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THE POTENTIAL OF CANDIDA BIOFILM PROTEIN AS BIORECEPTOR FOR CANDIDIASIS IMMUNOASSAY

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Abstract: Candidiasis or infection that is caused by *Candida* has become a new list of the therapeutical problems recently. The difficulties in diagnosing are the main cause of the unsatisfactory results from common therapies and diagnosis methods. This has urged researchers to find alternative ways in candidiasis diagnosis such as serology-based detection using antigen or antibody development. The aim of this study was to evaluate the potential of protein derived from *Candida albicans* biofilm as bioreceptor on candidiasis immunoassay through Dot Blot method. The research method used descriptive method with the following stages: (1) preparation of *Candida albicans* biofilm (2) extraction of *Candida albicans* protein through enzymatic and mechanical methods, (3) determination of protein molecular weight with SDS-PAGE (4) production of polyclonal anti- candida and (5) analysis of protein extract as bioreeceptor on dot blot. Profile of biofilm proteins on SDS-PAGE analysis were shown on molecular weight 27,42; 29,89; 38,10; 44,90; 48,75; 52,92; 55,14; 59,86; 70,56; 87,36; 102,54;115,05; 130,14;143,14;181,53 kD. There were differences in the intensity of dots in the control group (44070) and treatment groups (63170.5). It is noticeable that biofilm protein extract of *C. albicans* can be used for induction of anti-*Candida* polyclonal antibody production as the potential candidate of bioreceptor in candidiasis immunoassay.

Keywords: SDS-PAGE, polyclonal antibody, immunoassay, dot blot, biofilm

INTRODUCTION

Candidiasis is an infection caused by parasitic fungus from genus *Candida* on the mucosal layer and the other tissues. It is normal that *Candida* is found as flora in the mucosal membrane of the oral cavity, respiratory tract, and genitalia organs. The population of *Candida* inside the body is regulated by the immune system, but some conditions such as consumption of antibiotic, chemotherapy, organ transplantation and immunodeficiency will lead to the overgrowth of *Candida*.[1,2] Notably, eighty percent of the human population show the formation of *C. albicans* biofilm on their oropharynx, gastrointestinal tract and vagina.[3]

The increasing of candidiasis cases has added a new list of the unsolved problem in medicine recently. There are a number of methods used to detect candidiasis, but they are difficult to deliver accurate results on pathogenic *Candida* detection.[4] For an example, the infected people got a negative result on the observation of their blood culture. This has encouraged researchers to find alternative solutions on candidiasis detection. In addition, the immunoassay-based diagnostic kit is promising to be developed as the alternative candidiasis detection. Using immunoglobulin molecule (antibody) that is specific to the certain antigen, this method will perform better sensitivity and specificity also less time consuming than immunoassay method.

RESEARCH METHODS

Biofilm was produced according to modified Samaranayake *et al* (2005) and Merrit *et al* (2005) methods.[5] *C. albicans* biofilms were growth on cellulose nitrate membrane with 25 mm of diameter and 0.22 μm of pores on the surface of spider solid medium.

The cellulose membrane was sterilized and then was put on the surface of biofilm medium using sterile pinset. Following this, 50 μl of *C. albicans* inoculum with optical density (OD) 0.5 on 469 nm was dropped on cellulose nitrate membrane. Spider medium with cellulose nitrate membrane was incubated on 37^{O}C for 1 hour. After all, petri dish was flipped upside down for 24 hours of incubation.

Formed biofilm was separated from the membrane to be resuspended with sterile PBS. The suspension was centrifuged at 1000 rpm for 15 minutes. Biofilm of *C. albicans* was accumulated as a pellet, which then was stored in the refrigerator.

C. albicans biofilm was resuspended with 100 mL KCl 0.6 M containing 0.5 mg/mL *Achatina fulica* enzyme. Resuspension was sonicated for 2 minutes. If the suspension starts to form bubbles, the power should be reduced until it stops to form bubbles. There were four steps of sonication that were done in cold temperature for each 30 seconds. Sonicated suspensions were centrifuged for 15 minutes at 10,000 rpm. Biofilm protein extract was on supernatant, which was then concentrated using lyophilization with freeze dryer.[6]

The profile of *C. albicans* biofilm protein extract was analyzed using SDS-PAGE.[7] On the one hand, compositions of separating gel were 4 mL of acrylamide-bisacrylamide solution, 2.5 mL tris-HCL solution pH 8.8, 0.05 mL 10% SDS solution, 0.1 mL 10% potassium persulfate solution, 0.01 mL TEMED and 3.4 mL aquadest. On the second hand, stacking gel compositions were 0.67 mL of acrylamide-bisacrylamide solution, 1.25 mL tris-HCL solution pH 6.8, 0.25 mL 10% SDS solution, 0.05 mL 10% potassium persulfate solution, 0.01 mL TEMED and 3.075 mL aquadest. Stacking gel was poured on the top of the separating gel, followed by comb assembly. When the gel was ready, it was removed from the plate and put on the electrophoresis apparatus and sunk on separating buffer. Protein sample

(10 μL) was mixed with sample buffer (2 μL), while protein marker (2 μL) was mixed with 10μL fermented solution. Each of protein sample and protein marker was loaded on the well of stacking gel. Electrophoresis buffer was then poured into the chamber. Protein was run on 100 V, 200 mA for about 1 hour. Protein bands were dyed using commasie blue.

Animal models were divided into two groups, negative control group and treatment group. The control group was injected intraperitoneally with 100 μL Complete Freund's Adjuvant (CFA) without antigen. On the other side, treatment group was injected intraperitoneally with 400 µg *C. albicans* (emulsified with CFA 1:1). Booster was done in the second week. The control group was injected intraperitoneally with 100 µl Incomplete Freund's Adjuvant (IFA). The treatment group was injected intraperitoneally 400 µg *C. albicans* (emulsified with IFA 1:1). Boster performed again at fourth and sixth week. Rats were dissected on the seventh week and their blood was collected directly from the heart. Blood was centrifuged on 3000 rpm 4^{o}C for 30 minutes. plasma was transferred into Eppendorf tube and stored in the freezer until observation.[8]

Candida antigen was diluted in azida PBS and dropped on nitrocellulose membrane for about 50 L. This membrane was degassed for about 30 minut and blocked with 5% PBS skim milk for 1 hour. Thus, the membrane was washed using 0.05% PBS-Tween three times for 3 minutes. In the shaker, the membrane was incubated with anti-*Candida* primary antibody for 2 hours then washed with 0.05% PBS-Tween three times for 3 minutes before incubation using secondary antibody. After all, the membrane was washed with PBS-Tween three times for 3 minutes followed by addition of western blue and incubation for 2 hours in a dark room with a shaker. At last, the membrane was washed with aquadest and dried for observation.[9] Data were tabulated and analyzed using *Image G* for dot thickness measurement.

RESULTS AND DISCUSSION

C. albicans protein was extracted through biofilm matrix and cell wall lysis using *Achatina fulica* enzyme and sonication. This enzyme extract has a protein content of 1,35mg/mL, activity of 2,649 units/ml and specific activity of 1,958 units/mg and has the ability to hydrolyze glucan and chitin as the main composition of biofilm matrix and cell wall of *C. albicans*.[6] The intracellular protein concentration of *C. albicans* biofilm was 1.2066 mg/ml.

Proteins of biofilm extract will be separated based on their molecular weight through SDS-PAGE (Sodium dodecyl sulfate – polyacrylamide gel electrophoresis) separation. SDS will bind to the hydrophobic site of protein that leads to protein unfolded. Due to this, protein will stay on linear conformation. The proportion of SDS-protein is related to the molecular weight, so the big molecule of protein will move slowly and will be fractionated in the first place (see figure 1).

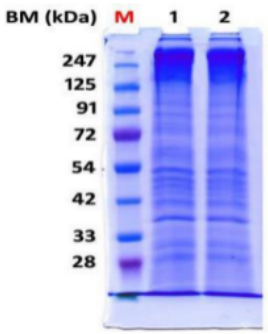


Figure 1. SDS-PAGE electrophoregram of *C. albicans* biofilm protein extract

The Rf (Retention Factor) and molecular weight of markers can be

analyzed on regression formula as seen in Table 1. The regression of molecular weight over Rf is y = -1.1429x + 2.3664.

Table 1. Rf score and molecular weight of marker components

a	b	Rf	BM	log BM
0,80	6,40	0,125	247	2,393
1,20	6,40	0,188	125	2,097
1,90	6,40	0,297	91	1,959
2,50	6,40	0,391	72	1,857
3,30	6,40	0,516	54	1,732
4,00	6,40	0,625	42	1,623
4,90	6,40	0,766	33	1,519
5,50	6,40	0,859	28	1,447

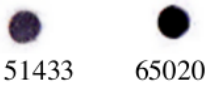
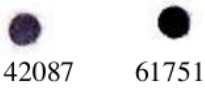
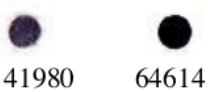
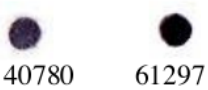
The molecular weight of proteins was obtained from Rf input to regression formula. It is noticeable that there were 15 intracellular proteins found from *Candida* biofilm extract with molecular weight of 181.53, 143.33, 130.14, 115.05, 102.54, 87.36, 70.56, 59.86, 55.14, 52.92, 48.75, 44.90, 38.10, 29.89 and 27.42 kDa. A previous study has shown four protein profiles with a molecular weight around 84.4, 69.5, 67.1, 62.5kDa.[10] It can be expected that different extraction method delivers different results. Sonication that replaced β-merkaptoetanol will allow *Achatina fulica* enzyme works optimally.

Based on SDS-PAGE results, it can be seen that intracellular protein of *Candida* biofilm is potential to be used as antigen for immunomodulator. Most of the effective immunogenic agent has molecular weight around 10,000 Da, but there are several exceptions such as insulin with molecular weight 500 Da and glycogen with molecular weight 4600 Da.[11]

Anti-*Candida* polyclonal antibody was formed as the immune reaction for a foreign antigen that enters into the body. High affinity and avidity antibody will be formed if the memory cells have been formed.[12] Stimulation of immune system can be done by addition of adjuvant. There are several types of adjuvant that can be used for immunomodulation, namely CFA that contains *Mycobacterium tuberculosis* for the first modulation. The existence of *Mycobacterium tuberculosis* will exacerbate immune system reaction. On the second modulation or booster, it uses IFA without *Mycobacterium tuberculosis* as the prevention of hypersensitivity.[13] Primary immune response happened after the first injection markedly by IgM production on the sixth and seventh day after injection.[14] The highest concentration of IgM was on the tenth until the fourteenth day after injection. On the following days, IgG is produced replacing IgM.

Dot blot is a semi-quantitative method that is used for antigen/antibody detection. Resulted dots have represented the interaction between antigen and antibody. This method is commonly used for screening or regular test with many samples.[15] On this dot blot research, we use antigens from intracellular protein extract biofilm and polyclonal antibodies from rat plasma (P). A solution of 9% PBS and 1% NaN3 have used as blank. Dot density can be measured using Image G software as shown in Table 2.

Table 2. Dot blot of Anti-*Candida* antibody against *Candida* biofilm protein antigen

Sample	control	P
1	 51433	65020
2	 42087	61751
3	 41980	64614
4	 40780	61297
Mean Density	44070	63170.5

It is noticeable that serum of animal models show positive reaction onto C. *albicans* biofilm protein extract, present on the black dot as seen in Figure 1. Dark blot results from the interaction of antigen, primary antibody and a secondary antibody that is labeled with alkaline phosphatase (AP), which alkaline phosphatase reacted with its substrate.[8] On the contrary, blank solution is presented in weak intensity dot due to there was no recognition of protein serum epitope. The interaction of antigen-antibody is correlated to lock and key interaction, the antibody will only bind to the specific protein. The antibody will be produced according to the amino acid structure of antigen that is recognized by the immune system.

The interpretation result of dot intensity shows that biofilm extract has higher density (63170.5) than the negative control group (44070). It refers to the existence of polyclonal antibody on rat serum. The polyclonal antibody has high affinity, avidity, and specificity that is shown by contrast dot in the membrane. The more interaction happens between antibody and antigen, the more contrast dot that will appear in the membrane. It correlated to the concentration of antibody in the serum. It also correlated to the antigen concentration that is shown in table 3. High reaction intensity between antigen and antibody is still detected on the 1/40 dilution of protein extract, but it is relatively lower than the negative control. The measurement of antigenic molecules should be done in further study through another method, such as western blot analysis.

Table 3. Do blot intensity on various antigen dilution

Various antigen dilution	Repetition I	Repetition II
Blanko	11798	12460
1/10	 100090	 130050
1/20	 75604	 56629
1/40	 40508	 119740
1/60	 37563	28721
1/80	22808	21941

CONCLUSIONS

Antigenic protein extract of *Candida* biofilm is able to induce the production of anti-*candida* polyclonal antibody, so it can be used for the development of candidiasis kit diagnostic.

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