Mass spectrometry as a useful tool for identifying new therapeutic targets on the cell surface of pathogenic fungi from the genus *Candida*

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Mass spectrometry (MS) is a universal technique with a wide range of applications, including proteomic studies of different organisms, particularly the characterization and sequencing of proteins isolated from specific cellular compartments. It is used for the identification of elements exposed on the cell surface of microbial pathogens, which are involved in the initial contact with the human host, and then in the further development of infection.

Given the increasing frequency of invasive fungal infections caused by pathogenic yeast from the genus *Candida*, especially among patients with severe immunological impairments, it appears advisable to study the diversity of cell wall proteins that arise during subsequent stages of infection and that are responsible for several important phenomena correlated with pathogenesis.

This study employed a liquid chromatograph-coupled mass spectrometer equipped with an electrospray ionization source (ESI), and an ion trap analyser. For tandem mass spectrometry, two approaches for fragmentation of ions - collision-induced dissociation (CID) and electron transfer dissociation (ETD) - were used to analyse the mixtures of peptides generated after tryptic digestion of fungal cell wall proteins (i.e. the "bottom-up" approach). Several surface proteins from *Candida* spp. were identified which could be potential drug targets and candidates for vaccine development.

Key words: proteomics, pathogenic fungi, candidiasis, mass spectrometry

Introduction

Currently, one of the most important problems facing medicine is the increased incidence of invasive fungal infections among immunocompromised patients, as a consequence of acquired drug resistance by pathogenic fungi and the shortage of effective prevention methods. At present, the dimorphic fungus *Candida albicans,* responsible for serious systemic infections, is still the most frequently isolated fungal pathogen in humans, albeit that the prevalence of infections caused the so-called "non-albicans" *Candida* species is showing a tendency to increase (Nawrot et al., 2013). Therefore, there is urgent need to find new approaches to control the growing incidence of candidiasis, with a particular priority being focused on the development of antifungal vaccines (Vecchiarelli, Pericolini, Gabrielli, and Pietrella, 2012).

As it is an interactive interface between the host and the pathogen, the cell wall from the fungal cell plays a significant role in the development of infections owing to its constant contact with the host. The candidial cell wall is a resistant, but easily adaptable and flexible structure, consisting of different types of proteins (often highly O- and N-mannosylated) and branched polymers of glucose residues containing β-1,3 and β-1,6 linkages (β-glucans), unbranched polymers of N-acetyl-D-glucosamine containing β-1,4 bonds (chitin) and lipids (Chaffin et al., 2008). Proteins are anchored in different ways to the elastic framework of microfibrillar polysaccharides (Pitarch, Nombela, and Gil, 2008).

If the proteomic approach is accompanied by other techniques, this enables the identification of proteinaceous components located within the fungal cell wall, which are responsible for the interactions with both host proteins and tissues and the immune response during infection. Currently, the proteomic methods used have been based on the chemical extraction of proteins from the cell wall with the use of SDS (sodium dodecyl sulfate) and DTT (dithiothreitol) or β-mercapthoethanol as denaturing and reducing agents, respectively, during incubation at 100°C. Other methods are based on HF-pyridine or mild alkali treatment (Castillo et al., 2008). However, it should be remembered that proteins obtained using above-mentioned procedures lose their tertiary and secondary structure and most probably also their function. Consequently, another approach was employed, comprising the direct tryptic digestion of proteins connected with the fungal cell surface and the subsequent analysis of the resultant peptides via mass spectrometry (MS) (Vialas et al., 2012).

In the present study, the enzymatically isolated native and functional candidial cell wall proteins (CWPs) were separated via protein gel electrophoresis, digested with trypsin and then the particular proteins were identified using LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry). The use of such an extraction method enabled the release of the cell surface proteins in a form suitable for simultaneous analysis in terms of interactions with various ligands. The bottom-up proteomic approach, which consists of both the generation of mixtures

of peptides after tryptic digestion of fungal proteins and their separation via ultra high performance liquid chromatography (UHPLC), was carried out with a Bruker Daltonics HCT Ultra ETD II mass spectrometer, equipped with an electrospray ionization source (ESI) and an ion trap analyser extended with an electron transfer dissociation/proton transfer reaction (ETD/PTR) module. Using this novel approach, several CWPs were identified from unicellular ("yeast-like") forms of *C. albicans*.

Materials and methods

Yeast strains and culture conditions

C. albicans strain 10231 was purchased from the American Type Culture Collection. It was precultured in Petri dishes with YPD agar (1% yeast extract, 2% tryptone, 2% glucose and 1.5% agar). Then, a single colony was transferred to liquid medium and unicellular yeast forms were grown at 30˚C for 16 h in YPD broth (1% yeast extract, 2% soybean peptone and 2% glucose) to a stationary phase, as confirmed by optical density at 600 nm. All reagents used were purchased from Sigma, St. Louis, MO, USA.

Isolation of C. *albicans* cell wall proteins

C. albicans cell wall proteins were isolated as previously described with slight modifications (Karkowska-Kuleta, Kozik, and Rapala-Kozik, 2010). Briefly, unicellular forms of *C. albicans* (3.8 g wet weight) were collected by centrifugation, then washed twice with 10 mM Tris-HCl buffer with 0.9% NaCl, pH 7.4 and twice with 50 mM Tris-HCl buffer, pH 7.5. To release the CWPs, cells were resuspended in 2 ml of 50 mM Tris-HCl buffer, pH 7.5, containing a 40 mM -mercaptoethanol, protease inhibitor cocktail (Roche, Basel, Switzerland) and incubated with 500 U (0.4 g) of -1,3-glucanase (Sigma, St. Louis, MO, USA) for 1 h at 37˚C. Then, the cells were centrifuged and the supernatant was collected and dialyzed against PBS, pH 7.4, for 48 h at 4˚C. SYTOX® Green (Invitrogen Life Technologies, Carlsbad, CA, USA) staining was used to verify that after this treatment at least 95% of *C. albicans* cells remained viable. The protein concentration was measured according to the Bradford method (Bradford, 1976). The isolated proteins were separated by SDS-PAGE in a Laemmli system (Laemmli, 1970) and visualized via Coomassie Brilliant Blue R-250 staining.

Protein identification via mass spectrometry

To identify the content of protein bands obtained after SDS-PAGE separation of isolated CWPs (see above), the gel pieces were manually excised, destained at 37˚C by sequential washing with water, 100 mM ammonium bicarbonate (NH_4HCO_3), 50% acetonitrile and 100% acetonitrile, and after that subjected to reduction with 10 mM DTT in 100 mM NH_4HCO_3 at 60˚C for 1 hour and alkylation with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 45 minutes at room temperature in the dark. Any excess of reagents was washed out with 100 mM NH_4HCO_3. Then, the gel pieces were dehydrated in 100% acetonitrile, dried in an Alpha 1-2 lyophilizer (Christ, Osterode, Germany) for 15 minutes and then dissolved in 15 µl of trypsin (Promega, Madison, WI, USA) solution (15 µl of 0.1 µg/µl in 50 mM NH_4HCO_3 with 5 mM $CaCl_2$) and incubated on ice for 45 minutes. Afterwards, additional 20 µl of 50 mM NH_4HCO_3 with 5 mM $CaCl_2$ were added. Digestion was carried out at 37˚C overnight. Peptides were extracted in a sonication bath with 50% acetonitrile and 2.5% formic acid at 37˚C. Then, the obtained peptides were analysed via mass spectrometry (MS) using a Dionex UltiMate 3000 UHPLC system (Dionex, Carlsbad, CA, USA) coupled with an HCT Ultra ETD II mass spectrometer (Bruker, Bremen, Germany). For this analysis, the peptides were separated on a 100 mm x 2.1 mm Accucore C18 column (particle size 2.6 µm) (Thermo Scientific, Carlsbad, CA, USA) using a 38 min gradient of 80% acetonitrile with 0.1% formic acid (1-45%). The mass spectrometer was operated in standard MS/MS mode with simultaneous fragmentation of most intensive precursor ions with CID and ETD. The resulting lists of peaks were used to search against the SwissProt protein database with a taxonomy restricted to Fungi, using an in-house Mascot server (v.3.0, Matrix Science, London, UK). The following search parameters were applied: enzyme specificity – trypsin, permitted number of missed cleavages – 1, fixed modification – carbamidomethylation (C), variable modifications – oxidation (M), protein mass – unrestricted, peptide mass tolerance – ±0.3 Da, and fragment mass tolerance – ±1.3 Da. Accepted proteins were those which had a Mascot score above 100. Assignment to the specific functional categories was made with the use of information included in the Candida Genome Database (CGD).

Results

Candida albicans yeasts in their unicellular forms were mildly treated for one hour with β-1,3-glucanase, which resulted in the release of a collection of proteins derived from cell walls. This enzyme allowed the hydrolysis of the glycosidic bonds in the β-1,3-glucan component of the fungal cell wall without prejudice to the proteins, while β-mercaptoethanol facilitated the release of proteins from the polysaccharide network, and the mixture of protease inhibitors protected proteins from proteolytic degradation. The staining with SYTOX® Green showed that almost all cells remained viable after this treatment, without any adverse permeability of cellular membranes. The concentration of proteins obtained from 3.8 g of wet cells was 800 µg/ml. Fig. 1 shows the scheme of the applied approach.

After electrophoretic separation of 16 µg of CWPs in the Laemmli system and staining with Coomasie Brilliant Blue, ten pieces of gel with different protein bands were ex-

cised and subjected to digestion with trypsin (A-J). The range of the molecular masses of the tested proteins was from ca.15 kDa up to 200 kDa, as shown in Fig. 2.

The two most frequently used methods in the proteomic approach are the "top-down" procedure, in which direct analysis of intact proteins is performed, and the "bottom-up" procedure, used in this work, which consists of an analysis of the separated mixture of peptides after tryptic digestion. Although one of the weaknesses of the second method is its relatively low sequence coverage, its high sensitivity in terms of peptide detection is its main advantage (Wu, Hühmer, Hao, and Karger, 2007). Prepared digested proteins were analysed using on-line LC-MS/MS treatment. Use of formic acid during chromatographic separation allowed the performance of positive, multiple ionization of distributed peptides in an electrospray ion source, which, thus, enabled the analysis of fragment spectra of the tested compounds in two different modes: CID and ETD, which can be simultaneously performed in the ion trap analyser. Conventional CID fragmentation enables collisions between precursor ions and helium atoms and prefers weak bond cleavages mostly generating b- and y-ions (peptide backbone cleaved between peptide bonds). This type of fragmentation is best suited for fragment spectra of the 2+ ions and the identification of peptides with a molecular mass up to 2.5 kDa. Thus, the analysis was greatly aided by the ETD module, which allowed for the identification of a number of peptides with a molecular mass larger than 3 kDa, as this type of fragmentation prefers charge stages above two. The main assumption of the fragmentation of peptides via ETD is the generation of an excess of radical anions from fluoranthene ($C_{16}H_{10}$) in a negative chemical ionization (nCI) mode. These accumulated anions are introduced to multiply charged peptide cations, previously isolated in the ion trap. In contrast to CID fragmentation, the ETD reaction strongly correlates to the charge state, but not to m/z ranges. The faster the reaction, the higher the charge multiplicity is. In consequence, this reaction generates large stretches of c- and z-type fragment ions (peptide backbones cleaved between a nitrogen atom and a carbon atom, but not at the peptide bond), which in combination with CID fragmentation results in an improved sequence alignment of proteins. It is noteworthy that as the molecular mass of the peptide be-

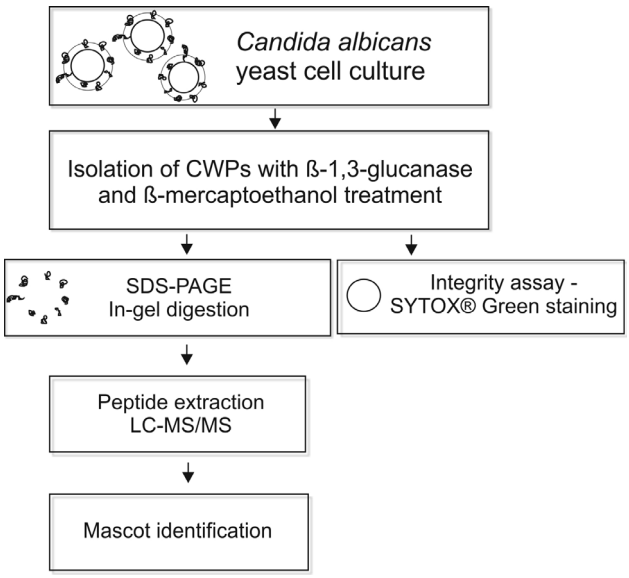

Fig. 1. The workflow of the procedure used for the identification of cell wall-connected proteins from C. *albicans* yeast cells

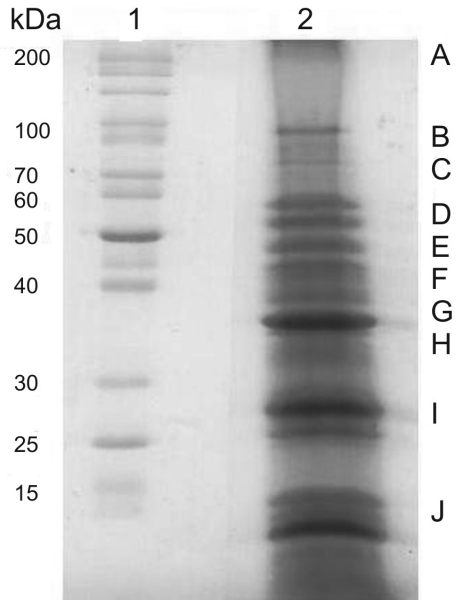

Fig. 2. The SDS-PAGE separation of a protein mixture obtained from *C. albicans* cell walls after treatment with β-1,3-glucanase. Proteins once extracted were separated on 12% gel of the Laemmli SDS-PAGE system and visualized by Coomassie Brilliant Blue staining. Excised bands were marked as A-J (Lane 2). Lane 1 presents the separation of molecular mass standards

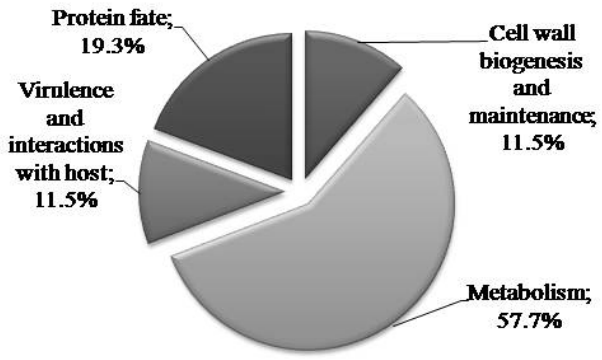

Fig. 3. The functional classification of identified cell wall proteins. The functions were assigned on the basis of information from the Candida Genome Database (CGD)

comes larger, the required charge state of the peptide for effective ETD fragmentation increases (Wu et al., 2007).

After efficient separation of peptides, 24 proteins were identified with Mascot scores higher than 100 and an additional two with lower scores, but with a high sequence coverage or a high number of identified peptides. Table 1 presents a list of identified proteins. The listed proteins possess different functions, starting from cell wall biogenesis and organization, metabolism and cell rescue, up to virulence and interactions with the host. Some are equipped

Table 1. Proteins from the cell wall of *C. albicans* yeast cells, identified via LC-MS/MS. After SDS-PAGE, particular protein bands (A-J, as marked in fig. 2) were excised and analysed with a Dionex UltiMate 3000 UHPLC coupled with a Bruker HCT Ultra ETD II mass spectrometer, and identified by searching the SwissProt protein database through a Mascot server.
MM – molecular mass. SC – sequence coverage. The functional classification was based on CGD: M – metabolism, CW – cell wall biogenesis and maintenance, V – virulence and interactions with the host, P – protein fate.

Band/ protein role	Accesion	Gene name	Protein	MM	pI	Scores	SC (%)	# peptides
A								
V	ALS2_CANAL	ALS2	Agglutinin-like protein 2	188.4	4.2	132.22	4.8	2
CW	CHI2_CANAL	CHT2	Chitinase 2	61.6	4.5	79.28	24.7	1
B								
M	METE_CANAL	MET6	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	85.8	5.3	1802.6	73	35
M	APE2_CANAL	APE2	Aminopeptidase 2	104.9	5.1	1232.78	56.1	30
V	PLB1_CANAL	PLB1	Lysophospholipase 1	66.9	4.5	224.65	14.2	3
CW	PHR2_CANAL	PHR2	pH-responsive protein 2	59.5	4.3	87.83	20.8	3
C								
M	METE_CANAL	MET6	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	85.8	5.3	1602.74	73.5	34
M	APE2_CANAL	APE2	Aminopeptidase 2	104.9	5.1	659.23	48.8	18
V	PLB1_CANAL	PLB1	Lysophospholipase 1	66.9	4.5	325.88	30.2	8
V	ALS2_CANAL	ALS2	Agglutinin-like protein 2	188.4	4.2	124.44	5.8	3
D								
M	G6PI_CANAL	PGI1	Glucose-6-phosphate isomerase	61.6	6.0	1529.92	55.5	24
M	METE_CANAL	MET6	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	85.8	5.3	794.54	28.8	17
M	DUG1_CANAL	DUG1	Cys-Gly metallodipeptidase DUG1	53.8	4.9	763.65	39.2	13
M	HXKB_CANAL	HXK2	Hexokinase-2	53.8	5.3	288.71	20.5	8
M	FIMB_YEAST	SAC6	Fimbrin (by homology)	71.9	5.3	228	5	4
M	EIF3B_CANAL	PRT1	Eukaryotic translation initiation factor 3 subunit B	84.4	6.0	104.39	10.4	4
E								
M	HXKB_CANAL	HXK2	Hexokinase-2	53.8	5.3	1657.64	63.2	20
M	METE_CANAL	MET6	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	85.8	5.3	343.07	12.5	8
M	TPS1_CANAL	TPS1	Alpha.alpha-trehalose-phosphate synthase [UDP-forming]	54.7	5.8	207.99	17.6	6
F								
M	ENO1_CANAL	ENO1	Enolase 1	47.2	5.4	986.45	55	14
P	EF2_CANAL	EFT2	Elongation factor 2	93.9	6.0	848.15	30	20
M	SAHH_CANAL	SAH1	Adenosylhomocysteinase	49.7	5.3	496.76	25.3	8
M	TKT1_CANAX	TKT1	Transketolase 1	73.8	5.5	329.79	18.6	6
P	HSP75_CANAW	SSB1	Heat shock protein SSB1	66.6	5.3	132	6	2
G								
P	HSP71_CANAL	SSA1	Heat shock protein SSA1	70.5	4.9	1234.63	40.1	21
M	ENO1_CANAL	ENO1	Enolase 1	47.2	5.4	1213.70	55	15
P	HSP72_CANAL	SSA2	Heat shock protein SSA2	70.2	4.8	1144.94	36.9	19
H								
M	ALF_CANAL	FBA1	Fructose-bisphosphate aldolase	39.4	5.7	1053.19	67.1	13
M	GRP2_CANAL	GRP2	Putative NADPH-dependent methylglyoxalreductase	37.6	6.0	679.31	73	16
M	METE_CANAL	MET6	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	85.8	5.3	334.46	18.3	6
M	IPYR_CANAL	IPP1	Inorganic pyrophosphatase	32.2	5.0	322.69	53.5	9
M	ENO1_CANAL	ENO1	Enolase 1	47.2	5.4	319.98	20.7	6

I								
M	ENO1_CANAL	ENO1	Enolase 1	47.2	5.4	1630.2	54.8	17
P	EF2_CANAL	EFT2	Elongation factor 2	93.9	6.0	901.60	20.4	13
M	EBP1_CANAX	EBP1	Probable NADPH dehydrogenase	46.1	6.0	357.44	19.9	6
M	G6PI_CANAL	PGI1	Glucose-6-phosphate isomerase	61.1	6.0	339.31	18.4	6
CW	BGL2_CANAX	BGL2	Glucan 1,3-beta-glucosidase	33.7	4.4	317.77	23.4	5
J								
M	PMGY_CANAL	GPM1	Phosphoglycerate mutase	27.2	5.8	1046.77	64.1	16
M	ENO1_CANAL	ENO1	Enolase 1	47.2	5.4	1040.89	40.9	11
M	TPIS_CANAL	TPI1	Triosephosphate isomerase	26.9	5.7	701.58	63.7	10
M	TKT1_CANAX	TKT1	Transketolase 1	73.8	5.5	454.48	14.8	6
M	HXKB_CANAL	HXK2	Hexokinase-2	53.8	5.3	346.53	17.8	6

with the signal peptide responsible for participation in the classical pathway of secretion through the endoplasmic reticulum and Golgi apparatus, and some have the glycosylphosphatidylinositol (GPI) attached during post-translational modification, which anchors the protein to the cell membrane (Chaffin, 2008). Example of proteins which possess both a signal peptide and a GPI anchor include Cht2p, an important enzyme for the remodelling of chitin (McCreath, Specht, and Robbins, 1995), and glycosidase Phr2p. Both these proteins are involved in fungal cell wall biosynthesis and maintenance. Another identified protein with a similar function is 1,3-beta-glucosyltransferase Bgl2p; however, this protein has the signal peptide, but is devoid of the GPI anchor. The typical fungal adhesin Als2p, which is a GPI-linked protein (De Groot, Hellingwerf, and Klis, 2003), plays a significant role in adhesion and biofilm formation (Hoyer, 2001); therefore, it is significantly involved in fungal virulence. The last, but not least identified virulence factor was Plb1p, an enzyme secreted from the hyphal tip during the infection of host tissues and involved in host cell penetration (Ghannoum, 2000).

The most abundantly represented group contained proteins responsible for cellular metabolism, often with the expected cytoplasmic localization and evolutionarily conserved functions in terms of glycolysis and gluconeogenesis or other metabolic cycles (Fig. 3). Nevertheless, they are also frequently localized on the cell surface with quite different assigned functions. Therefore, this group of proteins is often called "moonlighting proteins" (Chaffin, 2008). Their secretion occurs through still unrecognized export pathways (Nombela, Gil, and Chaffin, 2006). It should be emphasized that within this group many proteins possessing antigenic functions have been observed, e.g., Eno1p, Met6p, Pgi1p, Hxk2p, Sah1p, Tkt1p, Fba1p, Grp2p, Ipp2p, Gpm1p, and Tpi1p (Fernández-Arenas et al., 2004; Pardo et al., 2000; Pitarch et al., 2004).

The second largest identified group contained proteins involved in protein synthesis and folding. In addition to translation and elongation factors, this group also comprised the heat shock proteins from the Hsp70 family: Ssb1p, Ssa1p, and Ssa2p. The latter is present at the cell surface as well as at the outer surface of the plasma membrane, extending through the cell wall and, occasionally, appearing to reach the cell surface via channels (López-Ribot, Alloush, Masten, and Chaffin, 1996).

Finally, the two last excised bands (I, J) contained products of the proteolytic degradation of larger identified proteins. Proteolytic processing can also be proposed as an explanation of the presence of proteins with a theoretically larger molecular mass in bands of smaller apparent molecular mass, although it is conceivable that some proteins might be composed of subunits. On the other hand, some proteins of lower molecular mass can be identified in higher molecular mass bands, possibly owing to the formation of poorly dissociable aggregates.

Discussion

Among currently used antifungal drugs, four main groups can be distinguished, based on their mechanism of action and specific target localization within the fungal cell. Polyene and azole antifungals target ergosterol located in the cell membrane, echinocandins inhibit the synthesis of glucan in the cell wall and 5-fluorocytosine inhibits the synthesis of nucleic acids in the nucleus (Sanglard & White, 2006). At the moment, no commonly used drug is directed against proteins exposed at the fungal cell surface, which often play a substantial role in interactions with the host by spreading pathogens within an organism and developing infection. The most recent suggestions for new proteinaceous targets in the candidial cell wall have emerged from studies on the effects of potential drugs on the expression and exposition at the hyphal surface of a few important proteins responsible for biofilm formation and adhesion to the host. These mainly include Hwp1p (hyphal cell wall protein) and Als3p proteins. For instance, it has been shown that phorbasin H, a diterpene acid of a bisabolane-related skeletal class isolated from the marine sponge *Phorbas* sp., is able to decrease hypha-specific *HWP1* and *ALS3* mRNAs (Lee et al., 2013). Furthermore, strategies based on either suppressing the expression of these proteins via the activity of cis-2-dodecenoic acid (BDSF, a diffusible signal factor from *Burkholderia cenocepacia*) and trans-2-dodecenoic acid (trans-BDSF) (Zhang et al., 2011) or the down-regulation of *ALS3* genes in response to the addition of EFG1-specific 19-nucleotide siRNA (Efg1p is the main positive regulating transcription factor) (Moazeni et al.,

2012) offer a promising approach to prevent fungal adhesion and further development of infections. In contrast, there is a lack of corresponding data in relation to Als2p, another protein from the agglutinin-like sequence protein family identified in this work at the cell surface of unicellular forms of *C. albicans* and engaged in interactions with human epithelial and endothelial cells (Sandovsky-Losica, Chauhan, Calde-rone and Segal, 2006; Zhao, Oh, Yeater and Hoyer, 2005). Therefore, further investigations and search for substances capable of interfering with Als2p functions might be a promising way to counter disseminated candidiasis, since unicellular forms of yeasts are responsible for the spread of pathogens within a host organism.

Discovering safe, fast and effective methods for the prevention of fungal infections, especially in the field of vaccinations, is another problem. Advanced technologies, such as the LC-MS/MS technique with proteomic tools, may be useful instruments in the search for new potential targets of immunization. Nowadays, a few major proteinaceous candidates for vaccines are under consideration, e.g., secreted aspartic proteinase 2 (Sap2p), Als3p, Hsp90, Eno1p and Tpi1p (Vecchiarelli et al., 2012; Fernández-Arenas et al., 2004). However, continued identification of new proteins exposed at the fungal cell surface is the key challenge in the development of novel methods for the treatment of candidiasis. Preclinical studies in mice and on-going clinical trials have established that Als3 (the main antigen in the NDV-3 investigational vaccine) can induce a protective immune response against *C. albicans*. As has been mentioned, several proteins identified in our study, including Eno1p, Met6p, Pgi1p, Hxk2p, Sah1p, and Fba1p, show antigenic functions, so they might act as potential vaccination targets to be tested in the future. A quite promising candidate seems to be enolase, identified at the surface of *C. albicans* single cells, which could potentially be used as a construct for an oral vaccine against candidiasis (Montagnoli et al., 2004; Shibasaki et al., 2013).

A number of proteomic studies have already been conducted for the above-mentioned purposes, although with the use of different extraction methods than the one used in this work, in which native and functional proteins are obtained, enabling their further functional analysis. The total number of proteins extracted from fungal cell walls reported by different groups varies greatly depending not only on the protein isolation method employed, but also on the method and type of mass spectrometer used to analyse peptides obtained after tryptic digestion. One previously used approach comprised the direct digestion of exposed fungal proteins during a short treatment of intact cells with trypsin, followed by peptide separation and identification with the use of nano-LC, off-line MS/MS and MALDI TOF/TOF instruments (Hernáez et al., 2010; Vialás et al., 2012). Vialás et al. identified a total of 131 proteins among all tested conditions and different morphological forms: 38 proteins specific only to yeast cell forms, 26 specific proteins for hyphae forms and three specific proteins for biofilm. In these groups of proteins, some proteins were also found in our study, e.g., Cht2p, Bgl2p, Eno1p, Gpm1p and Met6p, which definitely confirms their presence in the cell wall. Interestingly, among the proteins identified by Vialás et al., there was a large group, including ribosomal proteins, involved in the synthesis of other proteins. However, their role on the cell surface is so far unclear. Another approach involves the preparation of cell walls by breaking the whole hyphal cells or unicellular yeast forms with glass beads, followed by extraction of cytoplasmic contaminants and tryptic digestion of the proteins remaining within the cell wall and analysis with an ApexQ FT ion cyclotron resonance mass spectrometer equipped with a 7T magnet and a CombiSource coupled to an Ultimate 3000 HPLC system (Heilman et al., 2011). This assay allowed for the identification of 21 GPI-linked proteins, including Cht2p and Als2p. In this study, adhesin Als2p was also identified at the surface of yeast cells. Given that adhesins are significant in terms of the *C. albicans* virulence mechanisms, identification of their localization and function appears to be essential for the discussion and design of their further use in the treatment of candidiasis. It is worth pointing out that many proteins which were originally assigned to the group responsible for metabolism ("moonlighting proteins") and non-covalently attached to the cell wall are also involved in the adherence to host proteins and cells and particular ligands are identified for them. A good example of these proteins is enolase, which is abundant at the surface of many types of prokaryotic and eukaryotic cells and is responsible for plasminogen and kininogen binding (Jong et al., 2003; Seweryn, Pietkiewicz, Szamborska, and Gamian, 2007; Karkowska-Kuleta et al., 2011). Moreover, another protein we identified, Fba1p, was indicated in a screening for putative cell wall proteins that bind to human plasminogen (Crowe et al., 2003). In addition, Gpm1p is described as a cell surface protein that binds host complement Factor H, FHL-1, plasminogen and kininogen (Poltermann et al., 2007; Karkowska-Kuleta et al., 2011). The ability possessed by pathogens to hijack and activate important proteolytic cascades responsible for homeostasis in a host organism, i.e. the fibrinolysis system and the contact (kinin-forming) system, and to affect this directly through their secreted proteinases or other surface-connected components involved in the adhesion to different host proteins can facilitate invasion and promote disease. Thus, our results shed some light on the important problem of the identification of native and functional proteinaceous components of cell walls derived from unicellular morphological forms of *Candida* spp., participating in the first stages of infection and invasion. This study is also part of a current trend in the search for and identification of the individual constituents of the cell wall in order to help create a complete picture of this important compartment of fungal cells.

In conclusion, we have established a strategy to study the cell wall proteome of *Candida albicans* yeast cells, but this approach could be successfully applied to other, "non-albicans" *Candida* species and different morphological forms, e.g. hyphae and pseudohyphae. A major advantage and novelty of this method is that it offers a possibility to perform further studies on the relationships between particular cell wall proteins and their functions through simultaneous analyses with the use of other techniques. After fractionation of cell wall proteins with different chromatographic techniques, particular sets of identified proteins originating from the same sample and from the same cell culture might be useful for testing its ability to interact with a variety of host proteins, i.e. kininogen or extracellular matrix proteins. For these purposes different experimental methods can be employed, e.g. enzyme-linked ligand sorbent assays (ELLSA), ligand blotting or label-free interaction analyses via surface plasmon resonance measurement systems. This enables a major current challenge to be tackled, i.e. the discovery of new targets for the development of antifungal drugs and effective vaccines.

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