinin peptides released in human plasma after incubation for 60 minutes at 37°C served as a control for data presented in (B) and was subtracted from the values obtained for each sample. Bars represent the mean values of 3 determinations ± standard deviation, with significant differences between consecutive samples indicated with $^{**}p < 0.01$, $^{***}p < 0.001$, or ns for "not significant".

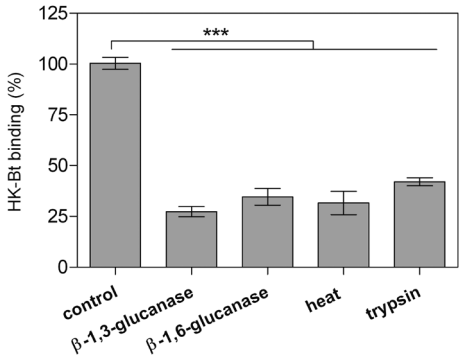

Figure 2. Binding of biotinylated HK to the *C. tropicalis* pseudohyphal forms that were pretreated with β-1,3-glucanase, β-1,6-glucanase, heat, and trypsin.
Fungal cells (5×10^8) were incubated in RPMI 1640 medium for 72 hours at 37°C to obtain the pseudohyphal forms. These were placed in Eppendorf tubes and treated for 1 hour at 37°C with β-1,3-glucanase (100 U in 100 µl total volume), β-1,6-glucanase (0.2 U per 100 µl), or trypsin (10 U per 100 µl) or heated at 80°C for 20 minutes. Then the cell suspensions (100 µl) were incubated with 50 nM HK-Bt for 1.5 hours at 37°C. The amount of bound HK-Bt was determined using the SA-HRP/TMB detection system. The level of HK-Bt binding to nontreated cells was considered 100%. Bars represent the mean values of 3 determinations ± standard deviation. Statistical significance levels against the control are marked with ***for $p<0.001$. The differences between pre-treated cells were statistically insignificant.

of a relative contribution to the total HK-binding capacity of *C. tropicalis* cells (Fig. 2). It was found that the β-1,3-glucanase-treated cells retained less than 30% of their original HK-binding ability. A comparable level of residual HK binding was determined in the pseudohyphae that were extracted with β-1,6-glucanase, which removes cell wall-associated proteins without destroying the β-1,3-glucan layer of the cell wall (Kapteyn *et al.*, 2000). Also, the treatments designed to destroy the proteinaceous cell wall components such as heating at 80°C for 20 min or extensive digestion with trypsin, decreased the HK-binding capacity by about 70%, i.e., to the similar level as that for β-1,3-glucanase extraction. Besides the quality control of β-1,3-glucanase-extracted fraction, these experiments suggested a predominant role of proteins in HK-binding by *C. tropicalis* pseudohyphae.

Candidate HK-binding proteins in the cell walls of *C. tropicalis*

To identify the particular HK binders in the whole mixture of proteins released from the cell wall using β-1,3-glucanase, affinity chromatography on HK-coupled agarose gels was used (Karkowska-Kuleta *et al.*, 2011). After washing out the unbound proteins, the tightly adsorbed proteins were eluted from the gel by boiling in the presence of SDS. The eluted proteins were then separated by SDS-PAGE, and the bands of interest were excised from the polyacrylamide gel. Only those bands were analyzed, which did not appear on the control gel prepared without the immobilized HK and whose reactive groups were blocked with ethanolamine. The particular proteins were identified using LC-MS/MS analysis. The database accession numbers, the abbreviated and full names of the proteins, their theoretical molecular masses, and the parameters of the MS/bioinformatics search such as the score, number of unique peptides, and the relative coverage of protein amino acid sequence by the matching peptides are presented in Table 1. Only

Table 1. Mass spectrometric identification of HK-binding *C. tropicalis* proteins isolated using affinity chromatography.
The results from a representative experiment are presented. After SDS-PAGE, particular protein bands were excised and digested with trypsin. Peptides were analyzed by LC-MS/MS using the UltiMate 3000RS LC nano System coupled with a MicroTOF-QII mass spectrometer. The obtained lists of peaks were used to search against the nonredundant SwissProt and NCBI protein databases with taxonomy restriction – Fungi.

Accession	Protein	Molecular mass (kDa)	Scores	Number of peptides	Sequence coverage (%)
gi|255722852	predicted protein CTRG_05838 (Hyr) (*Candida tropicalis* MYA-3404) Hyphally regulated cell wall protein, N-terminal fragment	194.8	115.0	2	3
gi|240134900	elongation factor 2 CTRG_01315 (Eft2) (*Candida tropicalis* MYA-3404)	91.8	177.2	4	4.7
gi|240133679	6-phosphogluconate dehydrogenase CTRG_03660 (Gnd1) (*Candida tropicalis* MYA-3404)	56.5	378.8	7	18.8
gi|240133182	enolase 1 CTRG_03163 (Eno1) (*Candida tropicalis* MYA-3404)	47.0	254.5	5	16.6
gi|240134564	phosphoglycerate kinase CTRG_02937 (Pgk1) (*Candida tropicalis* MYA-3404)	44.5	218.5	4	12.7
gi|255732698	fructose-1,6-bisphosphatase CTRG_05570 (Fbp1) (*Candida tropicalis* MYA-3404)	40.5	357.7	7	23.8
gi|240136434	transaldolase CTRG_00726 (Tal1) (*Candida tropicalis* MYA-3404)	35.5	244.9	5	24.8
gi|240134761	phosphoglycerate mutase 1 CTRG_01175 (Gpm1) (*Candida tropicalis* MYA-3404)	27.5	100.0	3	20.6

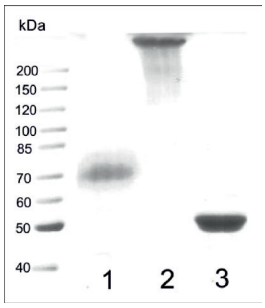

Figure 3. Electrophoretic characteristics of *C. tropicalis* purified cell wall-extracted proteins: Gpm1 (1), Hyr (2), and Eno1 (3).
Individual purified fungal proteins (3 μg each) were separated by SDS-PAGE under reducing conditions in the Laemmli system using 10% separating gel and then visualized with Coomassie Brilliant Blue R-250 staining. A pattern for molecular-mass marker mixture is shown in the leftmost lane.

proteins with a score >100 are listed. The eight major HK-binding proteins were identified in the proteinaceous mixtures obtained with the use of β-1,3-glucanase. These include both a typical cell wall protein covalently bound to the cell wall polysaccharide scaffold and atypical proteins, more loosely associated with the cell surface and known as "moonlighting proteins" (Karkowska-Kuleta & Kozik, 2014). In the latter group, elongation factor 2, 6-phosphogluconate dehydrogenase, Eno1, fructose-1,6-bisphosphatase, phosphoglycerate kinase, transaldolase, and Gpm1 were indicated as the major components that attract HK to the fungal cell surface. Importantly, a GPI-linked predicted protein CTRG_05838, which possesses an N-terminal fragment of the hyphally regulated cell wall protein (Hyr), was also identified on the cell surface of *C. tropicalis* as one of proteins responsible for HK adhesion. This protein belongs to the Iff adhesin-like protein family.

Purification of Eno1, Gpm1, and Hyr by ion exchange chromatography

Three *C. tropicalis* putative HK-binders were purified from the whole mixture of the surface-extracted proteins using ion exchange chromatography on a Resource™ Q column. They were selected for the purification attempts on the basis of the finding from affinity chromatography performed previously on HK immobilized in agarose beads. The two atypical ("moonlighting") *C. tropicalis* cell wall-associated proteins, Eno1 and Gpm1, were eluted with the linear gradient of NaCl into separate fractions, whereas Hyr was collected at the beginning of separation as unbound material eluted in the void volume. After confirming the purity of these proteins by SDS-PAGE (Fig. 3), their identity was confirmed with LC-MS/MS. Although the theoretical molecular mass of Gpm1 is reported as 27.5 kDa in Table 1, the electrophoretic separation confirmed its existence in the polymeric form (White *et al.*, 1993). As the quality and concentrations of Eno1, Gpm1, and Hyr were acceptable and sufficient, the protein preparations obtained were then used in the tests, directly demonstrating their HK binding ability.

Competition between *C. tropicalis* cell surface and purified soluble fungal proteins for HK binding

The involvement of Hyr, Eno1, and Gpm1 in binding of HK by *C. tropicalis* pseudohyphae was confirmed with a competitive assay based on the displacement of HK-Bt bound to the surface of *C. tropicalis* pseudohyphae by soluble purified Hyr, Eno1, and Gpm1 added at the

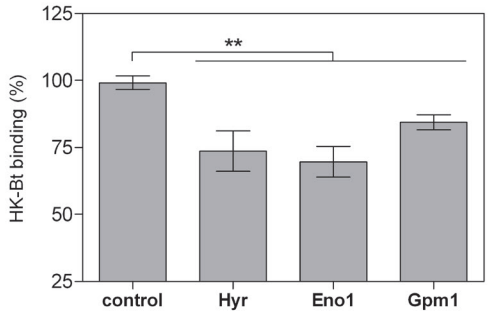

Figure 4. The displacement of HK-Bt binding from the cell surface of *C. tropicalis* pseudohyphal forms by Hyr, Eno1, and Gpm1.
The pseudohyphal forms of *C. tropicalis* (1×10^6) adsorbed in the wells of MaxiSorp microtiter plates were incubated with 50 μl of a solution containing 40 nM HK-Bt and 12-fold molar excess of Hyr, Eno1, or Gpm1. After washing-out the unbound protein, the amount of bound HK-Bt was detected with the SA-HRP/TMB system. Bars represent the mean values of 3 determinations ± standard deviation. Statistical significance levels against the control are marked with **for $p < 0.01$. The differences between the samples containing Hyr, Eno1, and GPM1 were statistically insignificant.

12-fold molar excess relative to HK-Bt (Fig. 4). The presence of each soluble fungal protein in the HK solution resulted in a decrease in the binding level by 20–30%, thus indicating their significant participation in HK adsorption to the whole fungal cells.

Testing the HK-binding to fluorescein-prelabeled *C. tropicalis* proteins with a microplate assay

The results obtained after affinity chromatography on the agarose-coupled HK suggested an important role of three *C. tropicalis* proteins – Eno1, Gpm1, and Hyr – in HK binding. This hypothesis was directly proven on the purified proteins using a simple semi-quantitative method. For this purpose, the microplate assay was used, in which fluorescein-prelabeled fungal proteins (at increasing concentrations) were bound by microplate-immobilized HK (Fig. 5). By measurements of fluorescence intensities and graphical analysis of the saturable plots obtained, the $K_{0.5}$ parameters (which can be considered

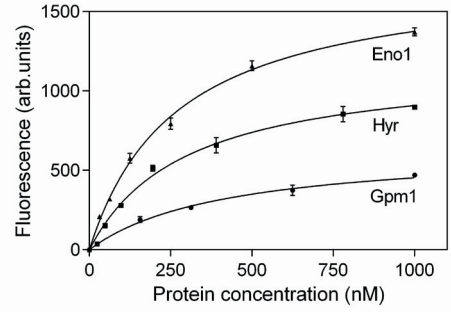

Figure 5. Binding of fluorescein-labeled *C. tropicalis* Eno1, Gpm1, and Hyr to microplate-immobilized HK.
Microplate wells coated with 1 pmol HK were filled with 50 μl of fluorescein-labeled fungal proteins at increasing concentrations in PBS. After washing out the unbound material, the fluorescence intensities of bound proteins were measured with the use of the microplate reader Synergy™ H1, with an excitation wavelength of 490 nm and an emission wavelength of 525 nm. The relative total binding of fungal proteins to microplate-immobilized HK is plotted after correction for "nonspecific" binding. Bars represent the mean values ± standard deviation (3 determinations). The solid lines represent data fits with the one-site binding model obtained with the use of GraphPad Prism software.

Table 2. Kinetic and thermodynamic parameters of HK binding by Eno1, Gpm1, and Hyr, determined by SPR measurements.
The binding parameters for interactions between fungal proteins (ligands) and HK (analyte) were determined after global fitting the data with a simple Langmuir model (1:1) with a drifting baseline.

Protein	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)
Eno1	$4.04\times10^{5}\pm2.02\times10^{4}$	$5.72\times10^{-2}\pm2.11\times10^{-4}$	$1.42\times10^{-7}\pm1.69\times10^{-9}$
Gpm1	$2.72\times10^{4}\pm1.21\times10^{3}$	$1.58\times10^{-2}\pm3.61\times10^{-4}$	$5.81\times10^{-7}\pm2.57\times10^{-9}$
Hyr	$3.93\times10^{5}\pm1.09\times10^{4}$	$8.66\times10^{-2}\pm5.27\times10^{-4}$	$2.20\times10^{-7}\pm3.42\times10^{-9}$

as a rough approximation of the dissociation constants, K_D) were estimated at 2.4×10^{-7} M for Eno1, 4.8×10^{-7} M for Gpm1, and 2.8×10^{-7} M for Hyr.

Kinetic and thermodynamic characteristics of the interactions between HK and *C. tropicalis* Eno1, Gpm1, and Hyr determined with surface plasmon resonance measurements

C. tropicalis Eno1, Gpm1, and Hyr were analyzed in terms of HK binding kinetics, using an arrangement in which human protein was injected over the sensor chip with an immobilized fungal protein. In the BIACORE 3000 system, the CM5 chip with carboxymethylated dextran was used, to which proteins were covalently coupled via amine groups. The sensograms shown in Fig. 6 as well as the kinetic and thermodynamic parameters of binding specified in Table 2 unequivocally confirm the ability to bind HK demonstrated by all the three purified fungal proteins. The equilibrium dissociation constants of the order of 10^{-7} M were determined for complexes of HK and Eno1, Gpm1, or Hyr.

DISCUSSION

The gathering of a variety of host proteins at the microbial cell surface is an important phenomenon that affects the virulence of many prokaryotic and eukaryotic pathogens (Tronchin *et al.*, 2008; Sardi Jde *et al.*, 2014) and is critical for initiating the contact between the host and pathogen, which involves adherence of the microorganism to the mucosal membranes and epithelial cells.

The binding and activation of proteins that constitute the contact system, one of the major proteolytic cascades involved not only in blood coagulation, but also in the initiation of inflammation (McKay, 1972), reportedly occurs at the surfaces of several species of pathogenic bacteria, including *Streptococcus pyogenes*, *Escherichia coli*, and *Porphyromonas gingivalis* (Ben Nasr *et al.*, 1995; Ben Nasr *et al.*, 1996; Rapala-Kozik *et al.*, 2011) and pathogenic *Candida* spp. yeast (Rapala-Kozik *et al.*, 2008; Karkowska-Kuleta *et al.*, 2010). Considering the latter genus, which contains the major fungal pathogens to humans, only the most widespread species *C. albicans* has been studied in terms of the molecular characteristics of the protein-protein contacts involved in this phenomenon (Karkowska-Kuleta *et al.*, 2011; Seweryn *et al.*, 2015).

The adsorptive concentration of HK on the pathogen's cell surface can play an important role in the development of infections. The production of bioactive kinin peptides from HK by host or microbial proteinases

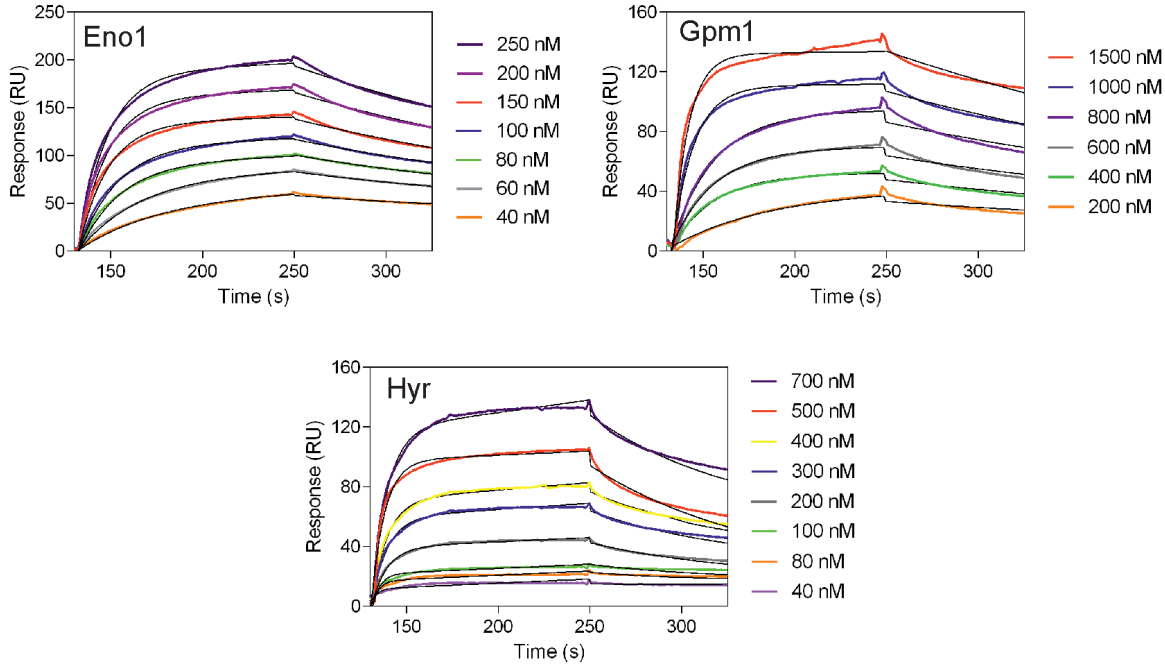

Figure 6. SPR analysis of HK binding to Eno1, Gpm1, and Hyr
The individual SPR sensograms for the interactions between HK and fungal protein represent the responses to HK solutions in the concentration range of 40–1500 nM that were injected at a flow rate of 30 µl/min for 2 minutes over the surface of the CM5 chip, which contained the immobilized *C. tropicalis* proteins. Black lines illustrate the global data fits with the Langmuir 1:1 binding model (with a drifting baseline).

contributes to the propagation of inflammation and the development of its typical symptoms, including pain, increased temperature, redness, enhanced vascular permeability, edema, and, consequently, the influx of immune cells to the site of infection (i.e., the mobilization of host defenses) (Calixto *et al*., 2001; Couture *et al*., 2001). However, these mechanisms, to some extent, might also be beneficial for invading bacteria and fungi by promoting the spread of pathogens within the host organism (Tapper & Herwald, 2000). As *C. tropicalis* is now regarded as a clinically very important and high-risk pathogen, particularly among immunocompromised patients in whom it causes serious bloodstream infections (Giri & Kindo, 2012), the key issue is to reveal the molecular mechanisms underlying the interactions between individual human plasma proteins and this opportunistic pathogen.

Here, we show that remarkable amounts of kinins can be formed by plasma kallikrein from HK that is preadsorbed on the cell surface of *C. tropicalis* (Fig. 1). The yield of kinin production was comparable to that determined in similar experiments on pathogenic bacteria (Persson *et al*., 2000; Mattsson *et al*., 2001; Ben Nasr *et al*., 1997; Rapala-Kozik *et al*., 2011). Nanomolar concentrations of kinin released from plasma (Fig. 1B), where HK occurs at a concentration of about 670 nM, exceeds the kinin levels under physiological conditions (i.e., 0.025 nM) by two orders of magnitude (Scicli *et al*., 1982; Bhoola *et al*., 1992). This observation strongly supports the pathophysiological and proinflammatory significance of strong HK adsorption, which is followed by further activation of contact system zymogens (i.e., FXII, pHPK) at the fungal cell surface.

Therefore, by taking into account these findings, this work aimed at identifying the HK-interacting components located in the cell walls of *C. tropicalis*. So far, there is only limited information about the organization of the *C. tropicalis* cell wall; however, some data concerning the proteins that are truly exposed at the cell surface have appeared recently (Lee *et al*., 2014; Karkowska-Kuleta *et al*., 2015). In this study, the mixture of *C. tropicalis* cell surface-associated proteins, which were released with β-1,3-glucanase, was assessed for individual HK-binding proteins using affinity adsorption on agarose-immobilized HK and LC-MS/MS analysis for the eluted fractions (Table 1). The features, localization, and significance of the individual proteins were mostly determined based on similarity to *C. albicans*, mainly because there are no *C. tropicalis* data from extended proteomic approaches, while the full genome of this species has only recently been sequenced (Butler *et al*., 2009). Of the typical adhesins covalently bound to the *C. tropicalis* cell wall, we assigned the HK-binding to one protein similar to members of the Iff/Hyr-like family (similarly to *C. albicans*, as reported by Karkowska-Kuleta *et al*., 2011). The other HK-binding proteins identified in the current study belong to the heterogeneous group of "moonlighting" proteins that are originally involved in evolutionarily conserved metabolic cycles. Elongation factor 2, 6-phosphogluconate dehydrogenase, Eno1, and Gpm1 were identified here as HK-binding proteins, and they had been previously classified as kininogen-binding proteins in *C. albicans* as well (Karkowska-Kuleta *et al*., 2011). Moreover, *C. albicans* enolase (Eno1) and phosphoglycerate mutase (Gpm1) were reported to possess plasminogen-binding activity, and Gpm1 was additionally suggested to be a receptor for complement factors H and FHL-1 (Jong *et al*., 2003; Poltermann *et al*., 2007). In *C. albicans*, both the abovementioned proteins, together with elongation factor 2, bind also two other components of the contact system, namely pHPK and FXII. This might suggest a shared role of these proteins in the assembly of the contact system proteins at the candidal cell surface, also for species other than *C. albicans*.

Since *C. tropicalis* proteins identified as interacting with agarose-coupled HK are considered here as putative HK-binding proteins, there was a need to explicitly confirm their interactions with this human protein. After effective purification of three fungal proteins Eno1, Gpm1, and Hyr to sufficient homogeneity and concentration, we first clearly showed by a competitive binding test (Fig. 4) that these proteins significantly contribute to HK binding to the cell surface of *C. parapsilosis* pseudohyphae. However, neither an individual protein nor all the three proteins together could account for the total HK-binding capacity of fungal cells, leaving a space for significant roles of the other proteins listed in Table 1. Then, the actual pairwise interactions of Eno1, Gpm1, and Hyr with HK were directly proven with a simple and quick fluorescent assay using microplate-immobilized HK. These two tests were followed by more sophisticated SPR measurements, in which kinetic and thermodynamic parameters of binding for the interacting fungal protein-human protein pairs were determined. For all the three investigated fungal proteins, the dissociation constants were in the range of 10^{-7} M, indicating a rather moderate strength of these interactions. Comparable values of this parameter were obtained for *C. albicans* proteins involved in HK binding (Seweryn *et al*., 2015). What is important, for the other two contact system proteins – pHPK and FXII – the binding strength expressed as K_D is also similar when investigating *C. albicans* Eno1 and Gpm1. This observation strongly suggests the important role of exactly these proteins in HK adsorption at the fungal cell surface, followed by contact system activation and generation of kinins. Moreover, in this work, the kinetic and thermodynamic binding parameters were determined for the complex of human protein with Hyr purified from *C. tropicalis*, therefore affirming the role of the latter as a fungal adhesin. This protein belongs to a large Iff/Hyr protein family, which in *C. tropicalis* contains putative typical cell wall proteins (Butler *et al*., 2009).

In conclusion, we described here the interactions between the cell surface of *C. tropicalis* and HK, an important human plasma protein. The identification of fungal proteins responsible for HK binding is important for further improvement of our knowledge of the mechanisms of candidal pathogenesis. These findings could also contribute in the future to the search for new therapeutic strategies (e.g., strategies that block mutual host-pathogen contact), an issue that is especially important in light of the progressively increasing resistance of *Candida* species to the commonly applied antifungal drugs (Tan *et al*., 2016). Therefore, there is an urgent need to accurately identify the molecular basis of the interactions between host defense mechanisms and the surface of non-albicans *Candida* species in order to introduce more effective methods for prevention and treatment of infections caused by these emerging dangerous pathogens.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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