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Evaluation of Effect of Secreted Aspartyl Proteinase 2 from *Candida Albicans* on Macrophages Activity against Blastoconidial *In-vitro*

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ABSTRACT

Background: *Candida albicans* is a fungus that alters into a pathogenic organism under appropriate conditions and results in fungal infection. Secreted aspartic proteinase (Sap) activity is considered an important virulence factor in such infections. We examined the *in vitro* effect of Sap2 *Candida albi*cans on NO production by murine peritoneal macrophages.
Methods: The methylotrophic yeast *Pichia pastoris* was chosen as an expression system for preparing substantial amounts of Sap2 isoenzyme (the most important Sap in pathogeneses). In this study, Sap2 was produced as a high-level expression and active recombinant enzyme without any post-translation change. Then, the effect of the produced protein on macrophage activity (ingestion, Nitric oxide release) was evaluated against blastoconidia in Balb/c macrophages. In order to examine the effect of Sap2 on macrophage ingestion, 10^6 log-phase *candida* blastoconidia were added to 2×10^5 mouse peritoneal macrophages. To swallowing assessment, the supernatant from macrophage lysate inoculated on SCC medium and colonies were counted.
Results: The result of *candida* colony number showed that the ingestion of macrophage treatment with Sap2 was 68٪ less than that of the negative control (group untreated with Sap2). We examined the *in vitro* effect of *Candida albicans* Sap2 on NO production by macrophages. Nitric oxide assay was also performed using the grass method. Sap2 of Candida albicans also inhibited NO production.
Discussion: Our results suggest that *Sap2* may evade host defense mechanism(s) through NO production suppression—mediated mechanism of stimulated macrophages.The present work assessed the ability of Sap2 to inhibit the macrophage in response to the ingestion and NO production of *C.albicans* blastoconidial.
KEY WORDS: *Candida albicans*, cloning, Sap_2, Macrophage, ingestion, No production.

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 (1). 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 (2, 3).

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 (4,5) and secretary immunoglobulin A (IgA) (6). Among yeasts of the genus *Candida*, those in the species *C. albicans* show the highest number of genes (10 genes) of the SAP family (7). 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 (8). 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 maybe of importance in systemic disease (9–11). 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/over expression 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 (12) or HIV infection (13) was significantly higher than that of isolates from asymptomatic carriers. Fungal antigens may stimulate specific cell mediated and humoral immune responses. It

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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. Phagocyte cells such as neutrophils and macrophages are potential components of the immune defense that protects mammals against *C. albicans* infection (14). Investigations have demonstrated that IFN-γ-activated macrophages required reactive nitrogen intermediates to exhibit effective fungicidal activity against yeast and hyphal forms of *C. albicans* (15). Using *Pichia pastoris* as a high expression system, we produce high amount of active recombinant Sap2. Also we show here that Sap2 can reduce of macrophage membrane in *Candida* blastoconidial ingestion.

Experimental procedures

Strains and plasmids

Escherichia coli XL1 Blue was used for transformation with competent cells and propagation of the recombinant plasmid. The *Ecoli-P.pastoris* shuttle vector pGAPZαA (3100bp) was provided from invitrogen. In this plasmid BamH1 site is flanked for linearization of the vector before transformation into *P.pastoris* genom. Zeocin gene was a selective factor. Monoclonal Antibody was used for western blotting provided of Takara Company (Japan). *P.pastoris Gs115 His- Mut+* used as a host for homologus recombinant and *Candida albicans* strain was obtained of the Persian type culther collection (PTCC).

Cloning of Sap2 in pGAPZαA

PCR was performed using homologues primers derived from DNA sequences of the Sap2 gene including the regions which encode their pro-peptide. Pairs of PCR primers employed were provided by Bioneer.

Forward: 5′-CACGAATTCACTCCAACAACAACCAAAA-3′

Reverse: 5′-CATCCGCGGAGGTCAAGGTCAAGGCAGAAATACA-3′

The PCR was carried out in thermal cycler (with a initial denaturation of 2 min at 94° C, followed by 25 cycles of Annealing at 48°C for 1′:20″ and Elongation at 72° C for 2 min. Second denaturation at 94° C for 30 s. PCR was completed by a final elongation step at 72° C for 10 min.

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 5′of forward and Reverse of primers respectively. PCR product with cloning sites *SacII* and *EcoRI* was inserted in pGAPZaA vector. Reaction performed by ration 5/1 of pGAPZαA concentration 50 ng/µl and PCR product 10 ng/µl using *$DNAT_4$ 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.

P.pastoris GS115 was transformed with 10µg of *BspH1*-linearized pGAPZaA+Sap2 vector by the Litium acetate/ ssDNA / PEG method (16). Sap2 gene was accomplished by homologous recombination into yeast genome. Transforming colonies was screened on YPD agar containing yeast extract at 1% (w/v), peptone at 1% (w/v), and dextrose at 2% (w/v) with zeocin as a selective antibiotic containing 100 mg/µl zeocin in 30° C for 72h. The selected transforming colonies were grown in 2ml of YPD without zeocin at 30°C. After 2 days incubation, 10µl of supernatant was loaded on SDS-PAGE gels to identify colonies expressing Sap2. Also for confirm of true homologous recombination, the true transforming colonies was screened for insertion of the construct at the AOX1 site by PCR (according to the recommendation of the manufacture- invitrogene) (17). Transforming *Pichia* genome was used as DNA template for the PCR reaction.

Forward: pGAP 5′- GTCCCTATTTCAATCAATTGAA-3′

Reverse: AOX1 5′- GCAAATGGCATTCTGACATCC-3′

Difference between sizes of bands confirmed insertion of sap2 in *Pichia* genom.

Purification of recombinant protein

One selected colony was grown in 10 ml YPD at 30° for 48h. After incubation for desalting, the culture supernatant was dialyzed against a 100-fold volume of 10 mM citric acid/NaOH pH 6.8(18). Dialyzed solution was concentrated to 5 mL using an Amicon concentrating cell (10 kDa MWCO). Produced Sap2 was purified by Ni-NTA (Nikle His- select, Amersham Pharmacia Biotech) affinity chromatography column with flow rate of 1ml/min. The recombinant Sap2 isoenzyme was eluted with gradient imidazol concentration. Optimal Elution buffer was 250 mM imidazole buffer pH 7.0. 10 µl of eluted solution was loaded on SDS_PAGE gel 12% stained with coomassie brilliant blue (Bio-Rad). Protein concentration was measured by the method of Bradford (19).

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 for10 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 A_{280}. For practical purpose, one unit of enzyme activity was defined as the amount of enzyme causing a ΔA280 of 0.1 in 1 h (20).

Western blotting

Sodium dodecyl sulfate- polyacryl amide gel electrophoresis (SDS-PAGE) of purified Sap2 performed in 12% polyacryl amide. 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 di aminobenzidine (DAB) as a substrate (21).

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 2×10^5cells, 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 10^6 *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 h, 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.

NO Assay

NO is unstable and rapidly converts to nitrite and nitrate. Accordingly, we estimated the level of NO synthesis by macrophages via measuring the amount of nitrite accumulating in the cultures using the method of Stuehr & Nathan [22]. Briefly, 100 ml of supernatants were mixed with the same volume of Griess reagent, and absorbance was read at 550 nm using an automated microplate reader. The concentration of nitrite was estimated from a NaNO2 standard curve.

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 pGAPZαA vector (Fig1). Construct was confirmed by digestion with two restriction enzymes of *EcoR1* and *Sac11* which were used for cloning (Fig2). 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).

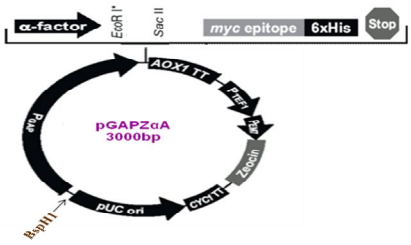

Fig 1. Map of plasmid pGAPZαA; *Zeo,* zeocin antibiotic gen; $P_{GAP,}$ galactose promotor. 5 *AOX1*, p.pastoris alchol oxidase; *CYC (TT)* cyctein terminator gene.

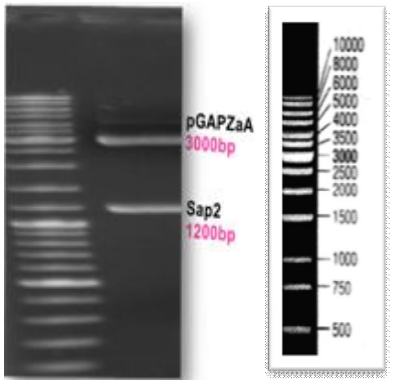

Fig 2.Double digestion of pGAPZαA+Sap2 construct by *EcolR1* and *Sac11* Double digestion of pGAPZαA+Sap2 construct by *EcolR1* and *Sac11* loaded on 1٪gel agarose. DNA marker 100-10000bp (5µg/µl).

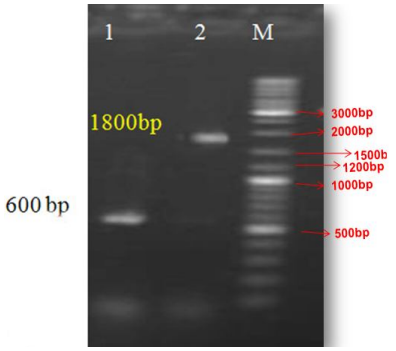

Fig 3: PCR of *pGAP, AOX1* primer was used for confirm of insert of construct in *p. pasturis*.

Sap2 Protein Purification

Sap2 from culture supernatant was purified with Ni-NTA. The efficiency of purification was analyzed by SDS-PAGE / Comassi blue staining. Western blotting showed one band at 48kDa. This protein secreted as a single proteinas (Fig4a, 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 (Fig5). This protainase was inhibited by pepstatin, the classical aspartyl proteinase inhibitor.

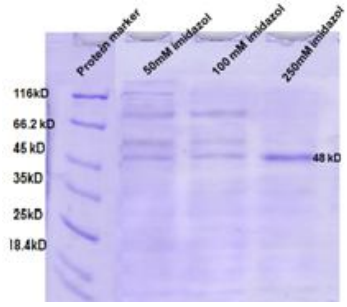

Fig 4a: protein profile of the culture supernatant of *P.pastoris* producing Sap2.The protein in 10 µl of supernatant was loaded onto 12٪ SDS-PAGE. The gel was stained with comassie brilliant blue. Protein marker: 116, 66.2,45,35,25,18.4,14.4KD).(Fermentase#RD0431). Single band were seen with 250mM imidazol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

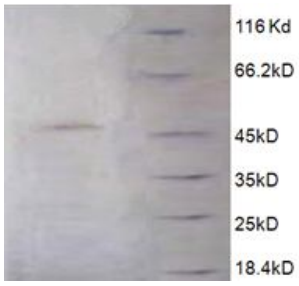

Fig 4b: Western blotting of the monoclonal Anti Sap2 with proteinase antigen Sap2 expressed in P.pastoris.0.5 µgml^{-1}proteinase antigen was reacted with Sap2 mAb. Single band in 48kD visualized by DAP.

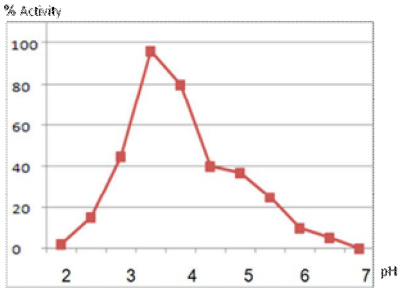

Fig 5: Activity assay of *C.albicans* Sap2 with BSA in KCl buffer. One unit of enzyme activity was defined as the amount of enzyme causing a ΔA280 of 0.1 in 1 h. The activity is depicted as a percentage (٪) of activity at the optimum pH.

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 lyses of macrophage. At the end of the phagocytosis 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 lyses macrophage treated with Sap2 were significantly ($P < 0.001$) lower than those of control groups(Fig6). 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.

Table1.

groups	Mean number of colony ٪
Treatment with Sap2	297 ± 5.5*
Control group	684 ± 3

* Significant differences with control group ($P < 0.001$)

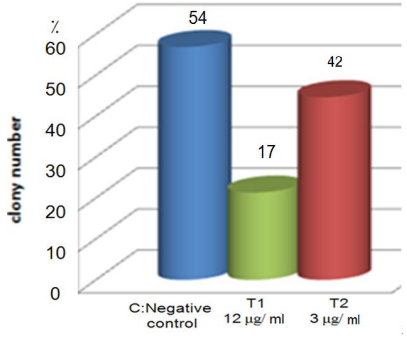

Fig 6: The results of colony number of blastoconidial after ingestion by peritoneal macrophage in test group and negative control. The percentage of the colony was measured in a CFU assay. The fungal colonies were counted after at least 24h of growth.

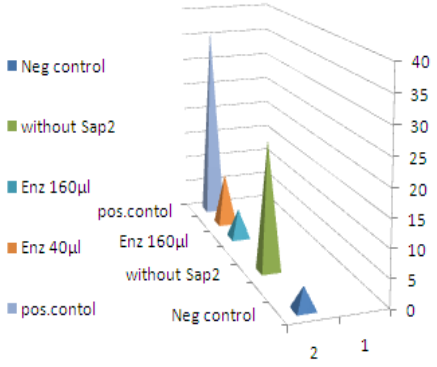

Fig7. Sap2 C.alb has an inhibitory effect on NO produce

Reduction of NO Production by Sap2 in MQ

We initially stimulated murine peritoneal macrophages by Sap2 (3 µg/ml and 12 µg/ml) for 5 hours. To examine the effect of *Candida albicans* on NO production, a variable number of *Candida albicans* was added to macrophage cultures. Then measured nitrite content in the culture supernatant. As shown in Fig7 and Table 2, *Candida albicans* showed a dose-dependent inhibition of NO production and caused an almost complete inhibition at 12µg/ml concentration of Sap2. NO production was strongly inhibited when Sap2 activated peritoneal macrophages were cultured with *Candida albicans*. In contrast, in the same chamber, when these cells were cultured separately in the absence of Sap2, no inhibition was noticed from NO production.

Table 2: Treatment with Sap2

Groups	Frequency	Averag
Enz 3µg	3	9.6
Without Enz	3	23.3
Enz 12µg	3	5.4
Pos Control	3	35.8
Neg Control	3	4.08

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 (23). Some of the most important virulence factors associated with invasive candidiasis, are extracellular acid proteinases, as well as hydrolytic enzymes produced by *Candida* (24) which allow the proteolytic invasion of tissues by these yeasts and interfere with the host cell membrane integrity leading to dysfunctions in their normal activities (25). The acid proteinase seems to facilitate the adherence, colonization, growth, and invasion of these microorganisms on the skin and mucosa (26). Consequently, the production of extracellular proteolytic enzymes may serve as a pathogenicity marker for these microorganisms (27). In this study, we described the cloning and the over expression of the *Sap2* gene in *P. pastoris*, that led 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's lower (28). The produced *Sap2* was highly active compared to the control group and had also a pH optimum at 3.5. Western blotting analyses of the Sap2 detect Sap2 proteinase as a single band on 48kD. After the treatment of Sap2 on macrophage, this organism had less ability to ingest *Candida* blastoconidial.

C. albicans represent mechanisms of immune evasion that contribute to the virulence (29). 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 (30). 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(31) favors the activity of the host acid lysosomal hydrolyses. 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* (32). Another possibility is that it could also affect some key enzymes of the macrophage oxidative metabolism which is important for an optimal microbicidal activity (33). Also, the phagosome-lysosome fusion enhances the activity of potential phagogenic factors as observed previously for *T.cruzi* or *M.Tuberculosis* (34). Howere, the activity of Sap2 enzyme would be enhanced by the change in pH that occurs during the phagosome-lysosome fusion. After 4 hours of ingestion of *Candida* by macrophage, the phagocytosed blastoconidia started to resist their phagocytes by forming germ tubes (35). Moreover, this organism elicited a weaker respiratory burst. We also examined the *in-vitro* effect of *Candida albicans* Sap2 on NO production by macrophages. Sap2 also inhibited NO production. The reduction in the production of NO was not due to the cytolysis effect of the culture supernatant, because the number of viable macrophages, estimated by trypan blue exclusion method, was not significantly different at the end of cultures (percentage of viable cells 80·263·5%). Since NO-mediated mechanisms play a central role in macrophage-mediated killing of *Candida albicans* [36], it may be possible that the effects of *Candida albicans* described in the present study represent putative mechanisms for the evasion of the host defence systems and multiplication of the fungus in host tissues.

It has recently been demonstrated that *Cryptococcus neoformans*, another fungal pathogen, also inhibits NO production by macrophages [37]. However, based on the results of double-chamber experiments, *Cryptococcus neoformans*-induced suppression of NO production was mediated to a large extent by a direct contact of the organism with macrophages rather than by a soluble factor.

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 (38). Our results also confirm this idea and showed that Sap2 isoenzyme is an important factor that can contribute to the inhibition of the macrophage and influence the pathogenesis of invasive *Candidiasis*. According to the findings of the present study, sap2 alters the ingestion blastoconidial by macrophages. Based on this information, it may be suggested that the reduction of *Candida* ingestion is due to protein alteration and probably the reduction of receptor expression in the macrophage cell membrane. The results also indicate that Sap2 plays a clear role in the inhibition of the immunity system and may constitute a novel target immunotherapy that is important for the production and improvement of new drugs.

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