The Effect of Candida Albicans Secreted Aspartic Proteinase 2 Expressed in Pichia Pastoris on Macrophage Ingestion

1Mahmoodi Elaheh, 2Yadegari Mohammad Hossein, 3Sadeghizadeh Majid and  
2Zahir Hassan Mohammad

1Karaj University of Medical Sciences, Alborz, Iran.  
2Department of Mycology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran.  
3Department of Genetic, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran.  
4Department of Immunology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

Abstract: Background: Candida albicans secrete aspartyl proteinases (Sap), which are of particular interest as virulence factors and had prominent role in Candida adherence, invasion, and pathogenicity. Methods: The methylotrophic yeast Pichia pastoris was chosen as an expression system for preparing substantial amounts of Sap2 isoenzyme (the most important Sap in pathogenesis). The expression protein was purified by Ni-NTA affinity chromatography column. Sap2 was produced as high-level expression and active recombinant enzyme and don’t need any post translation change. At the next step, Produced protein was added to Balb/C macrophage in In-vitro. In order to examine the effect of Sap2 on macrophage ingestion, 10⁷ log-phase candida blastoconidia were added to 2×10⁵ mouse peritoneal macrophages. For investigation of ingestion, supernatant from macrophage lysate inoculated on SCC medium and colony were counted. Results: The result of candida colony number showed, macrophage treatment with Sap2 had ingestion 68.7% less than the negative control (group untreated with Sap2). Discussion: The present work assessed the ability of Sap2 to inhibition of macrophage in response to ingestion of C. albicans blastoconidial.

Key words: Candida Albicans, Cloning, Sap2, Macrophage, Ingestion.

INTRODUCTION

Candida albicans is the most frequently isolated fungal pathogen in humans. The fungus C. albicans behaves as a common as well as a true pathogen of areas such as skin and mucosal surfaces. This organism forms part of the normal micro flora in the gastrointestinal tract and vagina even in individuals who do not have an apparent immunological dysfunction (Anassie, E.J., 2003). In general, superficial mucocutaneous candidiasis is frequent in patients with T-cell deficiencies, such as AIDS patients. The more serious, life threatening, deep-seated or disseminated candidiasis is normally found in a spectrum of severely immunocompromised patients (De Melo, AC., 2007; De Repentigny, L., 2000).

Sap2 is known to degrade many human proteins, including mucin, extracellular matrix proteins, numerous immune system molecules, endothelial cell proteins, and coagulation and clotting factors including molecules that protect mucosal surfaces such as mucin (Naglik, R., 2008; Schaller, M., 2000) and secretory immunoglobulin A (IgA) (Ray, T. and C.D. Payne, 1988). Among yeasts of the genus Candida, those in the species C. albicans show the highest number of genes (10 genes) of the SAP family (Kalkanci, A., 2005). This high number of genes may be related to the frequency of clinic isolates, but could also be associated with a higher capacity of colonization and consequent pathogenic potential of the species (Monod, M., 2004). Saps are differentially regulated during distinct stages of the infection process. Sap1–3 appears to play a role in the adherence and tissue damage of localized infection, whereas Sap4–6 may be of importance in systemic disease (Smolenski, G., 1997; Dabas, N. and J. Morschhauser, 2008; White, T. and N. Agabian, 1995). The different roles of the Sap1–3 and Sap4–6 isoenzyme subgroups are explained by variations in amino acid sequences as well as by different enzymatic characteristics, such as the optimum pH and the net charge. Selective expression/overexpression of genes encoding putative virulence attributes is an attractive strategy to overcome redundancy problems and clarify the contribution of each isoenzyme to virulence. The rationale for this approach is based on clinical studies which have shown that the Sap activity of C. albicans isolates from patients with vaginitis (De Bernardis, F., 1995) or HIV infection (Borelli, C., 2007) was significantly higher than that of isolates from asymptomatic carriers. Fungal antigens may stimulate specific cell mediated and humoral immune responses. It has been well documented that the host defense mechanism against mucosal infection with C. albicans is mediated mainly by cellular immunity and most invasive fungal infection occur in patients with defective cellular immunity. Phagocytic cells such as neutrophils and macrophages are potential components of the immune defense that protects mammals against C. albicans infection (Marcil, A., 2002). Investigations have demonstrated that IFN-γ-activated macrophages required reactive nitrogen intermediates to
Samples were cooled in an ice bath for 10 min and precipitated proteins were removed by centrifugation at 5000 rpm. Incubation at 37 °C, reactions were stopped by adding 700 μl of 10% TCA (w/v) (Tri chloride Acetic Acid). The PCR product was purified using a PCR purification kit (Bioneer). Subsequently purified PCR was digested by SacII and EcoRI (Takara 15U/μl) restriction enzymes designed previously at the 5ˊ of forward and 3ˊ of reverse of primers respectively. PCR product with cloning sites SacII and EcoRI was inserted in pGAPZaA vector. Reaction performed by ration 5/1 of pGAPZaA concentration 50 ng/μl and PCR product 10 ng/μl using DNAT, Ligase (fermentase 5unit/μl) in 16° C for 12hr. Reaction products transformed into E.coli XL1 Blue and grown in LB medium contain zeocin, 100mg/ml. The cloned fragment was sequenced. The sequenced result was confirmed the absence of PCR-induced errors. The cloned fragment was sequenced. The sequenced result was confirmed the absence of PCR-induced errors. 

**Activity Assays:**
30μl (2.25μg/ml) of Sap2 purified was mixed with 270μl 1% BSA (w/v) in 50mM KCl, pH 3.5. After 1 h incubation at 37 °C, reactions were stopped by adding 700μl 10% TCA (w/v) (Tri chloride Acetic Acid). Samples were cooled in an ice bath for 10 min and precipitated proteins were removed by centrifugation at 5000 g for 5 min. In this assay undigested BSA precipitated by centrifuge. The absorbance of the supernatant was measured in OD of A280. For practical purpose, one unit of enzyme activity was defined as the amount of enzyme causing a ΔA280 of 0.1 in 1 h (Simpson, R., 2008).
Western Blotting:

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) of purified Sap2 performed in 12% polyacrylamide. Following electrophoresis, proteins were transferred onto nitrocellulose membranes. Membranes were blocked with 2% (w/v) skim milk in Tris-buffered saline (TBS: 10 mM Tris / HCl, pH 7.5, 150 mM NaCl) and washed between steps with TBS containing 0.1% Tween 20 (TBST). Membranes were incubated for 1h with mouse anti-Sap2p mAb, (Takara/Japan). All of serum samples diluted by 1:100 and 1:200. Bounded proteins on the nitrocellulose reacted with anti-Sap2p mAb. The bound antibody was detected by react the blots with alkaline phosphate-conjugated goat anti-mouse IgG and visualizing by diaminobenzidine (DAB) as a substrate (Saeedinia, A., 2008).

Macrophage Culture and Stimulation:

Female inbred Balb/c mice (8 to 10 weeks old) were purchased from Pasteur Institute (Tehran, Iran). They were kept in animal house of Tarbiat Modares University, given sterilized water and autoclaved standard mouse pellet throughout the study. The animal study was approved by a local Ethics Committee.

Mouse peritoneal macrophages were recovered by rinsing with 10 ml of phosphate- buffered saline (PBS). The cells were washed twice in cold PBS, resuspended in cell culture medium (RPMI) supplemented with 10% FCS (fetal calf serum) and added to 96-well micro plates at a concentration of 2×10^5 cells. Followed by incubation at 37°C for 2hr under 5% CO2. During the course of this incubation, the macrophage was sticking to the cover slip. The non sticking cells were then removed by washing with PBS. More than 95% of adherent cells were macrophages. Then, the adherents macrophage were incubated for 5hr (37°C, 5% CO2) with Sap2 protein at two concentration of 3μg to 12μl with pH 6.9.

Ingestion Assay:

After incubation of macrophage with Sap2, supernatant were removed again and washed with PBS. In next step the macrophage cells were treated with 10^7 Candida blastoconidia in 1ml of RPMI1640. The ratio of yeasts to macrophages was 2:1. Culture plates were then centrifuged to facilitate contact between yeasts and phagocytes and were incubated for 3hr (37°C, 5% CO2). After this time, sterile water contain 1% BSA and 1% tween20 was added to macrophage cells and lysates were placed on sabouraud glucose agar. After incubation of the plates at 37°C for 24 hr, the number of colony-forming units (CFU) was determined as a measure of the number of ingested Candida cells present. The assay was compared with samples of yeast with macrophage without Sap2 under the same assay condition.

Statistical Analysis:

The significance of a difference between two groups was calculated using student's t-test with P < 0.05 used as the significant level. Each experiment was replicated three times.

Results:

Cloning of sap2 in Pichia Pastoris

PCR product with 1200bp (is coordinated in NCBI) encoding Sap2 protein was cloned into the downstream of pGAP signal peptide sequence of pGAPZaA vector (Fig. 1). Construct was confirmed by digestion with two restriction enzymes of EcoRI and SacI1 which were used for cloning (Fig. 2). The result of multiplied sequencing of product has no mutation on active site and binding site. P.pastoris GS115 was used as a host for transformation with BamH1 linerized DNA. Sap2-pGAP construct insert into P.pastoris genome via homologues recombination at the pGAP site. After transformation of vector into the yeast, colonies of yeast that were resistance against Zeocin selected and screened for Sap2 proteinase production. Also PCR of Pichia of selected colonies were done to confirm of homologues recombination (Fig. 3).

Fig. 1: Map of plasmid pGAPZaA; Zeo, zeocin antibiotic gen; P_galactose promoter. 5 AOX1, p.pastoris alcoh oxidase; CYC (TT) cytein terminator gene.
Fig. 2: Double digestion of pGAPZaA+Sap2 construct by EcolRI and SacI1 Double digestion of pGAPZaA+Sap2 construct by EcolRI and SacI1 loaded on 1% gel agarose. DNA marker 100-10000bp (5μg/μl).

Fig. 3: PCR of pGAP, AOXI primer was used for confirm of insert of construct in p. pasturis.

Sap2 Protein Purification:
Sap2 from culture supernatant was purified with Ni-NTA. The efficiency of purification was analyzed by SDS-PAGE/Comass blue staining. Western blotting showed one band at 48kDa. This protein secreted as a single proteins (Fig 4a, b). Sap2 concentration was obtained a yield of 75 ± 0.1μg /ml of culture supernatant calculated by the method of Bradford. The analysis of activity assays data showed that Sap2 had high activity compared to control group and also had a pH optimum at 3.5 (Fig 5). This proteainase was inhibited by pepstatin, the classical aspartyl proteinase inhibitor.

Macrophage Ingestion Assay:
In order to examine the effect of Sap2 on macrophage ingestion, 10^6 log-phase blastoconidia were added to 2×10^5 mouse peritoneal macrophages, which adhered to cover slips and already treated with different concentration of Sap2 isoenzyme. The ratio of infection was five blastoconidia per macrophage. Test was performed for 3hr that was enough to almost all blastoconidia ingested by the phagocytes. Because only ingestion was important, after this time immediately reaction was stopped with lyse of macrophage. At the end of the phagocytes period, samples were plated onto Sabouraud glucose agar. Negative control samples of yeast with peritoneal macrophage without effect of Sap2 were done in the same way. The fungal colonies were counted after 24hr of growth. A clear different in colony count was found between groups of test and control. The results illustrated in Table 1 indicated that number of C.albicans colony isolated from the lysis macrophage treated with Sap2 were significantly (P < 0.001) lower than those of control groups (Fig. 6). Also, these results revealed that Sap2 in 12μg/ml concentration had more effect on macrophage inhibition and reduce of ingestion in compared with 3μg/ml Sap2. The experiment was repeated three times, and similar results were obtained.

Discussion:
Although Candida albicans is the most frequently isolated yeast associated with human infection, changing patterns of the Candida species detected among clinical isolates in the last decade are evident. Therefore, rapid and reliable identification of Candida species producing certain virulence factors is important in routine clinical microbiology practice (Jochen, D., 2003). One of the most important virulence factors associated with invasive candidiasis are extracellular acid proteinases, hydrolytic enzymes produced by Candida (Schaller, M., 2001), which allow the proteolytic invasion of tissues by these yeasts and interfere with host cell membrane integrity, leading to dysfunctions of their normal activities (Morrow, B., 2006). The acid proteinase seems to facilitate the adherence, colonization, growth, and invasion of these microorganisms on the skin and mucosa (Chen, K., 2005). Consequently, the production of extracellular proteolytic enzymes may serve as a pathogenicity marker for these microorganisms (Staib, P., 2008). In this paper we described the cloning and the over expression of the
Sap2 gene in *P. pastoris*, that allowed to high-level expression with a yield of 75 ± 0.1 μg /ml. In contrast the yield of Sap2 produced in *Ecoli* was about 100 time lower (Smolenski, G., 1997). Sap2 produced had high activity in compared to control group and also had a pH optimum at 3.5. Western blotting analyses of the Sap2 detect Sap2 proteinase as a single band on 48kD. After treatment of Sap2 on macrophage, this organism had less ability in ingestion of *Candida* blastoconidial.

![Protein profile of the culture supernatant of *P. pastoris* producing Sap2.](image)

**Fig. 4a:** protein profile of the culture supernatant of *P. pastoris* producing Sap2. The protein in 10 μl of supernatant was loaded onto 12% SDS-PAGE. The gel was stained with comassie brilliant blue. Protein marker: 116, 66.2, 45, 35, 25, 18.4, 14.4KD. (Fermentase#RD0431). Single band were seen with 250mM imidazol buffer.

![Western blotting of the monoclonal Anti Sap2 with proteinase antigen Sap2 expressed in *P. pastoris*.](image)

**Fig 4b:** Western blotting of the monoclonal Anti Sap2 with proteinase antigen Sap2 expressed in *P. pastoris*. 0.5 μg/ml proteinase antigen was reacted with Sap2 mAb. Single band in 48kD visualized by DAP.

![Activity assay of *C. albicans* Sap2 with BSA in KCL buffer.](image)

**Fig. 5:** Activity assay of *C. albicans* Sap2 with BSA in KCL buffer. One unit of enzyme activity was defined as the amount of enzyme causing a ΔA280 of 0.1 in 1 h. The activity is depicted as a percentage (%) of activity at the optimum pH.

<table>
<thead>
<tr>
<th>groups</th>
<th>Mean number of colony (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment with Sap2</td>
<td>297 ± 5.5*</td>
</tr>
<tr>
<td>Control group</td>
<td>684 ± 3</td>
</tr>
</tbody>
</table>

* Significant differences with control group (P < 0.001).
Fig. 6: The results of colony number of blastoconidial after ingestion by peritoneal macrophage in test group and negative control. The percentage of the colony was measured in a CFU assay. The fungal colonies were counted after at least 24h of growth.

*C. albicans* represent mechanisms of immune evasion that contributes to the virulence (Taylor, B., 2005). Killed *C. albicans* could inhibit IFN-γ release by murine natural killer cells. IFN-γ production activates phagocyte cells, and up-regulates the fungicidal activity of these cells (Murciano, C., 2006). The uptake of invading microorganisms by phagocytes is followed by the fusion of cellular lysosomes containing hydrolytic enzymes with the phagosome containing microorganisms to form the microbicidal phagolysosomes. The pH within phagolysosomes, of the order of PH 4.7-4.8 (Ohkuma, S., 1978) favors the activity of the host acid lysosomal hydrolases However, it is also optimal for the enzyme activity of Sap2 as shown in this study.

A possibility of Sap2 activity is that Sap isoenzymes could act as cytolysins, as described for *Trypanosome cruzi*, *Listeria* and *Shigella* (Andrews, N., DA, 1994) other possibility is that it could also affect some key enzymes of the macrophage oxidative metabolism which is important for optimal microbicidal activity (Miller, R.A., 1997). Also the phagosome-lysosome fusion enhances the activity of potential phagogenic factors as observed previously for *T. cruzi* or *M. Tuberculosis* (Tradieux, I., 1992). Howere, the activity of Sap2 enzyme would be enhanced by the change in pH that occurs during the phagosome-lysosome fusion. After 4hr of ingestion of *Candida* by macrophage, the phagocytosed blastoconidia started to resist their phagocytes by forming germ tubes (Russel, D.G., 1995). Also this organism elicited a weaker respiratory burst. These results could be explained by the existence of fungal factors that are able to influence the oxidative metabolism of the phagocyte defense system negatively (Sasada, M., 1980). Our results also confirm this idea and showed that Sap2 isoenzyme is an important factor that could contribute to the inhibition of the macrophage and influence the pathogenesis of invasive *Candidiasis*. The results of this study show that sap2 alters ingestion blastoconidial by macrophages. Based on this information it may be suggested that reduction of *Candida* infection is due to protein alteration and probably reduce of receptor expression in the macrophage cell membrane. The results also indicate that Sap2 play a clear role in inhibition of immune system and may constitute a novel target immunotherapy that is important for improvement and production of new drugs.

REFERENCE


