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Isolation and Preparation for Sequencing of Hydrophobic *Candida albicans* Cell Wall Proteins by In-line Transfer from Continuous Elution Preparative Gel Electrophoresis to PVDF Membranes

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Introduction

A set of hydrophobic wall proteins from the opportunistic fungal pathogen *Candida albicans* has been identified.[1] Previous studies suggested that these hydrophobic proteins affect *C. albicans* adherence and virulence.[2–4] However, the low abundance of these proteins has meant that isolation of sufficient material for biochemical and sequence analysis has been problematic and a significant rate-limiting step in investigating the role of hydrophobic proteins in *C. albicans* pathogenesis. In addition, their hydrophobic nature results in adsorption of these proteins to equipment surfaces during purification. To minimize adsorption, it is necessary to limit the exposure times of native protein to as many surfaces as possible, such as tubing, dialysis membranes, and spin concentrator membranes. This means avoidance of many classical techniques, particularly those that involve sample dilution and require subsequent sample concentration. We have combined two advances in protein isolation and handling in a system which minimizes purification steps and the number of surfaces to which native proteins are exposed, and provides sufficient material for sequencing.

First, recent developments in mass spectrometry methods allow sequence determination from picomole quantities of protein immobilized to membranes such as polyvinylidene difluoride (PVDF).[5] Transfer of protein to such membranes is advantageous since it provides an inherent concentration step and an easy means for salt and detergent removal with minimal protein loss.[6] The obtained protein sequence can then serve as a basis for the synthesis of oligonucleotides to be used as probes in molecular cloning techniques. Expression of cloned protein would produce greater amounts than can now be achieved from wall digestion.

Second, the development of continuous elution preparative electrophoresis provided a useful means for protein isolation with minimal protein loss. Proteins can be isolated to homogeneity, in a single step in some cases, while minimizing exposure to surfaces. Typically, fractions containing proteins purified by preparative electrophoresis are subsequently run on slab form SDS-PAGE gels for transfer to PVDF or nitrocellulose membranes. We have modified this approach so that fractions from preparative electrophoresis runs can be collected directly to PVDF membranes in a dot blot apparatus. We have used this system to successfully isolate a 40 kDa hydrophobic protein from the *Candida albicans* cell wall in sufficient quantity for sequencing by tandem mass spectrometry.

Methods

Sample Preparation

A diagram of the overall preparation procedure is shown in Figure 1. Yeast cell walls were subjected to limited enzymatic digestion as described by Glee, Sundstrom, and Hazen (RCD).[2] Washed cells were suspended at a concentration of 1×10^9 cells/ml in 0.05 M sodium phosphate buffer (pH 7.5) containing protease inhibitors (1 mM EDTA, 0.2 mM PMSF, 1 µM leupeptin, 1 µM pepstatin A). β-1,3-glucanase (Lyticase, Sigma) was added to the suspension to a concentration of 250 U/ml. Digestion was carried out at 37 °C with occasional shaking until the concentration of released protein reached approximately 300 mg/ml. Protein release was monitored by a Coomassie® blue dye-binding assay. This method of digestion minimizes cytoplasmic contaminants based on the lack of ghost cells present at the end of the digestion period.

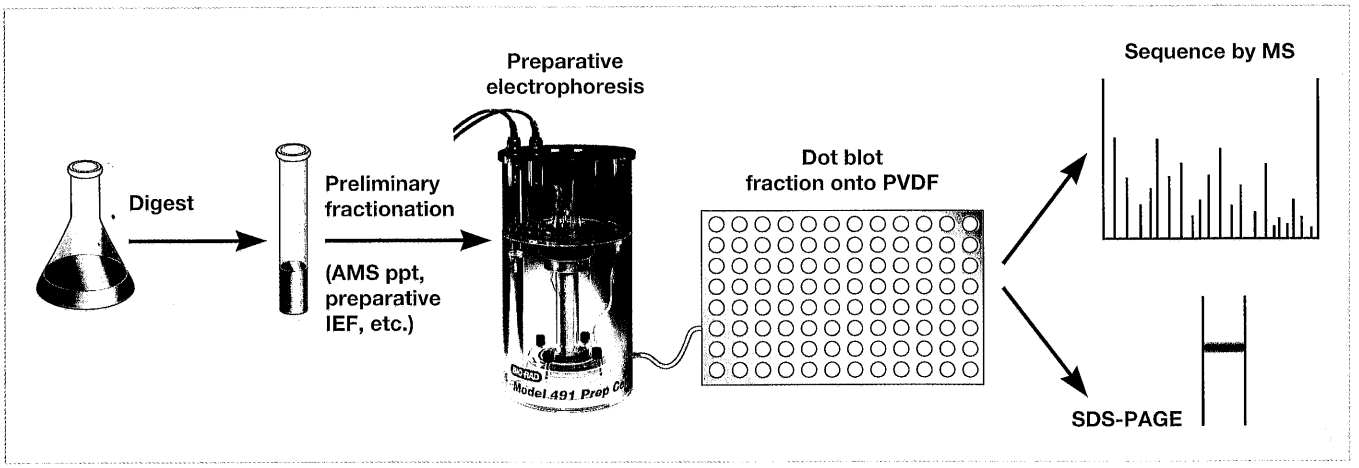

Fig. 1. Diagram of the protein isolation strategy presented here.

Proteins in the digest were first separated by ammonium sulfate (AMS) precipitation. The hydrophobic nature of the proteins, particularly in the 10% AMS cut containing the 40 kDa protein, made it difficult to solubilize the pellet in an aqueous solution without a detergent present. Because of this, an accurate determination of protein concentration was difficult. As a result, the entire pellet was solubilized in 0.5 ml of SDS-PAGE sample buffer, heated at 95 °C for 5 minutes, and loaded onto the preparative electrophoresis gel.

Preparative Electrophoresis

The Mini Prep Cell was chosen for continuous elution preparative electrophoresis because a smaller sample size could be used. The apparatus was assembled according to instructions. Electrophoresis was carried out using the discontinuous buffer system of Laemmli.[8] In optimization experiments using BSA, an 8% (30.0:0.8 acrylamide:bis-acrylamide, w/w) resolving gel was poured and allowed to polymerize overnight. For isolation of the *Candida albicans* wall protein described here, a 10% acrylamide resolving gel was used. In both cases the height of the resolving gel was 5 cm. A 1 ml stacking gel (4%) was layered over the resolving gel and allowed to polymerize. The running and collection conditions were according to the manufacturer's specifications.

Fraction Collection and Sampling

A dot blot apparatus (Bio-Dot®, Bio-Rad), containing PVDF membrane, was assembled and the wells washed three times with Dulbecco's Phosphate Buffered Saline (DPBS). After the final wash, 30 ml of DPBS were added to each well to keep the membrane wet during fraction collection. The assembled dot blot apparatus was then clamped to a fraction collector (Bio-Rad, Model 2128), which is able to deposit fractions into three 96-well plates (Figure 2). The top rack was removed and the tube support shelf raised to its highest position. Styrofoam blocks and elastic bands were used to hold the dot blot apparatus in place so that the wells of the apparatus correspond to those of the middle 96-well plate. The fraction collector was programmed to collect fractions in wells 97–192.

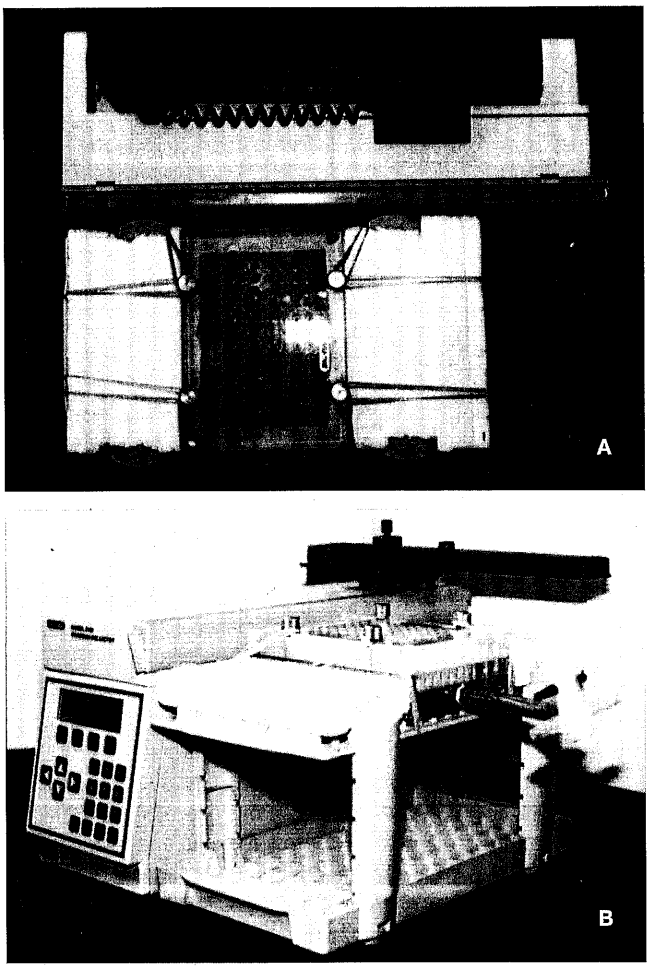

Fig. 2. Fraction collector showing attached dot blot apparatus

An 80 µl sample was drawn from each well and mixed with 40 µl of 3x electrophoresis sample buffer. When all the fractions had been collected, the remaining volume in the wells was passed through the membrane by gravity. The wells were washed three times, by gravity, with DPBS, and air-dried. The dots containing the protein of interest were identified by SDS-PAGE of the well samples.

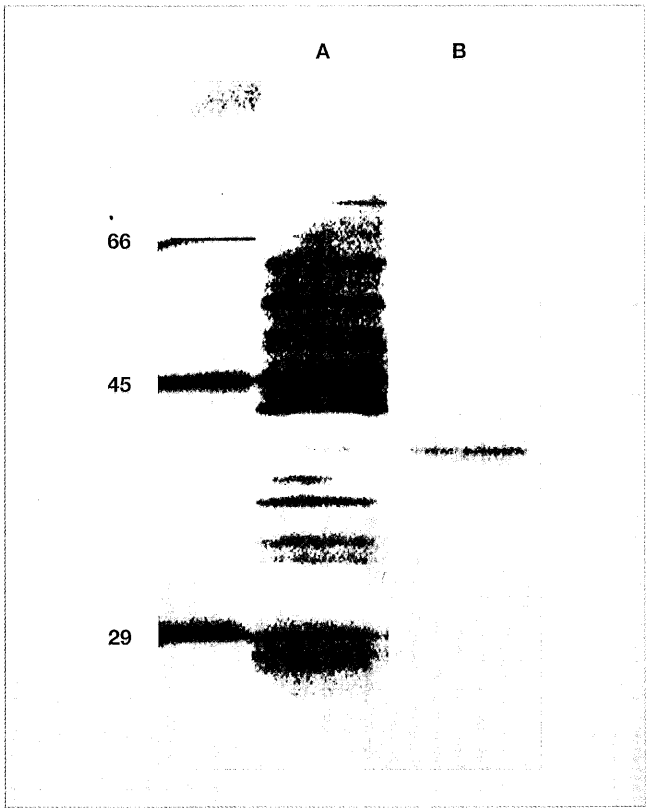

Fig. 3. SDS-PAGE gel of cell wall digest (lane A) and purified 40 kD protein (Lane B). Molecular weights of the standards (in kD) are given to the left.

Results

The operating parameters of the system, specifically protein recovery and required protein load, were determined using bovine serum albumin (BSA) as a test protein. With this system, a protein recovery of greater than 90% was achieved. Using slab SDS-PAGE to identify the wells containing the protein of interest, however, represents the limiting factor for the method. Preparative electrophoresis runs were carried out using decreasing amounts of bovine serum albumin. The results of these runs indicated that 20 µg of the protein of interest must be loaded onto the preparative electrophoresis gel in order for the protein in the well samples to be visibly stained using a silver stain,[9] 25 µg to be visibly stained by Coomassie brilliant blue.

Electrophoresis of the 10% AMS protein fraction was carried out as for the BSA samples. Fractions were collected and well samples taken as described above. The fraction containing the isolated protein was identified following SDS-PAGE of the well samples (Figure 3). The corresponding dot was cut from the membrane and submitted for sequencing by electrospray triple quadrupole tandem mass spectrometry.[10] The material from the dot has yielded sequenceable peptides, and the sequence of the peptides was used as a basis for the creation of degenerate oligonucleotide sequences to be used as probes for cloning (in preparation).

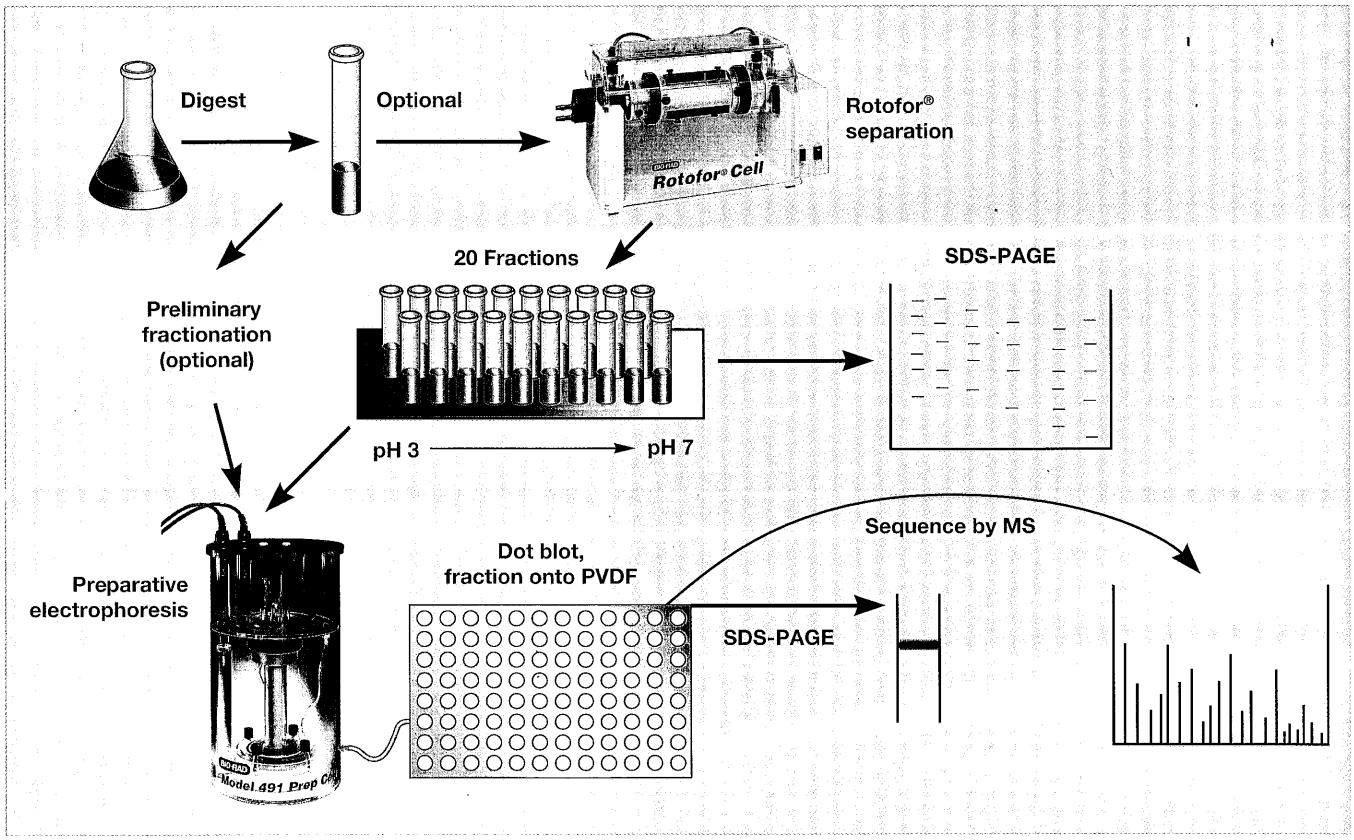

Fig. 4. Alternative strategy, using preparative IEF.

Conclusion

One benefit of this system, beyond minimizing protein exposure to surfaces, is the overall decreased time from digestion to sequenceable protein, regardless of whether or not the protein of interest is hydrophobic. The time frame for isolation of the 40 kD protein shown here is 12–15 hours. Another benefit is that the system has flexibility. If desired, samples can be delivered to tubes rather than the dot blot apparatus without altering any other part of the setup. Another alternative collection strategy is to transfer the well samples to a second dot blot apparatus and probe this second dot blot with antibody, if one is available. Finally, other separation strategies can be substituted for the 10% AMS cut. For example, we have isolated a 38 kDa protein from *Candida* cell walls using preparative isoelectric focusing (Rotofor, Bio-Rad) as the first separation (Figure 4).[11]

Acknowledgements

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