THE POTENCY OF PROTEIN EXTRACTS FROM*Candida albicans* AS BIORECEPTOR ON IMMUNOSENSOR FOR DIAGNOSIS OF CANDIDIASIS

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ABSTRACT

Currently diagnosis of candidiasis still usingthe traditional standard blood culture method. The traditional method were less sensitive andtime consuming. The purpose of this research were to develope the more sensitive immunosensor based method, and to examine the potency of C. albicans protein extract as bioreceptor to detect C. albicans and its biofilm in the blood of candidiasis patients.The research methods include: (1) preparation of digestive gland liquid of snail (Achatina fulica); (2) extraction of protein from C. albicans through enzimatis and mechanic methods and (3) analyzing the protein extract as bioreseptor through immunodot assay.The research results showed that the snail enzymes has protein content 1.35 mg/ml and specific activity 1.96 unit/mg. The snail enzyme hydrolyzed the cell wall of C. albicans with and without sonication, produced planktonic extracell protein extract (PEP) and biofilm extracell proteinextract (BEP), planktonic intracell protein extract (PIP), and biofilm intracell protein extract (BIP), with protein content 1.44; 1.29; 1.29 and 1.21 mg/ml respectively. The biofilm intracell protein (BIP) showed antigenic property toward antibody anti-Candida (positive control),giving red spot on imunodot assay. Immunodot assay can distinguish negative control serum (health man) and positive Candidiasis control by using antigen 1 ▪g/▪l and 50 ▪l serum.

Keywords: C. albicans, candidiasis, biofilm, immunodot assay.

INTRODUCTION

Candida spp is a dimorphic fungi normally found in the gastrointestinal tract, upper respiratory tract and genital mucosa of mamalia[1]. However, *Candida* overgrowth may cause a variety of diseases,ranging from mild to severe diseases. The *Candida* infection has increased therapeutical problem in recent years, such as autism, degenerative diseases etc. Species of Candida which is known cause many diseases in both humans and animals is*Candida albicans*.

The conventional laboratory diagnosis of candidiasis haven't given unsatisfied result yet.[2]The convensional standard blood culture method was less sensitive and takes 1-3 days. This case has motivatedmany researchers todevelop an alternative method, such as serological diagnosis through the detection of antigen or antibody.

The body's immune system will produce antibodies against antigenic proteins of *C.albicans*as anatural defense mechanism.The presence of *C. albicans* biofilm in the digestive tract would trigger the formation of specific antibodies as well. Antibodies can distinguish a variety of antigen determinants (epitopes), although it has a little difference in the structural configuration.[3]

Na and Song (1999) reported that the response of antibody Sap (secreted aspartyl proteinase) against *C. albicans* was able to be detected using ELISA (Enzyme-Linked Immuno absorbent Assay). The ELISA method has a sensitivity and specificity of 70% and76% respectively.[7]Beside antibodies, the diagnosis of *C. albicans* invasion can also be done through the detection of antigen. Oliveri (2008) did research using the Platelia *Candida* ELISA for the diagnosis of *Candida* invasion in the premature babies' patients. Detection of *Candida's*mannan (CM) antigen has shown higher sensitivity and specificity, which were 94.4% and 94.2%respectively.[4]

One of the new format to detect antigen or antibody is immunochromatographic biosensor.[5] Biosensor that uses antigen or antibody as bioreseptor is known imunosensor.[6] Gold Immunochromatographic Assay (GICA) is a new immunochromatography technique using nitrocellulose membrane as a carrier and colloidal gold labeled with antigen or antibody as a tracer. This technology has many advantages compared to other immunoassays, such as the simple procedure, rapid results, low price, do not require technicians with special skill so expensive equipment and can be used to detect antigen or antibody. This method has been widely used for the diagnosis of several diseases and detection of bioactive molecules, hormones, and haptens.[7]

In Indonesia, immunochromatography tests have been developed for the identification of a wide variety of antigens or antibodies, such as identification of *Helicobacter pylory* inantibody at Unit of Biomedical – Mataram District General Hospital, and identification of *Plasmodium vivax falciparium* at the Institute of Tropical Disease(ITD) - Airlangga University. Dewi*et al*. (2010) applied imunosensor to detect IgG against *Toxoplasma gondii* by using extracts ESA as bioreseptor and GICA method.[8] While, the immunochromatography test for the identification of *C. albicans* and its biofilm haven't been done..In this research have beenexplored the potential of extracts protein from *C. albicans* as bioreceptor on imunosensor-immunodot assay, as a preliminary studyto develope GICA (Gold Immunochromatographic Assay) method for candidiasisdiagnose.

The purpose of this research was to prove the potential of protein extracts form *Candida albicans* as bioreseptor on imunosensor to detect *Candida albicans* in the blood of patients with candidiasis, especially gastointestinal candidiasis.

MATERIALS AND METHODS

Materials

C. albicans was obtained from the laboratory of Microbiology, Faculty of Dentisty, AirlanggaUniversity. *Achatinafulica* was obtained from field around Kenjeran Complex.

Method

Preparation of *Candida albicans* Planktonic Cells Pellets

After shaking 24 hours, 100mL inoculum of *C. albicans* in YPD liquid medium (Yeast Peptone Dextrose) was centrifuged. The pellets were washed with 0.1 M PBS and was centrifuged again. The precipitate was called*C. Albicans* planktonic cell pellets.

Preparation of *Candida albicans* biofilm pellets

Production of *Candida albicans* biofilm pellets was did with metode Samaranayake, *et al* (2005) and Merritt, *et al* (2005) which have be modification.[9] The sterile cellulose membranes was placed on spider agar media. Furthermore, 50 mL inoculum of *C. albicans* with Optical Density (OD) 0.5 at λ 469 nm was dripped onto membrane and incubated for 24 hours. The Biofilm was separated from membrane and resuspended in PBS solution. The suspension was centrifuged for 15 min. The precipitate was called *C. albicans* cells biofilm pellets.

Isolation of Enzymes from *Achatinafulica*

The suture of *Achatinafulica* was cut and its liquid was taken at cold temperatures. The fluid was centrifuged and its supernatant was tested activity.[10]

Determination of activity and protein content of *Achatina fulica* Enzyme

Enzyme activity of β-1,3-glucanase was determined by measuring the amount of reducing sugars that was produced by hydrolysis of laminarin substrate (SIGMA). The protein content of enzyme was determined using the Lowry method.

Extraction of planktonic and biofilm *C. albicans*protein

Protein extraction was done using the method of Casanova[11]with modification.The*C. albicans* cell pellets were resuspended with 0,6M KCl and centrifuged. The residu was removed and resuspended in phosphate buffer and mixed with *Achatina fulica* enzyme . The mixture was slowly agitated at 28ºC for several hours and centrifuged at 2600 rpm for 10 minutes. Supernatant was removed and re-centrifuged at 20000 rpm for 30 minutes. The resulting supernatant named Protein of Extracellular Planktonic (PEP) extracts. While the pellet I and II werecombined and resuspended in phosphate buffer, and then sonicated. The suspension was centrifuged and its supernatant was removed and named Protein of Intracellular Planktonic (PIP) extracts. For extraction of Biofilms protein, pellets of biofilm were resuspended in PBS. The same method results Protein of extracellular Biofilm (PEB) extract and Protein of intracellular biofilm (PIB) extract.The extract of PEP, PIP, PEB and PIB was concentrated by lyophilization with a freeze dryer and stored at -20 °C.

Preparation of Candidiasis patient blood sample

The blood of patients candidiasis (patients with diabetes mellitus and HIV) werecollected. Blood was slanted on slopes SDA media aseptically, and the remainder is put in Vacutiner tubes. The blood that coats the surface of SDA allowed to grow mold and subsequently cultured.

Meanwhile, the blood in vacutiner was centrifuged to obtain serum and stored at -20°C for further research. The blood of healthy was used as a negative control.

Immunodot Assay

Optimization of antigen attachment

Antigen solution was diluted with use carbonate buffer pH 9.6 to obtain serial levels of protein 1.0; 1.5; 2.0 2.5; and 3.0 ng / uL. 3 uL of antigens was dripped on the surface of polystyrene plastic using a micropipette which will form colorless spots. After the wind dried, antigens were fixed in methanol for 5 min and dried again. For stabilization, section ofantigen was dipped into a solution of 10% sucrose, wind dried and stored at 2 ° C until used.

Optimization of serum volume

Serum of positive controlwas used for optimization of serum volume. The serum was diluted on variety of volum.

Test of anti-*Candida* with *distick* immunodot

50 uL of serum was put intomicroplateof flat-bottomed wells that have been filled with 150 uLTris-HCl pH 7.2. Then the tip of dipstick was put into the wells and incubated at room temperature for 30 minutes. After being washed 3 times using Tris-Cl buffer, the tip of dipstick was inserted into wells containing 200 uLof colloidal gold solution which bound Protein A and incubated for 15 min at room temperature. Than, the dipstick was washed 3 times. The presence of red spots was observed. The positive resultswasindicated by full red color spot formation.

RESULTS and DISCUSSION

Isolation of *Achatina fulica*Enzyme

Extract of enzyme has a protein content of 1,35mg/mL, activity of 2,649 units/ml and specific activity of 1,958 units/mg. Where the unit ofenzyme activityis defined as the amount of β-1,3-glucanase in1mlof extract which produces 1μmol of reducing sugars calculated as glucose per minute per mL at experimental conditions.

Extraction of *C.albicans* protein using combination of enzymatic and mechanical treatment

Extracts of *C.albicans* protein was obtained by lysis of biofilm matrix and the *C. albicans* cell wall using *Achatina fulica* enzymes and sonication. The process of enzymatic lysis by using *Achatina fulica* enzymes about 20 hours longer than zymoliase, which only 2 hours.[11] This case is due to zymoliase enzyme has much higher activity than the *Achatina fulica* enzyme extract.

This research produces four types of protein extracts, that are PEPextract (protein extracellular planktonic), PEB extract (biofilm extracellular proteins), PIP extract (protrein extracellular planktonic) and PIB extract (biofilm extracellular proteins). Based onFigure 1, the protein extractsof PEP and PEB have higher protein content than the protein extracts of PIP and PIB. This case is due to enzyme of *Achatina fulica* soluble and mixed with extracellular protein extracts at the centrifugation process.

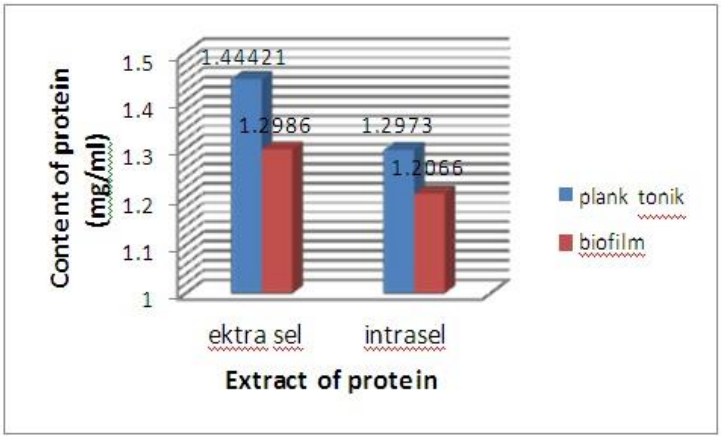

Figure 1. Content of Protein that obtained from lysis of biofilm matrix and *C.albicans* cell walls

ImmunodotAssay

The optimization of antigens attachment on polistyrena paper shows that protein extracts of PIP, PEP and PEB give a yellow brown color spots while the protrein extract of PIB donot give spot.The appearance of brown color spots may disturb the spot color which was generated by reagent signalat immunodot assay. Therefore protein extract of PIB with no spot wasselected asantigens inthe anti-*Candida* dipstick immunodottest.

Based on the results of antigen concentration optimization, protein extract of PIB gives a response in form of red spots against serum of candidiasis positive patient at concentration of1.0▪g/▪L. While the smallest concentration 0,5▪g/▪Ldo not give respond. Thusthe concentration of antigen used in this research was 1.0▪g/▪L with serum volume of 50uL.

The preliminary testof candidiasis diagnosis using dipstick immunodot had been successfully done by using some of ODHA patients and healthy individuals serum as shown in Table1.

Table1. Results of anti-*Candida*test with distickimmunodot

No. sample	Code	Spot of red	Result	Clinical specification
1	P1	there	Pos(+)	ODHA
2	P2	there	Pos(+)	ODHA
3	P3	there	Pos(+)	ODHA
4	H1	There but not intact	Neg(-)	Healthy
5	H2	no	Neg (-)	Healthy
6	H3	no	Neg(-)	Healthy

In this research, antigen was soaked together the patient's serum in a well, in order to enable the antibody-antigen binding and react with reagent signal (protein A labeled with gold colloidal). Protein A resembles secondary antibody which bounds the primary antibody Fc. Therefore in the diagnosis test by immunodot assay is not necessary using secondary antibody. Protein A binds to IgG derived mammals. Antigen binds to the antibody anti-*Candida* that present in the serum of patients forms a complex with the protein A, and gives signal in form of red spots. Red spot appears indicates that the serum of patients was infected by *Candida albicans*. The protein extract of PIB will be applied as bioreseptor on imunosensor GICA (Gold Imunocromatography Assay) in future research. The accuracy and sensitivity of the dipstick immunodottest has'nt been done yet in this research, so further research should also be done by comparing to other serological methods such as ELISA.

CONCLUSIONS

1. The extract of *Achatina fulicha* enzyme has potentionaslysis agent forcell wall and biofilm matrix of*Candida albicans.*
2. Protein extract of PIB (protein intracellular biofilm) has potentialasbioreseptoronimunosensor.
3. Dipstick immunodottest has been done with antigen concentration of 1,0·g/·L and serum volume of 50·L,

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