Original article

Assessment of carboxyl esterase activity in clinical isolates of *Candida albicans*

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Abstract
Introduction and objective: Esterase activity is used in evaluation and correlation of strains in fungi. Our previous study showed that *Candida albicans* has a kind of intracellular esterase activity in yeast extract, peptone, and glucose medium (YPG). The aim of this research was to study the qualitative and quantitative differences of this enzymatic activity among clinical isolates of this yeast.
Materials and methods: *Candida albicans* isolates which have been kept on Sabouraud dextrose agar medium by continuous passage were grown in YPG medium for 48h in order to induce enzymatic production. In the next step, yeast cells were collected and then broken with glass bead. Esterase activity of cytoplasmic extract of isolates was measured by colorimetric method. Besides, five synthetic substrates were used to assess the qualitative differences in this enzymatic activity.
Results: The cytoplasmic extract of 12 *C. albicans* isolates demonstrated an esterase activity to all used substrates and no significant qualitative and quantitative differences were found in this enzymatic activity. The average enzymatic activity of all isolates had a reversed relation to the number of carbon atoms in carboxyl substrates (except for alpha-naphtyle laurate). The amount of this activity for alpha-naphtyle acetate, beta-naphtyle acetate, alpha-naphtyle caprilate, alpha- naphtyle laurate, and alpha-palmitate were 14.4, 8.45, 0.94, 0.42, 0.75 unit (μM/mg protein in min), respectively.
Conclusion: The observed fluctuation in esterase activity of clinical isolates of *C. albicans* might be useful in tracking its sub species in epidemiological purposes.

Keywords: *Candida albicans*; Enzyme; Esterase activity; Alpha-naphtyle

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Introduction

All sub groups of one species have many common features but they may show some delicate differences which help to define sub species or strain. Determining the activity of carboxyl esterase of special infective species lead to a new quality and showing its range in a new way to figure out the mechanism of pathogenesis and unraveling the epidemiological factors which help the diagnosis and cure of the disease.

To define fungi strains such as *Candida albicans* numerous features have been evaluated till recent years and different methods have been used to show the range of features in subspecies. Some of them have older background such as resistotyping [1], morphotyping [2], typing by killer yeast [3,4], typing from electrophrosis pattern or wall protein imunoblating pattern [5], and lectin typing [6]. Therefore in this respect, methods like genotype have been introduced during recent years [7-12].

The quantitative [13,14] and qualitative [5,9,15-18] differences in enzymatic activity, have also been used in the evaluation of strain correlations in fungi. In this regard, proteinases and phospholipases have been used more than the other factors [16]. Schreiber *et al.* [17] studied the capability of producing a carboxyl acid proteinase in the clinical isolates of *C. albicans* and the other yeasts and Williamson *et al.* [13] tested the phospholipastic activity as a criterion for the typing of *C. albicans*. The method used in this study which is an appropriate method for biotyping is based on the research done by Bernhardt *et al.* [18] who measured fluctuation of 19 hydrolitic enzymes in *C. albicans*.

Because of significant variety in the characteristic of *C. albicans*, utilized method for separating sub species, and some of their downsides, not a single method can be adopted as a standard typing method. Hence, identifying new features of this important pathogen and its evaluation as a tool for classifying strain should be continued. Our previous study uncovered that cytoplasmic liquid of *C. albicans* shows a kind of esterase activity after growth in special environment which maximize after 90mins in pH=7.5 and 37ºC while facing with 1mM alpha-naphtyle acetate [19].

The aim of this study was to measure qualitative and quantitative fluctuation of this enzymatic activity by testing more samples of clinical isolates of this yeast. If this method reveals strain variation in *C. albicans* it can be an appropriate basis for further study in tracking sub species of this important pathogen.

Materials and methods

Candida albicans

The 12 isolates of *C. albicans* have been recovered from referees to a mycology lab and kept on Sabouraud dextrose agar (Merck, Germany) with continuous passage.

Inspiration of esterase activity

For inspiriting esterase activity, *C. albicans* isolates have been cultured in YPG medium (Yeast extract 1%, Peptone 1%, Glucose 2%, Merck, Germany) and incubated for 48 hours in 35ºC [19].

Making cytoplasmic extract

Grown yeast cells were collected and digested with glass bead after washing for three times [7]. Washed cells, glass bead with 0.5mm diameter, and breaking buffer (tris 62.5mM, dityotrytol 1mM, phenyl methyl solfonyl floride 0.2mg in each ml, glycerol 15%, pH=6.8); were mixed with 2:1:1 volume ratio, respectively in 20×150mm glass tubes and were shaken at

the speed of 2800rpm till more than 80% of cells were broken. Tubes were put in ice to avoid warming of the samples. Then, the suspension was ultra centrifuged in 1×10^5rpm in 4ºC for an hour and the surface liquid (cytoplasmic extract) was kept in -20ºC till measuring the enzymatic activity.

Substrate

To detect the amount of enzymatic activity, five types of synthetic substrates including: alpha-naphtyle acetate, beta-naphtyl acetate, alpha-naphtyle caprilate, alpha-naphtyle laurate, and alpha-naphtyle palmitate [3], were used. 100mM stocks from the mentioned substrates were made in N-propranol and kept in 4ºC till consumption.

Measuring the esterase activity

The esterase activity of isolates was measured as described in our previous study by colorimetery method in defined optimum situation [19]. In brief, substrate was added from 100mM stocks to a turning table containing phosphate 10mM buffers in 45ºC. 0.1, 0.2, and 0.3% Trayton X-100 were added to a buffer in order to solve alpha-naphtyle caprilate, alpha-naphtyle laurate, and alpha-naphtyle palmitate, respectively. After adjusting tube temperature, cytoplasmic liquid was added and the reaction volume arrived to 1ml with a buffer if required. After 90mins incubating, the reaction was ended by adding 50μl of sodium two decyle sulfate 10% solution. Then, 50μl of fast violet 2% solution were added and the absorption amount in 520nm wave lengths was measured by spectrometer.

Standard graph of enzymatic activity

Standard amounts of alpha-naphtole were used to draw graph considering esterase activity facing with utilized standard which leads to alpha-naphtyle release.

Computation of specified esterase activity

The unit of specified esterase activity is defined as the amount of enzyme in 1mg protein which can hydrolyze 1μM substrate and produce 1μM alph-naphtyle.

Data analysis

All the tests, from culture to evaluation of esterase activity were replicated two times and detecting enzymatic activity of isolates was replicated three times in the first period and two times in the second period. T-student test was used for variance test and $p<0.05$ was considered as statistical differences.

Results

Table 1 shows the results of five types enzymatic activity of *C. albicans*. All studied isolates had esterase activity while facing to tested substrates. The comparison among average enzymatic activity to five kinds of substrates demonstrates that they had the maximum level of activity to alpha-naphtyle acetate with 14.14Unit and the minimum level of activity pertains to alpha-naphtyle laurate with 0.42Unit.

The average enzymatic activity increased after facing with alpha-naphtyle laurate, alpha-naphtyle palmitate, alpha-naphtyle caprilate, beta-naphtyle acetate, and alpha-naphtyle acetate. Two way variance analysis showed that the difference among all the enzymatic activity was statistically significant (at 0.05 and 0.001 levels) except for the difference between the average of alpha-naphtyle caprilate and alpha-naphtyle palmitate. The other versatilities are significant, the difference of average activity to alpha-naphtyle laurate and alpha-naphtyle palmitate ($p<0.03$) and exposing other substrates ($p<0.0001$).

The average of esterase activity of isolates varies from 7.41 to 19.55Unit and the two way variance analysis showed that the observed differences in esterase activity

of isolates to beta-naphtyle acetate was statistically significant ($p<0.0001$). Moreover, the range of enzymatic activity of isolates to beta naphtyle acetate varied from 4.96 to 12.90Unit and the isolates with maximum and minimum enzymatic activity to this substrate were the ones which showed the maximum and minimum activity to alpha-naphtyle acetate, also showed that the perceived variations among activity of under studied isolates were statistically significant ($p<0.0001$).

The lowest and highest levels of activity to alpha-naphtyle caprilate were 0.66 and 1.35Unit, respectively; meaning statistically significant difference among isolates ($p<0.0001$). The minimum and maximum levels of activity to this substrate were 0.24 and 0.71Unit respectively, and the observed differences among isolate activity to this substrate was statistically significant ($p<0.0001$). Moreover, the range of activity to alpha naphtyle palmitate varied from 0.18 to 1.73 Unit and variance analysis showed the significant differences activity to this substrate ($p<0.0001$).

Table 1: Esterase activity in clinical isolated of *C. albicans* against substrates

C. albicans isolates	Substrate type and esterase activity rate (µM/mg protein in min)				
	α-Naphtyl palmitate	α-Naphtyl laurate	α-Naphtyl caprilate	β-Naphtyl acetate	α-Naphtyl acetate
No. 1	1.73	0.64	1.24	5.80	8.92
No. 2	1.51	0.71	1.35	12.90	19.55
No. 3	0.42	0.32	1	7.74	12.95
No. 4	0.82	0.5	1.05	12.26	19.22
No. 5	0.18	0.24	0.67	8.89	14.35
No. 6	0.62	0.30	0.80	8.60	13.02
No. 7	0.80	0.52	1.06	8.03	15.2
No. 8	0.32	0.24	0.75	7.47	14.73
No. 9	0.54	0.29	0.66	4.96	7.41
No. 10	0.81	0.40	0.78	7.86	14.13
No. 11	0.42	0.34	0.91	8.64	15.16
No. 12	0.80	0.55	1.03	8.27	15.07
Mean±SD	0.75±0.46	0.42±0.16	0.94±0.22	8.45±2.25	14.14± 3.49

Discussion

New and unknown features in a pathogenic species should be reported in order to find a method for classifying species. Then its fluctuation in a species population should be reviewed and the link between that feature and the other significant characteristics or diseases due to it, detected as the last step. Defining features of an organism sub species or typing makes careful tracking of sub species possible and will help preventing, diagnosis and curing diseases by picturing diseases epidemiology. This task will be possible by measuring criteria which change from one strain to another one.

More than ten differentiation methods have been introduced for sub species of *C. albicans* separation and some of their potentials have been evaluated for attaining epidemiological and non epidemiological goals. Detecting the link between the traits of the fungi sub species and indexes like source of the disease [4], clinical type of the

disease [1,2,8,9], geographical site [3,7,20], drug resistance [8,12,14,15], and existence of hospital infection are some examples of the mentioned goal.

There is much incoherence in the outcomes of different studies which had used this method and not a single sample of them has the appropriate criteria to be accepted as a standard typing method. Hence, it might be a good idea for detecting an acceptable and standard typing method to find unknown traits of this substantial pathogen and assess its potential for sub species differences.

The study of enzymatic activity of *Candida* has been done for which reasons including as a method for classifying sub species [13,18,21-23]. Indeed, there are limited reports on fluctuation of esterase activity in *Candida* sub species. Rudek [24] assessed the quality of esterase activity in the *Candida* species which often are segregated from human infections by using plates containing Tween then reported the sediment pattern of fatty acid reaction which were released from Tween with Ca ions of environment as a useful method for specification of some species. Moreover, Tsuboii *et al.* [25] showed the secretion of an extra cellular esterase in *C. albicans* species is more than pathogenic species.

Our previous study had unraveled a kind of intra cellular esterase activity of *C. albicans* which had not been reported before [19]. We had detected some of these features in the current study. According to our data this is the first scrutiny which study the intra cellular activity as a factor for differentiation of *C. albicans* sub species while previous limited surveys specified to study esterase secration which had been purposed to differentiate species but not sub species [24,25].

Conclusion

Results of current study express that *C. albicans* isolates show notorious qualitative and quantitative differences in each of tested substrates based on intra esterase activity. It sounds that these variations are useful as criteria for tracking sub species of this yeast and studying their strain link. Determination of enzymatic activity correlation with factors like virulence and *C. albicans* natural settlement is currently under study by our co-workers.

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