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A METHOD FOR EFFICIENT EXTRACTION OF CELL WALL PROTEINS FROM *CANDIDA ALBICANS*

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ABSTRACT

Candida cell wall proteomics is a challenging area of research. Extraction of cell wall proteins needs to be done carefully to obtain reproducible results. We used two different methods for extraction of candida cell wall proteins and documented the modifications required. In the course of extraction, we also evaluated efficacy of cell wall disruption by mechanical procedure of homogenization with glass beads to increase the protein yield and purity. For extraction of candida cell wall proteins, we utilized detergents like Sodium dodecyl sulphate (SDS), dithiothreitol (DTT), tris base and chitinase. This method was further optimized using homogenization of candida cells with glass beads over different time intervals. Amount of cell disruption at each time interval of bead beating using two varieties of beads was evaluated by gram stain, culture, protein estimation and SDS PAGE subsequently.

We observed that method using detergents and Chitinase yielded better quality proteins, less contaminated with salts. For homogenization, bead beating with glass beads of 0.5 mm diameter over 18 minutes was found to be superior. Our initial experiments revealed limits of both the methods in candida cell wall protein extraction. Mechanical disruption of the cells could be further optimized using routinely available vortex mixer to increase final protein yield and gave consistent and reproducible results.

Keywords: *Candida albicans*, Cell wall proteins, glass bead homogenization, SDS PAGE.

INTRODUCTION

Candida albicans is a polymorphic fungus, causing opportunistic infection in immunocompromised hosts. Generally, this yeast occurs as a commensal in the oral cavity, urogenital tract etc. of human beings[(1)]. The role of commensal flora leading to opportunistic infections needs careful investigations to understand their role of cell wall proteins in pathogenesis.

The cell wall proteins of candida and their receptors on mucosa play vital role in pathogenesis [(2)]. *C. albicans* cell wall proteins are being extensively studied to evaluate their role in variety of processes; adhesion to host cells, for antigenic studies, anti-mannan antibody preparation etc. [(3,4)]. As said in Pitarch et al (2002), Cell wall protein studies have been done using intact cells or after cell wall protein extraction using detergents or chaotropic agents, or by using, secretary products of developing cells from the culture filtrates [(5,10)]. In few studies along with detergents; enzymes like glucanases were used to extract cell wall proteins of candida [(6)]. In the present study we used one of the commonly used extraction method proposed by Pitarch et al, 2002[(6)] to obtain cell wall proteins with few modifications. The present study evaluate the method of extraction of cell wall proteins of *C.*

albicans described by Pitarch et al (2002) and the improvements made to the original procedure.

OBJECTIVES

The following objectives were pursued in the study:

1. To evaluate two different methods of candida cell wall protein extraction.
2. To standardize the glass bead homogenization technique.

MATERIALS AND METHODS

C. albicans growth conditions:

The study was conducted at SDM College of Medical Sciences & Hospital, Dharwad and Department of Chemical Engineering, National Institute of Technology Karnataka, Suratkal from August 2011 to November 2011.

C. albicans was grown in yeast nitrogen broth for 30 hrs at 35 ^{o}C in shaking incubator (Rotek, BOD Cooling Incubator Shaker ROSI-1) at 150 rpm, to limit the growth to stationary phase. The culture was centrifuged and candida cells were washed 3 times with distilled water in a cold centrifuge (Eppendorf India Centrifuge 5415 R) at 14,000 rpm at 4 ^{o}C. The washed cells were processed for cell wall protein extraction method by Pitarch et al. 2002 [(6)].

Cell Wall Protein Extraction Method by Pitarch et al. (2002) [(6)]:

Cells were washed initially with lysis buffer (10 mM Tris HCl, pH 7.4, containing 1 mM PMSF) 5 times and homogenized with glass beads for 10 minutes. The lysate was washed again with distilled water and centrifuged. The pellet was successively washed in 5%, 2% and 1% NaCl and then with cold distilled water 5 times each and then boiled in extraction buffer (50 mM Tris HCl, pH 8.0, 0.1 M EDTA, 2% SDS, 10 mM DTT) and cooled. Boiling step was again repeated similarly for one time. Tubes were cooled to room temperature (RT) and pellet was washed again with 0.1 mM sodium acetate buffer. Resulting pellet was suspended in extraction buffer and treated with chitinase (Sigma, Aldrich) (2 units, 0.2 ml) overnight (18 hrs) at 37 ^{o}C. The suspension was pelleted down by centrifugation and the pellet was washed in cold distilled water five times followed by 0.1 M sodium acetate buffer, pH 5.5 ten times. Finally, resulting proteins in the pellets were precipitated by tri-chloro acetic acid (6%).

Optimization of Pitarch et al (2002) [(6)] method, by standardization of Glass beads homogenization step:

The protein concentration of the extracts in method by Pitarch *et al.* (2002) was estimated by Folin Lowry's method using bovine serum albumin (BSA) as a standard. [11] The proteins were then subjected to gradient SDS PAGE (10-17%). Method by Pitarch *et al.* (2002) though gave better band quality; there were vertical streaks in the bands observed [(6)]. The protein extracted after gram staining showed the presence of intact cells remaining. Due to unavailability of bead beating instrument in our laboratory, we had used simple vortexer in initial extraction procedures. Therefore, method by Pitarch *et al.* (2002) [(6)] was further optimized using careful evaluation of glass bead beating homogenization procedure to aid the lysis of the tough candida cell walls. For standardization of homogenization with glass beads, one ml of washed pellet was again suspended in lysis buffer and adjusted to suggested turbidity for yeasts [(12)]. Ten ml screw capped sterile plastic tubes, were prefilled with beads up to roughly 10 mm height. To this 1 ml of the pellet adjusted to the required turbidity were added and tubes were cooled at -20 ^{o}C for 30 minutes before being subjected to disruption.

Two types of beads were used, a 0.5 mm glass beads (HiMedia), and 3-5 mm diameter glass beads (HiMedia), separately. Proper head space was left for the beads and pellet to move freely during vortexing. The tubes were labelled as 0 min, 3 min, 6 min, 9 min, 12 min, 15 min and 18

min, accordingly bead beating was done for 3, 6, 9, 12, 15 and 18 minutes for respective tubes. The experiments were carried out separately for both 3-5 mm and 0.5 mm glass beads. The first tube labelled as 0 minutes, was not subjected to glass bead homogenization. Second tube onwards, the tubes were vortexed continually for 3 minutes then kept in the ice box to cool for 2 minutes before next vortexing. This was to minimize warming up of the suspension during homogenization, so as to avoid thermal disruption of proteins in the suspension. A 100 µl of extracted sample from each tube of bead homogenization experiment were diluted 1:10 times using sterile normal saline. A 10 µl quantity of the diluted sample was plated on to SDA by semi quantitative streak culture method. The plates were incubated at 37 ^{o}C for 48 hrs. After incubation, number of colonies was carefully counted and candida colony count was estimated for 1 ml of direct sample. The results of both the techniques of bead beating were compared.

Pellets from all the different tubes were processed further with the next steps of extraction mentioned in Method by Pitarch *et al*. (2002) [(6)].

Analysis of protein extraction procedures

Representative samples from Method by Pitarch *et al.* (2002) [(6)], homogenized with glass beads for various time intervals were analyzed for protein estimation by Folin Lowry method and the quality of extracted proteins was assessed by SDS-PAGE (10-17%), (MONOKIN, Techno Source, Mumbai) and microscopy. All the tests were conducted in duplicate.

SDS Gel electrophoresis

Proteins were separated on SDS gel, according to the method explained previously with a few modifications [(13)]. Samples were suspended and vortexed for 1 minute in a SDS sample buffer containing Tris-HCl pH 6.8, Glyceine, β-Mercaptoethanol, bromophenol blue, kept in a water bath at 95 ^{o}C for 5 minutes and cooled to RT. Proteins were then separated in 10-17% polyacrylamide gel using the discontinuous constant voltage of 120V for stacking gel and 150V for resolving gel. Resulting gel was stained with silver stain method and bands were compared with molecular weight marker run in the gel.

Microscopic observation: Extracted samples from each set of experiments were smeared, stained by gram stain and observed under 100 X objective. Disrupted yeast appeared as dark "ghost" cells while intact yeasts were refractile. The percentage of disruption of cells was calculated by counting ratio of disrupted cells to total no. of cells in 25 oil immersion fields.

RESULTS

Table 1 shows effect of vortexing by two sizes of glass beads evaluated on two different strains of *Candida albicans* viz. RL-112 and CN-192. The parameters we used to evaluate the quality of cell disruption were percentage of disrupted cells protein concentration of the lysate, determination of viability of the cells and number of clear bands given by SDS-PAGE. The samples were initially homogenized by vortexing with glass beads. The resulting lysate was evaluated by gram stain and semi-quantitative culture on SDA and further subjected to extraction. The final yield was tested by protein estimation and SDS-PAGE electrophoresis.

The methods were intended to disrupt the cells maximally by increase in vortexing time. The percentage of disrupted cells increased proportionate to vortexing time. At 15 and 18 minutes the disruption was around 90% using 0.5 mm beads. Vortexing for all time periods showed that use of 0.5 mm beads was always better than 3-5 mm beads in lysing the cells. The lysates were pelleted and protein concentration was estimated. It is evident from table 1 that the protein yield also increased with vortexing time. The protein yield was highest at 18 min of vortexing.

With 3-5 mm beads percentage of cells disrupted was 76.9% (RL-112) and 78.8% (CN-192) at 18

minutes of homogenization. At 18 minutes of homogenization with 0.5 mm beads, 99.4% of cells in CN-192 and 92.0% in RL-112 showed disruption and the band quality was better.

Better lysis and better recovery of cell wall proteins at 18 min of disruption is reflected by scanty or no growth from the lysate on SDA and more number of bands on SDS-PAGE. The strain CN -192 gave no growth after 18 min of vortexing indicating complete lysis. The same was reflected on SDS-PAGE as the number of bands was 34, the maximum recorded in the present study.

DISCUSSION

Cell wall proteins act as major cell surface antigens that are recognized for adhesion by mucosal receptors to begin the infection process. Serotype A and B of *C. albicans* differ in cell wall mannoprotein structure [(14)]. Candida cell walls possess an electron dense outer layer having mannoproteins and the electron transparent inner layer formed of β 1-3 glucan and chitin. Mannoprotens give porocity to the cell wall. Specific manoproteins are synthesized during the hypheal mophogenesis. Chitin is glycosidecally linked to non-reducing ends of β-1-3 glucan and β-1-6 glucan [(14, 15)]. Hyphal forms carry significantly higher chitins as the organization of the cell wall is under the influence of morphogenetic codes [(2)].

Candida cell wall proteins have been extracted using different methods by different workers. The starting material and the method used for cell wall protein extraction influences final outcome or quality of extracted proteins. Studies have been carried out on various source materials like intact cells, cell wall extracted by cell disruption and proteins secreted into medium when protoplasts are synthesizing their cell walls [(5-9)]. The method used in the present study was based on breakage of cells to isolate cell wall protein.

In the method by Pitarch *et al*. (2002) [(6)] detergents were used to disrupt the cell wall. We observed excessive retention of intact candida cells in the lysate on storage which was indicating incomplete lysis of the cells by this method. The intact cells can act as artifact in SDS PAGE gels [(16)].The lysis of intact cells can release cytosolic proteins affecting the purity of the cell wall protein extract. Therefore, complete breakage of all intact cells is crucial in the cell wall protein extraction. Pitarch *et al.* (2002)[(6)] also have explained the need for complete mechanical disruption of the cells, not to leave behind intact cells, since subsequent enzymatic extraction in the later step can cause lysis of these intact cells and contaminate cell wall lysate with intracellular materials [(6)]. We, therefore, tried standardisation of bead beating step using 0.5 mm and 3-5 mm glass beads. We ran the beating over different time intervals and found that 0.5 mm glass beads used over 18 minutes in a simple vortexer gives best results. After homogenization the lysate was extensively washed with decreasing concentration of NaCl, to remove extracellular or cytosolic proteins attached through electrostatic forces [(6)]. The lysis of the candida cells was found to be >90% at 18 minutes of vortexing by 0.5 mm glass beads. The band quality and number on SDS-PAGE by the 0.5 mm bead beating was superior to using 3-5 mm beads over the same time period.

Mechanical methods are commonly used to disrupt fungal cell walls, with the combination of other methods. Klimerck *et al.* (2011) worked upon chemical, mechanical and osmotic shock in disrupting fungal cell wall and suggested that bead milling lead to better results in obtaining cell free extracts containing high concentration of soluble proteins, however for particular species further adjustments are required[(17)]. However, Okunghowa *et al.* (2007) suggested that the mechanical methods like sonication, French pressure cell press give good protein yield but glass beads provides a lesser quantity of protein and lead loss of protein activity [(18)].

Standardization of glass bead homogenization has to be done carefully with maintaining correct

quantity of cells subjected to homogenization. Ratio of cells Vs glass beads, size of the glass beads and time for vortexing needs to be carefully standardized. Kessler et al (1959) had obtained clean cell wall by beating the cells with glass beads in a blender for 90 minutes aided by treatment with 0.25 M sucrose solution [19]. In the original method of Pitarch *et al.* (2002) mechanical disruption of candida cells by bead beating, for 10 minutes with 30 S pulse in a bead beater was used [6]. We used simple vortex mixer, due to the unavailability of bead beater and sequentially increased homogenization time to test the efficacy of cell disruption.

Pitarch *et al.* (2002) have suggested overnight NaOH extraction for the release of enriched fractions of proteins directly linked to 1-3 glucan through their o-glycoside chains or other alkali sensitive linkages [6]. They also have suggested simultaneous the enzymatic treatment using quantazyme and exochitinase [7]. Since it was also shown by Kessler *et al.* (1959) that treatment with alkali can cause degradation of proteins linked with carbohydrates and lectin like proteins, we omitted the alkali extraction step [19]. The enzymes can release glycolytic enzymes tightly trapped within glucan-chitin complex [6]. In contrast to this, Klis *et al.* (2007) suggested use of high concentration of detergents to perturb plasma membrane and release cytosolic proteins. According to them, use of low concentrations of detergents, sodium phosphate buffer at pH 8 and extraction at low temperature avoids contamination of cell wall protein extraction with cytosolic proteins [20]. Feiz *et al.* (2006) did not use enzymes and detergents for the extraction but used instead, NaCl, low ionic strength buffer during extraction to prevent early release of cell wall proteins [8]. They showed that 78% proteins released by method by Pitarch *et al.* (2002), were intracellular proteins. However, Pitarch *et al.* (2002) demonstrated that, the proteins released by their method were from cell wall by showing failure to detect Sec 14p antigens, through immunoblotting procedure. Sec 14p is a marker of cytosolic contamination [6]. Casonova *et al.* (1992) biotinylated cell wall proteins during their growth stages, and performed extraction of cell wall proteins using β-mercaptoethanol and β-glucanses and confirmed extracted proteins to be of cell wall origin by doing extravidin alkaline peroxidase reaction in western blotting [5]. There are lot of researches conducted in the view of isolating cell wall proteins with avoiding cytosolic contamination. We intend to continue the studies with inclusion of a set of agents for detecting cytosolic proteins in our cell wall protein preparations and thus maximize the yield by continuous optimization of experiments.

CONCLUSIONS

With the above findings we conclude that one can use method by Pitarch *et al.* (2002)[6] for extraction of candida cell wall proteins with proper homogenization and lysis of the cells using glass beads. Since we used simple vortexer for homogenization this method can be easily adopted by laboratories not having sophisticated bead beaters or blenders or sonicators.

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Table 1: Glass beads homogenization procedure followed by extraction with Method by Pitarch *et al.* (2002).

Time	Strain	0 min		3 min		6 min		9 min		12 min		15 min		18 min	
Bead size (mm)		0.5	3-5	0.5	3-5	0.5	3-5	0.5	3-5	0.5	3-5	0.5	3-5	0.5	3-5
Grams stain; % of cell disruption	RL-112	0	0	26.1	9	65.1	40	78.2	60.7	74	66	89.4	70	92	76.9
	CN-192	0	0	35.1	7.5	33.3	13.8	37.8	63.0	85.0	62.5	93.2	64.0	99.4	78.8
Protein concentration µg/10 µl	RL-112	5	8.5	7..0	8.5	8.0	10	9.0	12.5	12.5	13.0	13.5	13.5	16.5	14.5
	CN-192	7.0	7.0	7.5	11.6	7.75	12.0	7.75	12.4	8.0	13.5	11.5	17.5	18.5	23.0
Growth on SDA	RL-112	+++	+++	++	+++	++	+++	++	+++	+	+	+	+	+	+
	CN-192	+++	+	+++	+	+++	+	+	+	+	+	0	+	0	+
SDSPAGE; Number of bands seen	RL-112	15	4	18	7	23	8	27	10	28	19	16	20	21	26
	CN-192	19	16	25	20	29	22	28	25	28	24	31	25	34	22

(+++) = > 500 colonies, (++) = > 200 and < 500 colonies, (+) = > 100 and < 200 colonies, < 100 is given as actual numbers