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Research Article

COMPARATIVE STUDY ON HYDROLYTIC ENZYMES PRODUCED BY DIFFERENT MORPHOLOGICAL FORMS OF *CANDIDA ALBICANS*

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ABSTRACT

In recent years, the incidence of fungal infections has been rising all over the world. The ability of *Candida albicans* to switch from yeast to hyphal growth is essential for its virulence. The aim of this comparative study was to biotype and characterize phospholipase, proteinase, phosphatase and haemolytic activities of yeast and hyphal forms of *Candida albicans*. The hyphal form of *Candida albicans* secrets high quantity of hydrolytic enzymes than yeast form, which helps in its virulence. These results suggest that pathogenic fungi produce larger amount of inducible hydrolytic enzymes than non-pathogenic fungi. In this investigation, plate methods were used to determine the phospholipase, proteinase and haemolytic activities and spectrophotometric method was employed for testing acid phosphatase activity.

Keywords: *Candida albicans*, Candidiasis, Hydrolytic enzymes, Haemolytic activity, Virulence.

INTRODUCTION

Candida albicans is dimorphic organism belonging to the genus *Candida* of ascomycetous like fungal species (Staniszewska *et al.,* 2012). *C. albicans* is the major causative factor of opportunistic human infections with very high morbidity and mortality rate of 30 to 40% (Staniszewska *et al.,* 2012, Barnett, 2008, Bialkova, 2006, Biswas *et al.,* 2000, Borg, 1998, Borges – Walmsely, 2000, Borman *et al.,* 2008, Gropper *et al.,* 2009, Silva *et al.,* 2010). *Candida albicans* is the most virulent species that is responsible to cause superficial and systemic infections especially in immunocompromised individuals (Bramono *et al.,* 2006). *Candida albicans* has the ability to switch reversibly between single celled yeast (blastospore) and an elongated filamentous form (Hyphae and Pseudohyphae) called as – "Morphological transition or morphological dimorphism" (Pei-Wen Tasi *et al.,* 2013). In *Candida albicans* morphological conversion of yeast to hyphae is well studied and can be induced in *in vitro* with several environmental factors known as "Inducers" (Ghosh *et al.,* 2009), *Candida albicans* differentially expresses various infection associated genes, cell surface and virulence proteins which contribute to its pathogenicity and function as "virulence factors" (Pei-Wen Tasi *et al.,* 2013). *C. albicans* secretes various hydrolytic enzymes such as acid phosphatases, phospholipases, proteinases, β-galactosidase which play an important role in candidal outgrowth (Bramono *et al.,* 2006, Fradin, 2003, Bramono *et al.,* 1994, Banno *et al.,* 1985, Ogawa, 1997, Naglik *et al.,* 2004, Tsang *et al.,* 2007). Hydrolytic enzymes help in adherence, tissue penetration and proliferation of fungi by causing invasion, destruction of host tissues, hence supplying degraded material to the organisms as nutrients (Bramono *et al.,* 2006, Fradin, 2003, Ogawa, 1997, Naglik *et al.,* 2004, Tsang *et al.,* 2007). Seven phospholipase genes have been identified, but only four of them are well characterized (Tsang *et al.,* 2007, Samaranayake *et al.,* 2006). Phospholipases contribute to pathogenecity of *C. albicans* by damaging the host cell membranes, which helps pathogen to invade host tissues. Saps are encoded by 10 SAP genes that play crucial role in *C. albicans* virulence (Tsang *et al*., 2007). Proteinases are capable of degrading epithelial and mucosal barriers of patients such as collagen, keratin, mucin, antibodies, complement and cytokines. Cloning and disruption of the genes for these enzymes showed their role in *Candida* virulence (Hube *et al.,* 1997, Sanglard *et al.,* 1997, Leidich *et al.,* 1998, Watts *et al.,* 1998, De Bernardis *et al.,* 1999). Acid phosphatase which is located in the cell wall of yeasts belongs to group of periplasmic enzymes secreted by *Candida albicans* (Vasileva- Tonkova *et al.,* 1993). These enzymes are glycoproteins and their content in yeast cell depends on the phosphate concentration of the growth medium (Vasileva- Tonkova *et al.,* 1993). Furthermore, Haemolysin is another important virulence factor thought to contribute to candidal pathogenesis (Tsang *et al.,* 2007). The ability of *C. albicans* to acquire elemental iron through haemolysin production is important in its survival and ability to cause infections in the humans (Tsang *et al.,* 2007, Weinberg, 1978). The secretion of haemolysin, lysis of the erythrocytes, followed by iron acquisition facilitates hyphal invasion in disseminated Candidasis (Tsang *et al.,* 2007, Odds, 1998). Expression of virulence factors helps to understand the epidemiology of infections, which result in improved therapeutic regiments. Intensive research is expected to identify pathogenic factors in fungi especially in *Candida albicans* for facilitating the diagnosis, treatment and prevention of Candidasis (Bramono *et al.,* 2006). The objective of this study was to comparatively measure proteinase, lipase, acid phosphatases amongst yeast and hyphal form of *Candida albicans* and try to correlate the role of these enzymes in fungal virulence. To our knowledge, the comparative study of different enzyme production by yeast and hyphal form is not yet been published before.

MATERIALS AND METHODS

The study was carried out on *Candida albicans*'*s* strain MTCC 227 in order to compare the production of different enzymes between yeast form cells (non-pathogenic) and hyphal cells (pathogenic). This study was performed in order to evaluate any possible difference in the secretion of hydrolytic enzymes of different forms of *Candida albicans*.

Materials

C. albicans strain

Candida albicans MTCC (ATCC 227), a quality strain was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained on Yeast extract Peptone Dextrose (YPD) agar slants at 4^{o}C.

Methods

Determination of Phospholipase activity

Candida albicans ability to produce extracellular phospholipase activity was determined by measuring zone of precipitation after growth on egg yolk agar (Samaranayake *et al.,* 1984, Tsang *et al.,* 2007, Sachin *et al.,* 2012). The egg yolk medium contained 13g Sabouraud's dextrose agar (SDA), 11.7 g NaCl, 0.11g CaCl2 and 10% sterile egg yolk (All in 184 ml distilled water) (Tsang *et al.,* 2007, Mohandas *et al.,* 2011, Sachin *et al.,* 2012). First, the components without egg yolk were mixed and sterilized, then the egg yolk was centrifuged at 500rpm for 10 min at room temperature and 20 ml of the supernatant was added to the sterilized medium (Sachin *et al.,* 2012, Tsang *et al.,* 2007). Standard inocula of the test (hyphal) and control (yeast) *Candida* (5 µl with 10^{8} yeast cells were deposited

onto the egg yolk agar medium and kept at room temperature (Sachin *et al.,* 2012, Tsang *et al.,* 2007). Each culture was then incubated at 37^{0}C for 48h (Sachin *et al.,* 2012, Tsang *et al.,* 2007, Mahmoudabadi *et al.,* 2010) after which the diameter of precipitation zone around the colony was determined (Sachin *et al.,* 2012, Tsang *et al.,* 2007, Mahmoudabadi *et al.,* 2010). Phospholipase activity (Pz value) was determined by taking ratio of the diameter of the colony plus the precipitation zone (in mm) (Price *et al.,* 1982, Sachin *et al.,* 2012, Tsang *et al.,* 2007, Mahmoudabadi *et al.,* 2010).

$$\frac{\text{Colony diameter}}{\text{Colony diameter + Zone of precipitation}} = Pz$$

Phospholipase activity of the isolate was considered positive when a precipitation zone was observed around the colony on the plate (Sachin *et al.,* 2012, Tsang *et al.,* 2007, Mahmoudabadi *et al.,* 2010). Pz value equals to 1, denotes no activity or negative for phospholipases, less than 1 (Pz < 1) indicates phospholipase activity. Lower the Pz value, higher is the enzymatic activity. Pz < 0.90-0.99 = weak phospholipase activity (+), 0.80-0.89 = poor phospholipase activity (++), 0.70-0.79 = moderate phospholipase activity (+++) and Pz < 0.70 = large phospholipase activity (++++) (Sachin *et al.,* 2012, Tsang *et al.,* 2007, Mahmoudabadi *et al.,* 2010). Reference strains of *Candida albicans* (ATCC10231 and ATCC 24433) were taken as positive control (Sachin *et al.,* 2012, Tsang *et al.,* 2007).

Statistical analysis: The assay was carried out in triplicate.

Determination of Proteinase activity

Extracellular proteinase activity was measured by using bovine serum activity. BSA as a substrate (Bramono *et al.,* 2006, Negi *et al.,* 1984, Tsuboi *et al.,* 1985, Tsuboi *et al.,* 1989). The activity was analyzed in terms of BSA degradation (Sachin *et al.,* 2012, Tsang *et al.,* 2007). In this, control (yeast) and test (hyphal) suspension of 1×10^{8}cells/ml was prepared and 200 µl suspension was inoculated onto 1%BSA medium (2% glucose, 0.1% KH_2PO_4,0.05% $MgSO_4$ 2% agar mixed after cooling to 50 ^{0}C with 1%BSA solution) (Sachin *et al.,* 2012, Tsang *et al.,* 2007). The plate was incubated for 5 days at 37 ^{0}C, after which the diameter of precipitation zone around the well was determined which indicated proteinase activity (Sachin *et al.,* 2012, Tsang *et al.,* 2007). Proteinase activity (Prz) was determined as the ratio of the diameter of the colony to that of the clear zone of proteolysis (in mm) (Sachin *et al.,* 2012, Tsang *et al.,* 2007, Akcaglar *et al.,* 2010). Reference strain of *Candida albicans* (ATCC 10231 and ATCC 10261) were taken as positive control (Sachin *et al.,* 2012, Tsang *et al.,* 2007).

Statistical analysis: The assay was carried out in triplicate.

Determination of Haemolysin activity

Haemolysin production was evaluated according to Manns *et al.,* method (Sachin *et al.,* 2012, Tsang *et al.,* 2007, Manns *et al.,* 1994, Luo *et al.,* 2001). Media was prepared by adding 7 ml fresh sheep blood to 100 ml SDA supplemented with glucose at a final concentration of 3% (w/v). The final pH of the medium was 5.6 ± 0.2 (Sachin *et al.,* 2012, Tsang *et al.,* 2007). The culture of both control (yeast) and test (hyphal) of *Candida albicans* (200 µl, 108 cells/ml saline) was inoculated into the well in the medium. The plate was then incubated at 37^{0}C in 5% CO_2 for 48h (Sachin *et al.,* 2012, Tsang *et al.,* 2007). After incubation, plates were examined and quantification of colonies was carried out. The haemolytic index (Hz value) was used to determine haemolysin activity of hyphal and non-hyphal cells (Sachin *et al.,* 2012, Tsang *et al.,* 2007). Hz was calculated by the ratio of the diameter of the colony to that of the translucent zone of haemolysis in mm (Sachin *et al.,* 2012, Tsang *et al.,* 2007). A reference strain of *Candida albicans* (ATCC 90028) was taken as a positive control (Sachin *et al.,* 2012, Tsang *et al.,* 2007).

Statistical analysis: The assay was carried out in triplicate.

Determination of Acid phosphatase activity

Determination of Acid phosphatase activity by *Candida albicans* was carried out with *p*-nitrophenyl phosphate (pNPP) as a substrate (Vasileva-Tonkova *et al.,* 1993). The reaction mixture contained 100 µl enzyme sample, 100 µl 0.1M-sodium acetate buffer (pH 5.5) and 100 µl 3.8 mM- pNPP (Vasileva-Tonkova *et al.,* 1993). After incubation at 37 ^{0}C for 15 min, the reaction was stopped by addition of 1 ml 0.2M NaOH (Vasileva-Tonkova *et al.,* 1993). The absorbance was measured at A400 (Vasileva-Tonkova *et al.,* 1993). One unit of phosphotase activity was defined as the amount of enzyme catalysing the formation of 1 µmol p-nitrophenol/min under standard assay conditions (Vasileva-Tonkova *et al.,* 1993). When some other substrate was used, the assay is carried out according to Lanzeta *et al.,* 1979 method (Vasileva-Tonkova *et al.,* 1993).

RESULT AND DISCUSSION

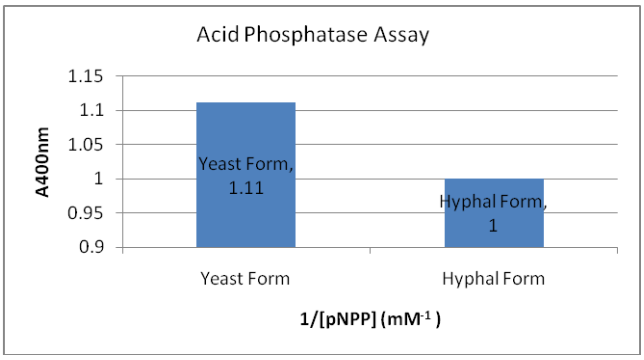
Phospholipase, Proteinase and Haemolysin Assay

Activity	Yeast Form (Value)	Hyphal Form (Value)
Phospholipase (Pz)	0.96 ± 0.03 (+)	0.69 ± 0.01(++++)
Proteinase (Prz)	0.94 ± 0.03 (+)	0.67 ± 0.01(++++)
Haemolysin (Hz)	0.86 ± 0.03 (+)	0.65 ± 0.01(++++)

(+ = weak, ++++ = strong)

Acid Phosphatase Assay

For, acid phosphatase activity when reading was taken at 400nm it was found to be 1.11 for yeast form and 1 for hyphal form



RESULTS AND DISCUSSION

The pathogenicity of *Candida albicans* depends on several virulence factors, including germination, adherence to host cells, phenotypic switching and production of extracellular enzymes (Sachin *et al.,* 2012). In this comparative study, it was found that hyphal form produces higher amount of phospholipase, proteinase, haemolysin and acid phosphatase enzyme as compared to yeast form. It supports the data that these enzymes also play role in the pathogenicity of *C. albicans* and helps for their virulence. To the best of our knowledge, this is the first attempt to compare the enzyme secretion between yeast and hyphal form of *Candida albicans*.

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