Published in final edited form as:

Electrophoresis. 2000 March ; 21(5): 956–961. doi:10.1002/(SICI)1522-2683(20000301)21:5<956::AID-ELPS956>3.0.CO;2-D.

Cell surface proteins of *Candida albicans*: Preparation of extracts and improved detection of proteins

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Abstract

We have reexamined the detection of the components in a β -mercaptoethanol and ammonium carbonate buffer extract of surface proteins of *Candida albicans* and the effects of postextraction manipulation of the extract on recovery of extract components. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), preferential staining of some moieties was observed when bands detected by a commercial silver staining method or a Coomassie Brilliant Blue (CBB) staining method were compared. Additional protein bands that were either not detected or poorly detected by a single method alone were readily observed by a combined silver-CBB staining method. This method also detected alterations in the profile of extracted proteins from organisms grown in the presence of galactose or hemoglobin rather than glucose. Two-dimensional electrophoresis (2-DE) gel analysis by double stain showed better detection of several acidic and basic protein spots. Less than 10% of the extract as determined by a dye-binding assay was lost following either or both lyophilization and dialysis. These manipulations of the extract did not change the protein profile following SDS-PAGE as determined by the combined staining or Western blot analysis of a 70 kDa protein. These observations suggest that soluble cell wall proteins are not unusually sensitive to procedures routinely used in protein purification. In addition, these studies suggest that a modified staining method that combines both silver stain and CBB stain provides improved detection of cell wall proteins compared to either method alone.

Keywords

Candida albicans; Cell wall protein; β -mercaptoethanol extract; Protein gel staining; Two-dimensional polyacrylamide gel electrophoresis; Double staining

1 Introduction

Candida albicans is an opportunistic fungal pathogen that, strictly speaking, is a polymorphic or pleomorphic organism, although the term dimorphic is commonly accepted. The cell wall, which is outside the plasma membrane, is responsible for determining the shape of the organism (for a recent review see [1] and references therein). This cell wall is

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primarily composed of carbohydrate as represented by three polysaccharides. Microfibrillar glucan and chitin polymers represent structural components of the wall. The third polysaccharide, mannan, is covalently associated with proteins. The cell wall proteins, which may also contribute to structure, have been the focus of many studies because of their role as adhesins. To study these proteins it is necessary to extract, separate, and detect the components. Earlier reports from this laboratory have shown that several cell surface proteins are extracted from *C. albicans* by treatment of intact cells with ammonium carbonate buffer containing β -mercaptoethanol (β ME) [2]. Preparation of this extract for subsequent analysis generally requires manipulations such as lyophilization and dialysis. A recent study reported that dialysis for 2 h of cell wall extracts obtained by partial glucan digestion resulted in a 40–60% loss of proteins, while overnight dialysis further increased the loss [3]. We have reexamined whether lyophilization and dialysis, alone or in combination, affect the recovery of protein and the profile of soluble (*i.e.*, without glucan hydrolysis) extracted proteins. We have increased the sensitivity of the analysis by examining methods of detecting proteins in SDS-PAGE and in 2-DE of the extracts.

The separation of complex proteins by high resolution 2-DE and the detection of protein spots are crucial methods in proteomics. A single method of protein gel staining may not yield sufficient protein detection, particularly when a complex protein is involved. CBB and silver stains are the most widely used staining techniques to detect proteins in polyacrylamide gels [4]. Several modifications have been developed to increase the sensitivity, selective detection, and better staining of different types of proteins and lipids in the same gel [5–7]. We have used silver and CBB staining alone and in combination in order to maximize detection of components.

2 Materials and methods

2.1 Organism and culture growth

C. albicans strains NCPF 3153 and ATCC 44807 were used in this study. Strain NCPF 3153 yeasts cells were grown in $4 \times$ yeast nitrogen base (YNB with amino acids; Difco Laboratories, Detroit, MI) plus 0.3 M galactose for 24 h at 37°C in a gyratory incubator shaker at 150 rpm. ATCC strain 44807 yeast cells were grown in YNB plus 0.3 M galactose, 0.1 M glucose, or 0.1% w/v human hemoglobin (Sigma, St. Louis, MO) plus 0.1 M glucose for 24 h at 23–25°C with shaking.

2.2 Preparation of β ME extracts

Yeast cells were collected by centrifugation and washed twice with sterile distilled water. β ME extract was prepared as described [2]. Briefly, washed cells were resuspended in ammonium carbonate (1.89 g/L) and 1% v/v β ME (1/10th of the culture volume) and were incubated at 37°C for 30 min. After centrifugation the extract was filtered (0.2 μ M filter) and divided into two equal portions. One portion was dialyzed against 5 mM Tris-HCl, pH 7.4, for 24 h at 4°C by using dialysis tubing (molecular weight cutoff 6000–8000; Spectrum; Gardena, CA). The other portion of the undialyzed β ME extract was stored at 4°C until further use. Half of the dialyzed or undialyzed extract was lyophilized to complete dryness.

Lyophilized samples were resuspended in sterile distilled water. Protein concentration was determined according to Bradford [8] by using γ -globulin as a reference.

2.3 SDS-PAGE and immunoblotting

SDS-PAGE (discontinuous) [9] was performed in the mini-gel format on 12.5% w/v separating and 4% w/v stacking slab gel at a constant voltage of 65 V. Three or more gels were prepared for simultaneous electrophoresis. Each lane contained 20 μ g of protein of an extract preparation. After electrophoresis, two gels were stained by silver as recommended by the manufacturer using the silver-stain kit from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). The third gel was stained with 0.1% w/v Coomassie Brilliant Blue R-250 (CBB; Sigma) dissolved in 40% v/v methanol, 7% v/v acetic acid for 1 h to overnight. Gels were destained in the same solution without dye. Double staining was performed by counterstaining the silver-stained gel with 0.1% w/v CBB as described above. Results presented here are representative of three individual experiments. Electrophoretic transfer of proteins to nitrocellulose paper and immunoblotting were performed by standard methods using wet transfer as described previously [10]. The membrane was blocked with 3% skimmed milk in TBS for 2 h and washed in TBS + 0.05% Tween-20 (TBST). The membrane was incubated with an IgG_{2a}-class mouse monoclonal antibody that recognizes 70 kDa heat shock protein (hsp) or heat shock constitutive (Hsc) protein (Stressgen Biotechnologies, Victoria, BC, Canada) used at 1:500 dilution. A peroxidase-conjugated goat anti-mouse IgG (1:1000 dilution; Boehringer Mannheim Biochemicals, Indianapolis, IN) was used as a secondary antibody.

2.4 2-DE

2-DE was performed basically as described by O'Farrell [11]. Protein samples (100 μ g in 5 μ L) were added to 45 μ L sample buffer and incubated at 37°C for 1 h before loading at the cathodic end of the tube gel (2 mm diameter, 110 mm long). The sample buffer contained 9 M urea, 0.2% CHAPS, 0.1 M DTT, 0.16% Bio-Lyte 3/10 carrier ampholyte, and 0.64% Bio-Lyte 5/7 carrier ampholyte (Bio-Rad). The first-dimensional gel (9 M urea, 4% acrylamide, 2% CHAPS, 0.4% Bio-Lyte 3/10 carrier ampholyte, 1.6% Bio-Lyte 5/7 carrier ampholyte) was prefocused at 15°C (200 V for 15 min, 300 V for 15 min, and 500 V for 30 min). The first-dimensional gel was electrophoresed at 15°C with sample for 16 h at 500 V and for an additional 1 h at 1000 V. The pH gradient of the focused tube gel was determined by incubating the gel pieces (1 cm long) in 1 mL distilled water overnight and measuring the pH of the solution. After focusing, the sample gel was removed from the tube and incubated in the equilibration buffer (Tris-HCl, 0.067 M, pH 6.8; 10% glycerol, 2.3% SDS) for 7–10 min. The first-dimensional gel was placed at the top of a 12.5% SDS-PAGE slab second-dimensional gel (1.5 mm thick) and electrophoresed. Pre-stained SDS-PAGE marker (Bio-Rad) proteins mixed in agarose gel pieces were coelectrophoresed until the blue dye reached the bottom. At least three gels were prepared and analyzed simultaneously. Gels were stained as described above. Protein spots were enumerated by placing each 2-DE gel on a white light box and marking each spot on a clear plastic film placed on top of the gel. Determinations were made on three gels. The observations were analyzed by analysis of variance (ANOVA) followed by Bonferroni Multiple Comparison Test with $p < 0.05$.

3 Results

3.1 Postextraction manipulation

β ME extracts of cell wall protein were prepared for analysis by dialysis, lyophilization, or a combination of both treatments. A small loss of protein, 10% or less, was associated with postextraction manipulation (Table 1). Each preparation was separated by SDS-PAGE. Multiple gels were prepared and each gel was stained either by CBB, silver, or by a double stain protocol (Fig. 1). Comparison of the staining protocols showed that sharp delineated bands were present in all samples throughout the molecular weight range. Generally, the high molecular weight moieties were more readily distinguished in the CBB-stained gel (Fig. 1C) and the low molecular weight species in the silver-stained gel (Fig. 1A). The combined silver and CBB stain showed improved detection of protein bands as both the high and low molecular weight species were clearly visible (Fig. 1B). Further, detection of some bands appeared improved compared to either single stain. Comparison of the protein profile of the untreated preparation (lane 4) with those of the treated preparations (lanes 1–3) showed that the treatment did not alter the protein profile. To increase the sensitivity to detect degradation, a single representative mid-size protein, an hsp70, was analyzed by immunoblotting. Immunoreactivity of hsp70 was not affected by dialysis, lyophilization, or the combination of both treatments as shown by sharp bands in all four samples (Fig. 2).

3.2 Growth condition

Growth of *C. albicans* on galactose rather than glucose or in the presence of hemoglobin is reported to alter the profile of cell wall proteins [12, 13]. To determine the ability to detect alterations in the protein profile, soluble cell wall proteins isolated from cells grown under these conditions were analyzed as described above. As shown in Fig. 3, differences in the profile of these preparations (lanes 2 and 3) were detected by all the stains used when compared to the profile of proteins from glucose-grown cells (lane 1). However, the double stain revealed some additional bands (Fig. 3B, asterisks) in the extract from galactose-grown cells that were not detected with silver alone (compare lane 2 of B to that of A).

3.3 2-DE

To assess the utility of double staining in 2-DE gels, we examined detection of the moieties in the extract from organisms grown on galactose separated by 2-DE and stained by each method. When the spots in each gel were enumerated, silver stain showed a slightly increased number of protein spots (140 ± 4.5) compared to CBB stain (129 ± 5.5), although the difference was not significant. Combined silver and CBB stain almost doubled the number of detectable spots (245 ± 4.5) compared to either single stain ($p < 0.001$). Some of the protein spots stained by silver were not stained by CBB (Fig. 4A, solid arrows). During the development process of the silver staining protocol, many acidic proteins stained faster than other proteins due not only to the relative abundance of these proteins but, perhaps, also to the nature of these proteins. This observation was further evidenced by CBB-stained gel where many of those proteins were unstained (compare the proteins marked by arrows in Fig. 4A to Fig. 4B). Many of the proteins between 30–206 kDa, particularly acidic proteins, were not readily detected by silver. However, CBB stains many of them (Fig. 4B, open arrows; compare to Fig. 4A). There were several other abundant proteins that were poorly

stained (hazy) by silver. Double staining increases the detection of total proteins in 2-DE gels (Fig. 4C). Some proteins that were weakly stained by silver or CBB alone were intensively stained with the combined protocol and appeared as blue (weakly stained by CBB) or bluish-orange (weakly stained by silver) spots. Although not photographically reproduced, even less abundant protein spots can be detected when double stained gels were viewed under white light background. A few additional protein spots that were not at all or only poorly detected by silver or CBB staining protocol were clearly observed by double stain (Fig. 4C, asterisks). This improved staining pattern agrees with our SDS-PAGE (I-D) observations.

4 Discussion

There is a growing body of experimental evidence indicating that the properties of cell wall proteins and glycoproteins such as expression, distribution, and biochemical characteristics depend on multiple factors [1]. These include the growth condition (*e.g.*, medium), and organism-related properties such as growth state, morphology, and strain. Cell wall proteins have been of particular interest in that they contain components that elicit a host immune response and that mediate adherence to the host. To investigate these proteins it is necessary to have reliable methods of extraction, analysis, and in some cases material for purification of these proteins. A number of extraction procedures have been used to remove proteins from isolated cell walls and intact cells. We have previously demonstrated the utility of extraction of nonglucan-bound proteins (soluble proteins) from intact cells by treatment with an alkaline ammonium carbonate buffer with β ME [2]. A variety of methods have been used to demonstrate that proteins present in the extract are *bona fide* cell wall proteins and that cytoplasmic proteins are not extracted during the procedure (reviewed in [1]). These methods include biotinylation of intact cells with membrane impermeable agents prior to extraction, analysis of extracts from mutant strains with cell wall and cytoplasmic markers, indirect immunofluorescence detection of antibody binding to specific proteins on whole cells, and immunoelectron microscopy of intact cells using protein-specific antibodies.

Recently, there was a report that the postextraction manipulations that are generally used to prepare cell wall extracts for analysis or purification of components resulted in substantial loss (40–60%) of material in a 2 h dialysis, with greater losses with longer periods [3]. In addition, degradation of components was noted. In our report, we have demonstrated that a small but generally acceptable protein loss of 10% (Table 1) is associated with removing extraction buffer components (24 h dialysis) and concentrating proteins. Further, the analysis of the protein profile by general gel staining (Fig. 1) or more specifically by immunoblot of a specific protein (Fig. 2) did not show evidence of degradation associated with broadening or fragmentation of the reactive band. These procedures differ from the previous report [3] in that only nonglucan-linked proteins were included in the extract and thus no glucanases were added to obtain the extract.

We have demonstrated that the analysis of protein profiles and detection of bands is improved by using a combination of silver staining following by CBB (Figs. 1, 3, 4). Using a combined double stain, increased sensitivity of protein staining has been reported for model proteins such as BSA, lysozyme, insulin, and erythrocyte membrane proteins [5, 6].

Staining SDS-PAGE-separated cell wall proteins, first with CBB, followed by silver, did not show improved bands (data not shown). In 2-DE, we showed preferential staining of acidic proteins by silver. It appears that there is a relation between SDS-PAGE (1-DE) and 2-DE in the pattern of staining proteins by silver or CBB. While similar numbers of proteins were detected by single staining in 2-DE, there were some differences in identity of the stained proteins. By combining silver and CBB staining methods the number of detected proteins was increased (Fig. 4C). This increase appeared to be derived from two sources. First, clear staining of both acidic and basic proteins was detected in the same gel. Secondly, we showed intensive staining of several spots poorly stained by each method alone. The mechanism for improved staining is not clear. The combined silver and CBB staining of the yeast cell wall protein offered two advantages: (i) detection of proteins that would be missed with a single method, and (ii) enhanced detection of some poorly stained proteins. Improved detection of proteins should prove useful, as we continue to define surface protein components of the *C. albicans* and to investigate how their identity and abundance is influenced by various parameters including strain and environment.

Acknowledgments

This work was supported in part by a Pfizer, Inc., Medical Education Grant and Public Health Service Grant AI23416 from the National Institutes of Health to WLC.

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Abbreviations

hsp	heat shock protein
βME extract	ammonium carbonate-β-mercaptoethanol buffer extract

Figure 1. SDS-PAGE analysis and gel staining of cell wall proteins of *C. albicans* (NCPF 3153). Several gels were run in parallel and each stained by a different protocol: (A) silver stain, (C) CBB, or (B) combined staining with silver followed by CBB. The extracts were prepared as described in Section 2.2. Lane (1) undialyzed and lyophilized; (2) dialyzed and lyophilized; (3) dialyzed and unlyophilized; (4) undialyzed and unlyophilized. The vertical bar (C) indicates bands poorly stained or unstained with CBB compared to (A) silver and (B) double stains. (A), (C) Arrowheads indicate weakly stained bands with a single stain. (B) Arrows indicate intensively stained bands by double stain.

Figure 2. Protein integrity monitored by immunoblot. SDS-PAGE-separated proteins from *C. albicans* (NCPF 3153) were transferred to nitrocellulose and hsp70 detected as described in Section 2.3. The extracts and lanes are the same as in Fig. 1.

Figure 3.

Application of improved staining to detect changes in cell wall protein profile. Proteins were extracted from *C. albicans* ATCC 44807 as described in Section 2.2. The extracts were dialyzed, separated by SDS-PAGE, and stained by (A) silver, (B) double stain, and (C) CBB. Asterisks indicate improved detection of bands. A 55 kDa band reported to be increased in extracts from cells grown with hemoglobin [12] is indicated by open triangle. Lane (1) extract from cells grown with glucose; (2) extract from cells grown with galactose; (3) extract from cells grown with glucose plus 0.1% hemoglobin. Broad-range SDS-PAGE standards (Bio-Rad) were (M, top to bottom, in kDa) myosin (200), β -galactosidase (116), phosphorylase *b* (97.4), BSA (66.2), ovalbumin (45), carbonic anhydrase (31), and trypsin inhibitor (21.5).

Figure 4.

Improved staining of acidic and basic proteins after 2-DE by combined double stain. Proteins extracted from *C. albicans* NCPF 3153 was used. Gels were prepared and processed in parallel and were stained by (A) silver, (B) CBB, or (C) double stain. Prestained SDS-PAGE standards (M; Bio-Rad), from top, are (in kDa) myosin (206), β -galactosidase (117), BSA (79), ovalbumin (48.3), carbonic anhydrase (34.7), soybean trypsin inhibitor (29.3), lysozyme (21.3) and aprotinin (7.6), on the right side of the second-dimensional gel. (C) The pH of the first-dimensional gel run in parallel is indicated at the bottom. (A) Solid arrows indicate proteins stained by silver but not by CBB. (B) Open arrows indicate proteins detected by CBB but not by silver. (C) Asterisks indicate additional proteins detected by double stain. Note that many other spots are stained intensively compared to silver-or CBB-stained gels.

Table 1
Protein recovery following postextraction manipulations^{a)}

Extract	Treatment		Protein loss (%)
	Lyophilization	Dialysis (24 h)	
1	–	–	–
2	+	–	7.5
3	–	+	7.5
4	+	+	10

^{a)} β ME extracts of cell wall protein were prepared for analysis by dialysis, lyophilization, or both treatments. Results are the mean recovery determined for two experiments.