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Departamento de Biologia Vegetal

A differential polypeptide approach to fight human fungal pathogens

Ana Margarida da Silva Pinheiro

Mestrado em Microbiologia Aplicada

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A differential polypeptide approach to fight human fungal pathogens

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MASTER THESIS

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Resumo

Os fungos patogénicos representam, à escala mundial, uma séria ameaça para a saúde humana, verificando-se, nas últimas duas décadas, um aumento significativo de infecções fúngicas graves devido a fungos patogénicos oportunistas. Apesar das notáveis melhorias que se têm verificado na descoberta de novos agentes antifúngicos nos últimos 10 anos, o diagnóstico de infecções fúngicas é ainda um problema devido à limitada disponibilidade de agentes antifúngicos e, consequentemente, as infecções fúngicas tornaram-se num importante factor de mortalidade e morbilidade e cada vez mais representam um fardo no sistema de saúde.

O aumento acentuado de fungos patogénicos menos comuns mas clinicamente importantes é uma consequência directa do aumento do número de pacientes que possuem um sistema imunitário deficiente, que por sua vez é uma consequência do aumento do número de casos de SIDA e dos avanços na tecnologia terapêutica. No entanto, enquanto que no passado as micoses oportunistas ocorriam tipicamente em hospedeiros imunocomprometidos, estas complicações são cada vez mais observadas em pacientes adultos doentes, cirúrgicos mas não-imunocomprometidos.

As leveduras são um dos grupos de microrganismos causadores de infecções fúngicas humanas, sendo a candidemia uma das principais causas de infecções nosocomiais. As espécies de *Candida* são leveduras comensais de humanos que causam infecções tanto superficiais como invasivas. Estes organismos ocorrem abundantemente no tracto gastrointestinal humano e na vagina, podendo ser isoladas a partir de fezes e de mucosas onde existem como organismos comensais. Quando se tornam patogénicos podem provocar candidíase disseminada, uma síndrome que pode colocar a vida em risco com uma mortalidade atribuída de 10-50%. De acordo com *ARTEMIS Global Antifungal Surveillance Program, C. albicans* é a espécie de *Candida* mais comum (63-70%) em infecções fúngicas invasivas, seguida de *C. glabrata* (44%), *C. tropicalis* (6%) e *C. parapsilosis* (5%).

Relativamente às opções de tratamento, o agente antifúngico ideal deve possuir um largo espectro de acção, baixos níveis de resistência, vias de administração flexíveis, causar poucos efeitos secundários e ter interacções limitadas com outras drogas. Actualmente existem quatro grupos de antifúngicos disponíveis para o tratamento de micoses sistémicas em humanos, os polienos, azóis, equinocandidas e a flucitosina. Nenhuma destas classes de antifúngicos possui todas as características anteriormente descritas o que em última análise conduz a falhas no tratamento. Dada a substancial mortalidade e morbilidade associadas com infecções fúngicas invasivas, o tratamento utilizando uma combinação de agentes antifúngicos é muitas vezes considerado. É também essencial identificar novos e potentes agentes antifúngicos, com novos modos de acção, de modo a combater o actual aumento de infecções fúngicas.

Foi desenvolvida uma pesquisa no Instituto Superior de Agronomia que culminou na descoberta de uma abordagem nova e original na luta contra os fungos patogénicos. Esta abordagem refere-se a um polipéptido singular, denominado BLAD (Banda de *Lupinus Albus* Doce, num gel de electroforese), que possui uma forte actividade inibitória na germinação e desenvolvimento de esporos de fungos patogénicos. A BLAD apresenta uma actividade antifúngica tanto preventiva como curativa, é activa contra uma ampla gama de fungos patogénicos ao mesmo tempo e o desenvolvimento de resistência por parte dos fungos é improvável. Apesar de todos os resultados

animadores obtidos até à data, pouco se sabe acerca do mecanismo de acção da BLAD, existindo apenas algumas evidências de que actua ao nível do envelope celular (membrana plasmática e parede celular).

Este plano de trabalho está inserido num projecto mais amplo cujo objectivo é determinar se a BLAD é eficaz no tratamento de infecções fúngicas humanas. Para tal, e tendo em conta que o objectivo final é aplicar a BLAD na área clínica, o primeiro passo passou por produzir este polipéptido através da sua expressão heteróloga, sob a forma recombinante. Actualmente existem inúmeros hospedeiros novos e sofisticados mas, considerando a ausência de resíduos glicosídicos no polipéptido BLAD e as muitas vantagens que o sistema de expressão usado pela *Escherichia coli* apresenta, este foi o microrganismo escolhido como célula hospedeira. O gene que codifica para o polipéptido BLAD já se encontrava inserido no plasmídeo pET 151 D-TOPO® e, portanto, o primeiro passo passou por clonar o plasmídeo em One Shot® TOP10 Chemically Competent *E.coli,* para confirmar a correcta inserção do inserto, e em BL21 Star™(DE3) One Shot® Chemically Competent *E. coli* para a expressão do produto. Fizeram-se vários ensaios de expressão de modo a perceber o tempo óptimo de indução necessário para a obtenção de uma maior expressão do produto, o que permitiu concluir que o maior rendimento era atingido ao fim de 6 horas de indução com IPTG *(Isopropyl β-D-1-thiogalactopyranoside*). De seguida procedeu-se à purificação da BLAD recombinante com recurso a colunas de Ni-NTA e *Dialysis step-wise*. Uma vez purificada a BLAD recombinante, realizaram-se diferentes *Immuno blots* para confirmar que se tratava de facto do polipéptido BLAD, com recurso a um anticorpo específico para o mesmo, e para verificar se este mantinha actividade de lectina (pela sua capacidade de reconhecer anticorpos inespecíficos), propriedade característica da BLAD. Para tal utilizou-se como anticorpo primário um anticorpo inespecífico. A presença de sinal em ambos os *Immuno blots* revelou que não só se tratava de facto da BLAD como esta mantinha a actividade de lectina. Por último testou-se a actividade antifúngica da BLAD recombinante em *C. albicans.* Os resultados obtidos demonstraram que esta possui forte actividade antifúngica visto que é necessária, para igualdade de outros factores, uma menor concentração para provocar o mesmo grau de inibição e morte do microrganismo que a BLAD purificada do tremoço. Apesar de se ter revelado um sistema de expressão bastante promissor e se terem obtidos excelentes resultados com a BLAD recombinante, não foi possível obter quantidade de proteína suficiente para o restante trabalho, pelo que o trabalho foi continuado com recurso à BLAD purificada do tremoço.

De seguida, foram avaliados os efeitos morfológicos e fisiológicos da BLAD, usando *C. albicans* como modelo de fungos patogénicos unicelulares. Começou por se determinar a Concentração Mínima Inibitória (MIC) e Mínima Fungicida (MFC) de BLAD, em diferentes meios de cultura, com diferentes densidades de inóculo. Prosseguiu-se o trabalho apenas com o meio em que a BLAD revelou ser mais letal (meio PDB) e com a densidade de inóculo óptima para os ensaios seguintes (10^5 células/mL), condições onde são necessários 125 µg/mL de BLAD para inibir o crescimento do microrganismo e 250 µg/mL para provocar a sua morte. Uma vez optimizadas as condições de crescimento e as concentrações de BLAD, realizou-se uma curva de crescimento de *C. albicans* exposta às concentrações inibitória e letal de BLAD, de modo a estudar o seu efeito no

crescimento da levedura. A curva obtida permitiu concluir que a adição de BLAD ao meio de cultura tem um forte efeito no crescimento de *C. albicans* visto que células expostas à concentração inibitória de BLAD apresentam uma diminuição na taxa de crescimento, quando comparado com a situação controlo, e as expostas à concentração letal tornam-se não viáveis ao fim de 4 horas de exposição. Em simultâneo foi avaliada a actividade metabólica e a integridade da parede celular de *C. albicans,* recolhendo amostras ao longo da curva de crescimento e visualizando-as num microscópio de fluorescência. Foram adicionados dois fluorocromos às amostras, FUN-1, indicador de actividade metabólica e, consequentemente, viabilidade, e *Calcofluor white*, marcador de parede celular. Os resultados obtidos sugerem que ao fim de 12 horas de incubação com a concentração letal de BLAD, as células tornam-se metabolicamente inactivas, não viáveis e não cultiváveis mas, no entanto, não se verificam alterações visíveis ao nível da integridade da parede celular de *C. albicans.* Por último determinou-se a localização celular da BLAD aquando da ocorrência de morte celular. Estudos anteriores realizados com Alexa Fluor® 488, composto fluorescente com elevada afinidade para o grupo amina das proteínas, demonstravam que a BLAD se situava no interior da célula. No entanto este método revelou-se inconclusivo e, como tal, recorreu-se à imunofluorescência que, numa primeira fase, revelou que a BLAD se liga ao envelope celular, sem destabilizar a parede celular, mas não entra para o interior da célula. Com o objectivo de esclarecer se a ligação da BLAD à célula se dava ao nível da membrana ou parede celular, realizou-se nova imunofluorescência com protoplastos de *C. albicans*, o que revelou que a BLAD se liga à membrana celular, sem vestígios da mesma no interior da célula.

Por último, procurou-se os alvos específicos da BLAD no envelope celular dos agentes patogénicos, utilizando para isso, proteínas da membrana dos protoplastos de *C. albicans*. Começou por se analisar o perfil proteómico da membrana celular de *C. albicans,* após remoção da parede celular, através da formação de protoplastos. De seguida, e com o objectivo de identificar os resíduos glicosídicos alvo do polipéptido BLAD, a fracção proteíca da membrana celular de *C. albicans* foi sujeita a três processos de desglicosilação: remoção dos oligossacáridos *N*-ligados a proteínas, dos oligossacáridos *O*-ligados a proteínas e de ambos. O perfil electroforético obtido revelou que, tal como esperado, existe uma variação ao nível do peso molecular das proteínas, resultante da remoção dos resíduos glicosídicos. É de salientar que após remoção dos oligossacáridos *O*-ligados a proteínas o perfil das proteínas de membrana de *C. albicans* foi drasticamente alterado, resultando no desaparecimento das proteínas de maior peso molecular. Após incubação da BLAD com cada tipo de membrana celular sujeita a diferentes processos de desglicosilação, verificou-se que o polipéptido possui elevada afinidade para a membrana celular de *C. albicans*, o que está de acordo com os resultados obtidos na imunofluorescência, uma vez que permanece ligada mesmo após o tratamento para a remoção dos oligossacáridos *N*- e *O*-ligados a proteínas em simultâneo. No entanto, não foi possível determinar o alvo específico da BLAD na membrana celular de *C. albicans* uma vez que esta apresenta elevada afinidade para todos os tipos de proteínas desglicosiladas testadas presentes na membrana celular. Este resultado pode ter como explicação o facto de a BLAD poder ter mais que um alvo ou, mais provavelmente, que os métodos usados para a *N*- e *O*-desglicosilação não terem sido

100% eficazes para estas condições e, portanto, podem não ter removido na totalidade os oligossacáridos das glicoproteínas presentes na membrana celular de *C. albicans*.

Palavras-chave: BLAD, antifúngicos clínicos, proteínas recombinantes*, Escherichia coli, Candida albicans,* oligossacáridos

Abstract

Pathogenic fungi represent, worldwide, a serious threat for human's health, being *Candida albicans* the most common cause of invasive fungal infections. All antifungal agents available present many disadvantages and, therefore, it is essential to identify new potent and safe antifungal drugs with novel modes of action. BLAD is a novel polypeptide derived from *Lupinus* which exhibits a powerful inhibitory activity upon germination and development of spores from human fungal pathogens and a major goal is to use BLAD in the clinical area.

With that in mind, the first objective was producing BLAD in a recombinant form, through its heterologous expression, using *Escherichia coli* as the host cell. The production and purification of recombinant BLAD was achieved, and it possess the same biological activities as its *"Lupinus"* form, such as a strong antifungal activity.

The second goal was assessing the physiological and morphological effects of BLAD on fungi, using *C. albicans* as a unicellular pathogenic fungal model. Upon exposure of *C. albicans* to lethal concentration of BLAD, the yeast became metabolically inactive, non-viable and nonculturable. Moreover, the results obtained suggest that BLAD passes through the cell wall and binds to the plasma membrane, but it does not enter into the cell.

Finally, the search for specific targets for BLAD in the pathogen cell envelope was assessed, using *C. albicans* membranes. Although having high affinity to all types of deglycosylated proteins from the cell membrane tested, the specific type of glycoprotein which is targeted by BLAD was not uncovered. This possibly means that BLAD has more than one target, and/or, more likely, the deglycosylation mehtods used in the present work did not fully remove either *N*-linked and/or *O*-linked oligosaccharides in these conditions from *C. albicans* cell membrane glycoproteins.

Key words: BLAD, clinical fungicide, recombinant proteins, *Escherichia coli*, *Candida albicans*, oligosaccharides

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Chapter I
Introduction

I.1 Increase of fungal infections in humans

Pathogenic fungi represent, worldwide, a serious threat for human's health with significant increase of severe fungal infections due to opportunistic fungal pathogens over the past two decades [1]. Since mid-1980s many hospitals reported that fungi were becoming common pathogens in nosocomial infections [2] and, in addition, a study published recently shows that the incidence of nosocomial fungaemia was 2 per 1000 hospital admissions, which is associated with 48% rate of mortality [3]. Moreover, the rate of sepsis due to fungal organisms in the United States increased 207% during the period between 1979 and 2000.

Despite the remarkable improvements that have been made in diagnostic modalities and antifungal agents in the past 10 years, the diagnosis of fungal infections is still difficult compared to the diagnosis of bacterial infections by conventional culture, and treatment remains a great challenge because of the limited availability of antifungal agents and of their questionable efficacy [4]. The recent epidemiological data underscore the high potential for and the threat of emerging and re-emerging pathogenic fungi to be transmitted in unexpected geographic and clinical settings likely due, at least in part, to present day sophisticated logistics solutions and easy travel around the globe, facilitating the transmission of microorganisms despite increased security measures and protection efforts. Fungal infections have thus become an important factor of morbidity and mortality and represent an increasing burden on medical systems [5]. The most common infections are bloodstream infections (BSI) and invasive fungal infections.

I.2 Rise in the number of patients with a dysfunctional immune system

The sharp increase of less common but medically important fungal pathogens is a direct consequence of the rising number of patients bearing a dysfunctional immune system [6]. Although recent progresses in medicine have improved control of infectious diseases, at the same time the advent of certain medical practices has actually favored the occurrence of microbial infections [5]. Consequently, the incidence of invasive fungal infections has increased and the population of patients at risk has expanded to include those with a broad list of medical conditions [7]. In this turn, the raise of immunocompromised patients is a direct cause of the AIDS epidemic, advances in therapeutic technology (including organ transplant development), the use of increasingly aggressive regimes of chemotherapy and the insertion of intravascular devices [8]. The incidence of invasive fungal infections has also increased significantly and has emerged as a worldwide healthcare problem, as a result of the rapidly increasing numbers of patients at risk over the past two decades [4]. However, whereas in the past, opportunistic mycoses typically occurred in immunocompromised hosts, these complications are increasingly observed in non-immunocompromised surgical and critically ill adult patients [9].

I.3 Most common human fungal pathogens

The etiology of invasive fungal infections has changed in the past decades. In the 1980s, yeasts (particularly *Candida albicans*) were the most causative agents of invasive mycoses. However, in recent years, molds have become more frequent in certain groups of patients, such as hematopoietic stem cell transplantation recipients [7].

Candida spp. are human commensals that cause both superficial and invasive infections [9]. These organisms occur abundantly in the human gastrointestinal tract and vagina. *Candida* species can be isolated in stool and from various mucus membranes where they exist as a commensal species [10]. One of the main reasons for *Candida's* virulence is its versatility in adaptation to various different habitats and the formation of biofilms that enhance its ability to adhere to surfaces and cause infections. *Candida* species become pathogens when the host's resistance to infection is impaired locally or systemically [11].

During the period 1979-2000 candidemia was reported to be the third most common cause of nosocomial bloodstream infection in critical adult patients, representing 11% of all BSI. The incidence of candidemia in U.S. hospitals during 2000-2005 increased from 3.65 to 5.56 episodes per 100,000 population [9]. Despite improved understanding of the pathogenesis of invasive disease and the advent of new diagnostic and therapeutic strategies, the attributable mortality at approximately 40% has essentially remained unchanged for the past few decades. Disseminated candidiasis is a life-threatening syndrome with an attributable mortality of 10 to 50% [12]. According to the National Nosocomial Infections Surveillance System (NNIS), non-*albicans* species were responsible for only 24% of nosocomial fungal infections involving *Candida* species in the 1980s. Within a decade, however, non-*albicans* species accounted for 46% of bloodstream infections due to *Candida* species [11].

The ARTEMIS Global Antifungal Surveillance Program showed that *C. albicans* was the most common (63-70%) candidal cause of invasive fungal infections, followed by *C. glabrata* (44%), *C. tropicalis* (6%) and *C. parapsilosis* (5%). However, geographical and institutional differences are widely reported [11]. The shift in *Candida* toward non-*albicans* species is likely due, at least in part, to selective pressures imposed by the increased utilization of antifungal agents [10].

In addition, invasive fungal infections caused by other non-*Candida* yeasts have been reported. *Trichosporon* species is the second most common cause of yeast fungaemia in patients with malignant haematological disease (after *Candida* species) and invasive trichosporon infection has been increasingly identified during the past 30 years. Species like *Rhodotorula,* non-*neoformans Cryptococcus* and *Geotrichum* have also been reported. Nevertheless, among yeasts, *Candida albicans* still is the predominant cause of invasive fungal infections [11].

In recent years, owing to the use of prophylactic or empirical antifungal treatment strategies, there has been a decrease in the frequency of *Candida* infections and a significant increase in the incidence of mould infections [13]. Among molds, *Aspergillus fumigatus* is the most frequent species of *Aspergillus* causing clinical disease, perhaps due to specific virulence factors unique to the organism. *Aspergillus* most commonly causes invasive pulmonary aspergillosis, often with subsequent

dissemination [14]. Moreover, reports of aspergillosis caused by the non-*fumigatus* species and infection caused by hyaline and black molds have been increasing in number [7].

I.4 Antifungal agents commercially available

The ideal antifungal agent should have broad antifungal activity, low rates of resistance, flexible routes of administration, few associated adverse events and limited drug-drug interactions [15].

Nowadays there are four groups of drugs available for the treatment of systemic mycoses in humans, which are, polyenes, azoles, echinocandins and flucytosine [16]. None of these classes of antifungal agents matches all the characteristics of an ideal agent [15], which ultimately leads to treatment failure.

The causes of treatment failure depend on fungus properties, antifungal drug properties and host factors. The treatment should be accordingly with fungal cell type and the size of fungal population. The use of an inappropriate dose of the antifungal agent, poor absorption, distribution or metabolism and drug-drug interactions may also contribute to lack of efficiency. Finally, it is also essential to consider the immune status of the patient, the presence of foreign materials and the site of infection [8].

Polyenes are broad-spectrum antifungal agents produced by the bacterial genus *Streptomyces.* Amphothericins A and B, both members of the polyene antifungal drug class were reported in 1955 but only amphotericin B was developed because of its superior potency [12]. It also presents broad spectrum of activity and there are relatively few examples of mycological resistance to the drug [17]. For many years this antifungal agent was considered standard therapy for serious fungal infections including invasive aspergillosis. However, multiple studies have established not only the unacceptable toxicity of this compound for serious infections but also demonstrate its lack of efficacy in high-risk patients with these infections [18]. Its mode of action is to bind to ergosterol, the principal sterol in fungal membranes, which leads to perturbations in membrane function and, ultimately, cause leakage of cellular contents. The structural difference between ergosterol and cholesterol, the major sterol in mammalian membranes, is sufficient to explain the greater binding affinity of amphotericin B for ergosterol over cholesterol and is the basis for the selectivity of this antifungal agent. However, this selectivity is low and suggests potential toxicity of amphotericin B for mammalian cells [19].

Like polyenes, azoles also exert antifungal activity by targeting ergosterol in the fungal cell membrane [20] and were first approved in 1980. Chemically, they all have either an imidazole or a triazole group joined to an asymmetric carbon atom as their functional pharmacophore [21]. Their main mode of action is to inhibit 14α-demethylation of lanosterol in the ergosterol biosynthetic pathway. As a result, ergosterol is replaced with unusual sterols and the normal permeability and fluidity of the fungal membrane is impaired. Additionally this brings consequences for membrane-bound enzymes, like those involved in cell wall synthesis [19]. The azole family of antifugal agents can be classified into two groups: the imidazoles (miconazole and ketoconazole) and the triazoles (fluconazole, itraconazole, voriconazole and posaconazole) [15].

The third class of antifungal agents with FDA (Food and Drug Administration) approval was the echinocandins in 2001. These agents, which include caspofungin, micafungin and anidulafungin, destabilize the fungal cell wall by depleting glucans, which are necessary to maintain its stability. This class has fungicidal activity against *Candida* spp., both *in vitro* and *in vivo*, and fungistatic activity against *Aspergillus* spp. [20].

Unlike polyene and echinocandin antifungal agents, azoles present higher solubility, lower toxicity, wider tissue distribution and availability for oral distribution. For this reasons the triazoles are the most widely used antifungal agents and have activity against many fungal pathogens [15]. However, clinical use of azoles is limited because of an increase of resistant strains, particularly during long-term treatments [1].

Flucytosine is a unique antifungal agent having no siblings or progeny in its antifungal class. It's a compound that mostly has some value as adjunctive treatment with amphotericin B in clinically difficult infections. Its antifungal specificity arises from the fact that fungi, but not human cells, possess the enzyme needed to take up flucytosine and convert it internally to 5-fluorouracil, a compound that is highly toxic to all eukaryotic systems. Fluorouacil becomes incorporated in fungal DNA and RNA and blocks synthesis of both these vital molecules, preventing cell proliferation [21].

Given the substantial morbidity and mortality related to invasive fungal infections, treatment with a combination of antifungal agents is often considered. Combined antifungal therapy approaches may be used to broaden the spectrum of activity, enhance the rate or extent of killing, minimize development of resistance or reduce toxicities. However, there are detrimental effects too, including attenuation of activity, increased resistance or toxicity, increased cost and drug interactions, which are hazards of combined therapy and must be carefully considered [20].

The introduction of these antifungal agents facilitated a more aggressive approach to the prophylaxis and treatment of fungal infections in the past decade leading to concerns about the emergence of resistant organisms [8]. Moreover, all of antifungal agents available present many disadvantages, mostly due to the appearance of fungal resistance, and the mortality rates remains relatively high. Therefore, it is essential to identify new potent and safe antifungal drugs with novel modes of action able to manage the actual raise of fungal infections.

I.5 The potential of BLAD polypeptide

Research was developed in Instituto Superior de Agronomia which allowed the development of a new and original approach in the fight against pathogenic fungi. This approach refers to a novel polypeptide, named BLAD (from the Portuguese/Latin *Banda de Lupinus Albus Doce*, meaning "band from sweet *Lupinus albus"* referring to a polypeptide band in an electrophoresis gel), which exhibits a powerful inhibitory activity on the germination and development of spores from fungal pathogens. *Lupinus* cotyledons were collected at various times, from the dry seed to 30-day-old senescing cotyledons, and their protein extracted, fractionated by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) and probed with polyclonal anti-ubiquitin antibodies (Ab). This experiment showed the appearance of an abundant 20 kDa polypeptide at 4 days after the onset of germination (DAG) and disappearance at 12 to 14 DAG (Fig. I.1).

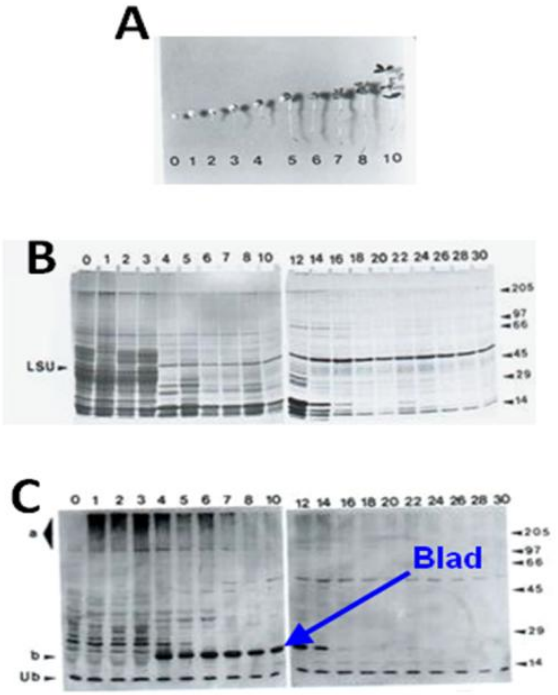

Figure I.1: Experiment which led to BLAD discovery back in 1991, as described in [22] and [23]. **(A)** *Lupinus albus* from the dry seed (time zero) until 10-day-old plantlets. **(B)** Electrophoretic (SDS-PAGE) analysis of total soluble polypeptides present in cotyledons, from the dry seed until their senescence (30 days after the onset of germination). **(C)** Immunoblotting analysis; the polypeptides present in the gels depicted in (B) were transferred onto a membrane and probed with polyclonal anti-ubiquitin antibodies. Days after the onset of germination are marked on top of gels and blots. Molecular masses (kDa) of standards are shown on the right. LSU: large subunit of ribulose bisphosphate carboxylase; Ub and a: free ubiquitin and large molecular mass ubiquitin-protein conjugates; b: BLAD.

Briefly, BLAD is isolated during growth of *Lupinus* seedlings and is a stable and intermediary breakdown product of β-conglutin catabolism, the major storage protein present in seeds of the *Lupinus* genus. It is a 20 kDa nitrogen-rich polypeptide, as part of a 210 kDa oligomer, composed of 173 amino acid residues, non-glycosylated but phophorylated. It is encoded by an internal fragment of the gene encoding the precursor of β-conglutin from *Lupinus* and occurs naturally in all *Lupinus* species examined to date, but in no other legume tested. BLAD exhibits extreme resistance to denaturation but is extremely sensitive to cleavage, either chemical or proteolytic. BLAD also presents lectin activity, binding to glycoproteins, and catalytic activity. In addition to the applicability in human health, BLAD has also potential to be used in the food industry, open air agriculture, organic farming and greenhouses.

This polypeptide has already been tested against a wide range of fungi (Table I.1) and its antifungal activity seems to be universal, as no negative results were obtained so far. Also, preliminary acute toxicological assays detected neither oral toxicity (in rats) nor dermal/allergenic toxicity (in guinea pigs).

Table I.1: *In vitro* activity of BLAD against different fungi.

Animal bacterial pathogens/ Food poisoning microorganisms	*In vitro* activity
Aspergillus niger	+
Pseudomonas aeruginosa	+
Listeria monocytogenes	+
Bacillus subtilis	+
Staphylococcus aureus	+
Salmonella thyphimurium	+

Food spoilage fungi	*In vitro* activity
Aspergillus niger	+
Yarrowia lipolytica	+
Saccharomyces cerevisiae	+
Zygosaccharomyces rouxii	+
Dekkera bruxellensis	+
Kluyveromyces marximianus	+
Zygosaccharomyces bailli	+
Penicillium sp.	+

Animal fungal pathogens	*In vitro* activity
Aspergillus fumigatus	+
Candida albicans	+
Candida dubliniensis	+
Candida glabrata	+
Candida lusitaneae	+
Candida parapsilosis	+
Candida tropicalis	+
Cryprococcus neoformans	+

When considering non-human applications, BLAD displays both preventive and curative antifungal activities, is active against a wide range of pathogens at the same time, and the development of fungal resistance mechanisms is unlikely. Contrary to the currently available antifungal agents, BLAD does not require safety interval between application and harvest, no protective equipment is required and needs lower economical costs and time required for certification when compared to chemical fungicides.

Despite all the very encouraging results obtained so far, little is known regarding BLAD efficacy against human fungal pathogens or its mode of action. In addition to the *N*-acetyl-β-glucosaminidase and chitosanase catalytic activities which allow BLAD to target the fungal cell wall, there is some evidence that BLAD interacts at the cell envelope level (cell wall and plasma membrane).

I.6 Objectives

This working plan is integrated in a broader project whose major goal is to assess BLAD efficacy in the treatment of human fungal infections. Hereupon, and given that the major objective is to use BLAD in the clinical area, the first step is to produce this polypeptide via heterologous expression, in a recombinant form, using *Escherichia coli* as host cell. Subsequently, the physiological and morphological effects of BLAD will be assessed, using *Candida albicans* as a unicellular pathogenic fungal model. Lastly the search for specific targets for BLAD in the pathogen cell envelope will be carried out, using *C. albicans* protoplast membranes.

I.7 References

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CHAPTER II

Recombinant production of BLAD polypeptide. Cloning and expression of BLAD in *Escherichia coli*

II.1 Introduction

The development of recombinant DNA technology in the early 1970s enabled the expression of heterologous genes into pro- or eukaryotic host which do not naturally harbor these pieces of DNA [1]. This allowed introducing traits for the production of desired compounds into non-natural producers.

Recombinant protein production was first employed for the production of human proteins in microbial cells, like insulin in *Escherichia coli* [2]. Since that, impressive progresses over the past decades have brought hundreds of therapeutic proteins into clinical applications [3] and allowed the development of several expression systems, both prokaryotic and eukaryotic. Consequently, recombinant protein production is, nowadays, a multi-billion dollar market, comprising biopharmaceuticals and industrial enzymes. Global sales for biopharmaceutical proteins reached US$87 billion in 2008, and is expected to rise up to US$169 billion in 2014 [2].

In the first attempts to produce recombinant proteins, the gene of interest is typically cloned into an expression vector, generally a plasmid. Expression vectors are extrachromosomal self-replicating DNA elements that contain an origin of replication (ori), a selection marker (usually antibiotic resistance), transcriptional promoters, translation initiation regions (TIRs) as well as transcriptional and translational terminators [1].

After cloning the gene of interest into a plasmid it is essential to choose the best host cell in order to maximize the expression of the product. The selection of the host cell will be strongly influenced by the type and use of the product, as well as economic or intellectual property issues. A number of new, sophisticated host-vector combinations have become available in recent years [4]. Nevertheless, *E.coli* remains the working horse of recombinant protein production [2] mostly due to the profound genetic and physiological characterization, the short generation time, the ease of handling, the established fermentation know-how and finally the high capacity to accumulate foreign proteins to more than 20% of the total cellular protein content [5].

The many advantages of *E.coli* have ensured that it remains a valuable organism for the high-level production of recombinant proteins, however, the major drawbacks of this expression system include the inability to perform many of the posttranslational modifications found in eukaryotic proteins, the lack of a secretion mechanism for the efficient release of protein into the culture medium and the limited ability to facilitate extensive disulfide bond formation [6].

One of the most useful systems for expression of recombinant proteins in *E. coli* is the pET vector series, which is based on the T7 phage RNA polymerase and uses the pBR322 origin of DNA replication. The expression of the recombinant protein using these plasmids is tightly regulated and, when induced, produces high levels of transcripts and recombinant proteins. Moreover, the presence of six consecutive histidine residues (6XHis-tag) permits the purification of the fusion protein on metal charged columns [7].

The possible use of BLAD into the clinical field, especially for the treatment of systemic fungal infections, will require the use of its recombinant form by heterologous expression. Considering the absence of glycosylated residues in the polypeptide BLAD and the ease handling of *E. coli* prokaryotic cells, this system is the most promising.

II.2 Materials and methods

II.2.1 Biological materials and growth conditions

II.2.1.1 *Escherichia coli*

The *Escherichia coli* bacteria were used to clone the gene that codifies for the polypeptide BLAD with the aim of expression. Therefore, two different strains were used: TOP10 strain and BL21 Star™ (DE3). TOP 10 was used for all routine cloning experiments whereas the second one was used for recombinant protein expression and was cultivated in LB (Luria-Broth) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl) at 37 °C.

II.2.1.2 *Candida albicans*

Candida albicans var. *albicans* (CBS 562) was grown at 35 °C for 24 h in Glucose Yeast Peptone (GYP) medium (1% (w/v) peptone, 0.5% (w/v) yeast extract, 2% (w/v) glucose, 1.5% (w/v) agar). For the antifungal susceptibility tests was used PDB medium (24 g/L Potato Dextrose Broth), buffered at pH 7.5.

II.2.2 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR), described in [8], was performed in a thermocycler "MasterCycler Gradient", *Eppendorf*. The polymerase used was "Platinum® Taq DNA polymerase-Invitrogen" and the set of primers was t7 (t7 forward "TAATACGACTCACTATAGGG" and t7 reverse "TAGTTATTGCTCAGCGGTGG"), specific for the pET151/D-TOPO® plasmid.

The master mix contained 10x PCR buffer, 10 mM dNTP mixture, 50 mM $MgCl_2$, 10 µM of each primer, 1 µL template DNA, 1 unit of Platinum® Taq DNA polymerase and autoclaved, distilled water to a final volume of 10 µL.

The tubes were then incubated in a thermal cycler at 94 °C for 30 s to complete denatures the template and activates the enzyme. The polymerase reaction was performed for 30 cycles: 94°C for 30 s to denature de DNA; 55°C for 30 s for primer annealing and 72°C for 1 min per kb for DNA synthesis. The PCR tubes were placed at 4 °C after cycling and stored at -20 °C until use.

II.2.3 Cloning of PCR products in *E.coli*

The gene that codifies for the polypeptide BLAD had already been cloned into pET151/D-TOPO® plasmid. This vector has an ampicillin resistant marker for selection, a sequence that encodes for *N*-terminal fusion tags for detection and purification of recombinant fusion proteins and a sequence recognized by "TEV-Tobacco etch virus" protease, that cuts the histidines tail. Has a T7l*ac* promoter for high-level IPTG-inducible expression of the gene of interest in *E.coli*.

TOPO® Cloning reaction was first transformed into One Shot® TOP10 Chemically Competent *E.coli* for characterization of the construct, propagation and maintenance. After being purified, the plasmid was cloned into BL21 Star™(DE3) One Shot® Chemically Competent *E. coli* for product expression. Both transformations were made according to the Invitrogen's protocol "Champion™ pET Directional TOPO® Expression Kits" [9].

II.2.4 Plasmid purification and DNA quantification

Before being cloned into BL21 Star™(DE3) One Shot® Chemically Competent *E. coli,* plasmids were isolated and purified according to the Promega's protocol "Wizard® *Plus* SV Minipreps DNA Purification System" [10].

After being purified, nucleic acids were quantified according to the manufacturer instructions in a BioTek's Take3™ spectrophotometer using the Gen5 program.

II.2.5 Recombinant BLAD expression

Preliminary assays were made in order to determine the optimal conditions of protein expression, according to the Invitrogen's protocol "Champion™ pET Directional TOPO® Expression Kits" [9]. In these tests, 500 µL of BL21 Star™(DE3) One Shot® Chemically Competent *E. coli* previously transformed with the plasmid containing the gene that codifies for the polypeptide BLAD were grown overnight in 10 mL of LB medium supplemented with 100 mg/mL ampicillin, at 37 °C, 150 rpm. The first step was to determine the optimal induction time for the greatest product expression. The overnight culture was refreshed and when the $OD_{640\ nm}$ reached 0.4 the culture was divided in two. One was induced with 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) and the other was kept as control. Aliquots were removed at 0, 4, 6, 12 and 48 h of induction for SDS-PAGE analysis. After being determined the optimal induction time the culture volume was raised for 1 L, in order to obtain more expressed product, and induced with 1mM IPTG. Finally, the culture was centrifuged at 3.000 *g*, 10 min at 4 °C and the cells were stored frozen at -80 °C until use.

II.2.6 Recombinant BLAD purification

Recombinant BLAD was purified under denaturing conditions using the Ni-NTA agarose according to the Invitrogen's protocol "Ni-NTA Purification system – For purification of polyhistidine-containing recombinant proteins" [11]. This system is based on the high affinity and selectivity of the Ni-NTA agarose for recombinant proteins tagged with six histidine residues. The cells were resuspended in 8 mL of denaturing binding buffer (8 M urea, 20 mM sodium phosphate pH 7.8, 500 mM NaCl) and then lysed by three cycles of freezing in liquid nitrogen, thawing and vortex. The cell lysate was centrifuged at 5.100 *g*, 20 min, 15 °C. The supernatant was transferred to a prepared purification column, previously equilibrated with 8 mL of 0.5 M NaOH, for 30 min with gentle stirring, and then twice with 8 mL denaturing binding buffer. The column was then washed three times with 4 mL of denaturing wash buffer (5 M urea, 20 mM sodium phosphate pH 6.0, 500 mM NaCl). BLAD was eluted with 4 mL of denaturing elution buffer (5 M urea, 20 mM sodium phosphate pH 4.0, 500 mM

NaCl) and stored at 4 °C until use. All the samples were collected for subsequent SDS-PAGE analysis.

Instead of proceeding directly to the full removal of the denaturing agent (Single step dialysis), urea was removed in steps, through a Dialysis step-wise method, with the aim of trying to avoid protein aggregation or destabilization. With this, protein tends not to aggregate as rapidly as in the single step dialysis, which leads to a higher solubility.

The first steps of the dialysis were performed along with the purification, passing from: 8 M urea, pH 7.8; 5 M urea, pH 6.0 and 5 M urea, pH 4.0. After the recovery of the eluted (in buffer composed of 5 M urea, 20 mM sodium phosphate pH 4.0, 500 mM NaCl), from the Ni-NTA column, it was placed in a dialysis membrane. Posteriorly was placed in 5 L of a buffer composed of 3 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.0, with gentle stirring, for 2 h. After that, the dialysis membrane was placed in 5 L of a buffer composed of 20 mM sodium phosphate, 500 mM NaCl, pH 7.0, overnight, at 4 ºC. In the end of the dialysis, the sample was recovered, quantified for subsequent immunoblot analysis and lyophilized for the antifungal susceptibility tests.

II.2.7 Immunoblotting

Proteins separated by SDS-PAGE were blotted onto a PVDF (Polyvinylidene fluoride) membrane, previously soaked in transfer buffer (50 mM trizma base, 3.7 M glicine, 0.04% (w/v) SDS and 20% (v/v) methanol) at 15 V for 45 min, using a semi-dry system “TransBlot Semi-Dry Transfer Cell” (Bio-Rad). After protein transfer, the polypeptides in the membrane were fixed for 5 min in a solution containing 10% (v/v) acetic acid and 25% (v/v) 2-propanol. Total polypeptides in the membrane were visualized with Ponceau S. The membrane was washed for 1 min with water, incubated for 15 min with 0.026 M Ponceau S, 1.8 M trichloroacetic acid and 1.2 M sulfosalicylic acid, and washed for 5 min with water.

The membrane, containing the fixed polypeptides, was incubated for 1 h with PBS (137 mM NaCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4 and 2.7 mM KCl) containing 0.05% (w/v) Tween 20, 3% (w/v) milk powder and 300 µg/mL of BLAD. After that, the membrane was incubated with the first antibody, 500-fold diluted in PBS containing 0.05% (w/v) Tween 20 and 3% (w/v) milk powder. After 1 h, the membrane was washed (2 x 5 min) with PBS containing 0.1% (w/v) Tween 20 and then with a salt solution (1 M NaCl, 0.01 M Na2HPO4 and 0.5% (w/v) Tween 20). Before being incubated with the second antibody, the membrane was once again washed, for 15 min, with PBS containing 0.05% (w/v) Tween 20 and 3% (w/v) milk powder. After these washes, the membrane was incubated for 1 h with the second antibody 1250-fold diluted in PBS containing 0.05% (w/v) Tween 20 and 3% (w/v) milk powder. The membrane was then washed (2 x 5 min) with PBS containing 1% (w/v) Tween 20 and once with the salt solution for 10 min. After that the membrane was washed (3 x 5 min) with PBS containing 0.1% (w/v) Tween 20 and once with PBS for 1 min. The membrane was always kept at 37 °C with gentle stirring.

According to the purpose of the study, two primary antibodies were used. A BLAD-specific and other unspecific, both produced in rabbit as described in [12], and a second antibody linked to peroxidase specific for both primary antibodies, produced in goat and bought from SIGMA.

For revelation of the signal, the membrane was placed in a "SuperSignal West Femto Maximum Sensitivity Substracte" solution, an extremely sensitive quimioluminiscent substrate for the detection of the secondary antibody linked to peroxidase. The signal was observed in a ChemiDoc™ XRS + (*Molecular Imager*, BioRad).

II.2.8 Antifungal susceptibility tests

The susceptibility testes performed in yeasts were made according to the CLSI - Clinical and Laboratory Standards Institute (former NCCLS - National Committee for Clinical Laboratory Standards) guideline M27-A3 [13], using broth microdilution method. According to this guideline, the cell suspension should be adjusted with a spectrophotometer (Shimadzu UV-1800) to an $OD_{640\ nm}$ = 0.05, in order to give an inoculum concentration of $1x10^6$ cells per mL. However, the calibration curve performed for strain CBS 562 indicated that the concentration of $1x10^6$ cells/mL is achieved with and $OD_{640\ nm}$= 0.15, and, therefore, this value was used throughout for inoculum preparation.

II.2.8.1 Antifungal agents

Both native and recombinant BLAD polypeptides were purified and stored lyophilized at room temperature. When needed both solutions of BLAD were prepared in mili-Q sterile water and 200 µL were added to the microplate's first line. A twofold dilution was made, twelve times, using mili-Q sterile water, in the 96-wells microplates. The final concentration of native BLAD, after the addition of the inocula, ranged from 1000 to 480 µg/mL and for the recombinant, from 312.5 to 0.153 µg/mL.

II.2.8.2 Minimum inhibitory concentrations (MICs) determination

Yeast cells were grown on GYP medium for 24 h at 35 °C and the inoculum suspension was prepared by picking colonies and resuspending them in 5 mL of sterile 0.9% (w/v) saline (NaCl). The resulting suspension was vortexed for 15 s and the cell density was adjusted with a spectrophotometer to an $OD_{640\ nm}$ =0.15. The final inoculum suspension was made by a 1:50 dilution followed by a 1:20 dilution with double-strength broth medium, which resulted in a final concentration of $1x10^3$ cells per mL. Two other final inoculum concentrations were tested: $1x10^4$ cells/mL, achieved by a 1:50 dilution followed by a 1:2 dilution with double-strength broth medium, and $1x10^5$ cells/mL, achieved by a 1:10 dilution with double-strength broth medium. The inoculum size was verified by enumeration of CFU obtained by subculturing on GYP plates.

Yeast inocula (100 µL) were added to each well of the microplate, containing 100 µL of the diluted BLAD solution (twofold). Final volume in each well was 200 µL.

The microplate was incubated at 35 °C and examined after 72 h. The MIC endpoints were the lowest drug concentration that showed absence of growth, as recorded visually.

II.2.8.3 Minimum fungicidal concentrations (MFCs) determination

MFCs were determined by two different methods. In one method, the inoculum size was 10^3 UFC/mL and after MIC determination, as previously described, 20 µl aliquots were subcultured from each well that showed no visual growth onto GYP plates [14].

In another method, described in [15], the inoculum was prepared as described in the CLSI guidelines, except for the inoculum size (10^4 and 10^5 CFU/mL). After MIC determination the content of each clear well was homogenized and the entire volume (200 µL) was subcultured onto two GYP plates (100 µL aliquots/plate). To avoid antifungal carryover, aliquots were deposited as a spot onto the agar plate and allowed to soak. After the plate was dry the cells were separated and removed from the drug source by streaking/ surface spreading.

In both cases, the plates were incubated at 35 °C for 24 h. The MFC was the lowest drug concentration that killed over 99.99% of the final inoculum.

II.2.9 General procedures

II.2.9.1 Electrophoresis

II.2.9.1.1 Agarose gel electrophoresis

Horizontal electrophoresis was performed in 1% (w/v) agarose gel in order to resolve nucleic acids. Agarose was dissolved in TAE buffer (400 mM Tris-acetate pH 8.0, 10 mM EDTA) 10-fold diluted, containing "Gel Red nucleic acid stain" (5 µL/100 mL). The molecular marker used was "1 kb plus DNA ladder" from SIGMA and "Gel loading buffer" (10x concentrated, composed of 0.21% (v/v) Bromophenol Blue, 0.21% (v/v) Xylene Cyanol F, 0.2 M EDTA, pH 8.0 and 50% (v/v) Glycerol) was added to each sample, to facilitate loading of the samples into the wells. The electrophoresis ran at 120 V.

II.2.9.1.2 Polyacrylamide gel electrophoresis in SDS-PAGE

The samples were precipitated with iced cooled 80% (v/v) acetone, at -20 °C during 30 min and then centrifuged at 15.000 *g*, 10 min at 4 °C. The pellet was ressuspended in sample buffer containing 0.08 M Tris-HCl pH 6.8, 0.1% (w/v) *β*-2-mercaptoethanol, 2% (w/v) SDS, 15% (w/v) glycerol and 0.006% (w/v) of a 1% (w/v) solution of *m*-cresol purple. After that the samples were vortexed and boiled for three minutes.

A polyacrylamide gel in denaturing conditions (SDS-PAGE) was used in a discontinuous system with a concentration and a separation gel, according to the method described in [16].

The electrophoresis was performed on a vertical system using mini gels, with the addition of a cathode buffer composed of 25mM Tris-HCl pH 8.8, 192 mM glycine and 0.1% (w/v) SDS. As a standard two different markers were used: Dalton Mark VII-L for SDS Gel Electrophoresis (SIGMA), a low molecular marker from SIGMA that range from 14 kDa to 70 kDa and Precision Plus Protein™ All Blue Standards marker (kDa), a marker from BioRad, that range from 10 to 250 kDa.

The electrophoresis ran at 30 mA and 200 V, using a power supply EPS 500/400 (Pharmacia/LKB). The polypeptide migration was interrupted when the *m*-cresol purple was near the lower end of the mini gel.

II.2.9.2 Protein Quantification

Protein content was determined according to a modification of the Lowry's method [17] using bovine serum albumin as the standard. The samples were read in a spectrophotometer, at 750 nm.

II.2.9.3 Protein Staining

The gels were stained with Coomassie Brilliant Blue R250 (CBB R-250). Polypeptides were fixed in TCA 10% (w/v) for 15 min. After that the mini gels were stained for a period longer than 3 h with a solution containing 0.25% (w/v) CBB R-250, 25% (v/v) 2-propanol and 10% (v/v) glacial acetic acid. The destaining solution composed of 25% (v/v) 2-propanol and 10% (v/v) glacial acetic acid was kept until the polypeptides could be visualised.

II.3 Results and discussion

II.3.1 Cloning of pET151 into competent cells and selection of recombinants

To start the procedure the pET151 D-TOPO® plasmid previously cloned by our investigation group with the gene that codifies for the polypeptide BLAD (519 bp) was used. In order to guarantee that the plasmid was correctly cloned with the gene, a PCR reaction was performed using the t7 set of primers and the resulting reaction was run in an agarose gel (Figure II.1).

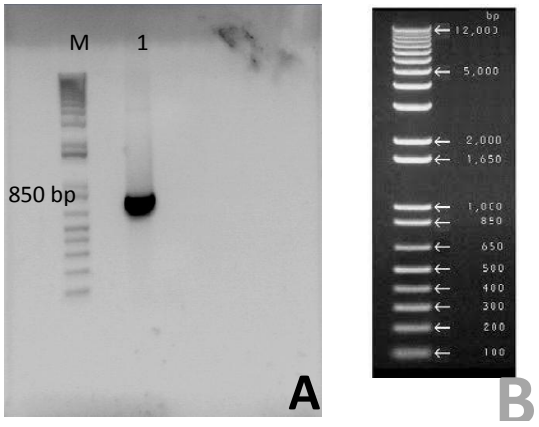

Figure II.1: Electrophoretic analysis in 1% (w/v) agarose gel. **(A)** Amplification with the t7 set of primers of pET151 D-TOPO® containing the gene that codifies for the polypeptide BLAD. **(B)** 1kb plus DNA ladder marker, Invitrogen.

The t7 set of primers is specific for the pET151 D-TOPO® plasmid t7 promoter and flanks a region of approximately 300 bp, in the multiple cloning site. The gene that codifies for the polypeptide BLAD was inserted in this region and, consequently, when an amplification reaction is performed with this set of primers is expected a fragment with approximately 800 bp.

The analysis of figure II.1 shows that the pET151 D-TOPO® plasmid was correctly cloned. A transformation was subsequently performed into One Shot® TOP10 Chemically Competent *E.coli.* In order to select the transformed colonies, the solution where the reaction took place was plated into LB plates supplemented with ampicillin. Then, 10 colonies were randomly chosen and analyzed by PCR using the t7 set of primers in order to select the positive recombinants and guarantee that the gene was correctly inserted (Figure II.2).

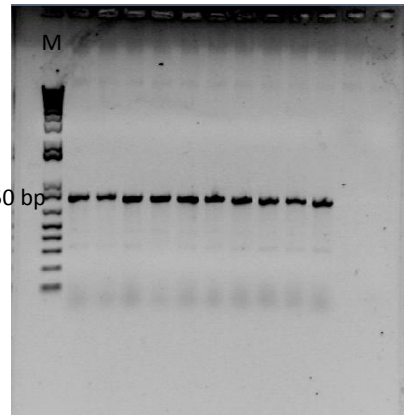

Figure II.2: Electrophoretic analysis in 1% (w/v) agarose gel. Amplification with the t7 set of primers of 10 colonies cloned with pET151 D-TOPO® containing the gene that codifies for the polypeptide BLAD. [M] 1kb plus DNA ladder marker, Invitrogen.

The figure II.2 shows that the fragment was inserted in all the selected colonies. To quantify the DNA content in each colony, they were grown overnight in LB medium with ampicillin (100 mg/mL) and purified by Minipreps, according to the manufacturer instructions [10]. Since the DNA content was similar in all the colonies, approximately 40 ng/µL, only one was selected to continue the work and subsequently sent to be sequenced.

II.3.2 Recombinant BLAD expression

After confirming the proper orientation of the gene, the plasmid containing the insert was cloned into BL21 Star™(DE3) One Shot® Chemically Competent *E. coli* for product expression.

The expression of the polypeptide BLAD was achieved using the Champion™ pET Directional TOPO® Expression Kits according to the manufacturer instructions [9]. To better understand the optimal induction time it needs to achieve the greatest product expression, first, a pilot expression protocol was performed. The culture was grown in LB medium supplemented with ampicillin and induced with 1 mM IPTG when $OD_{600\ nm}$ = 0.5. During the experiment a fraction of the culture was kept non-induced and aliquots of all samples were recovered after 0, 4, 6, 12 and 48 h (Figure II.3).

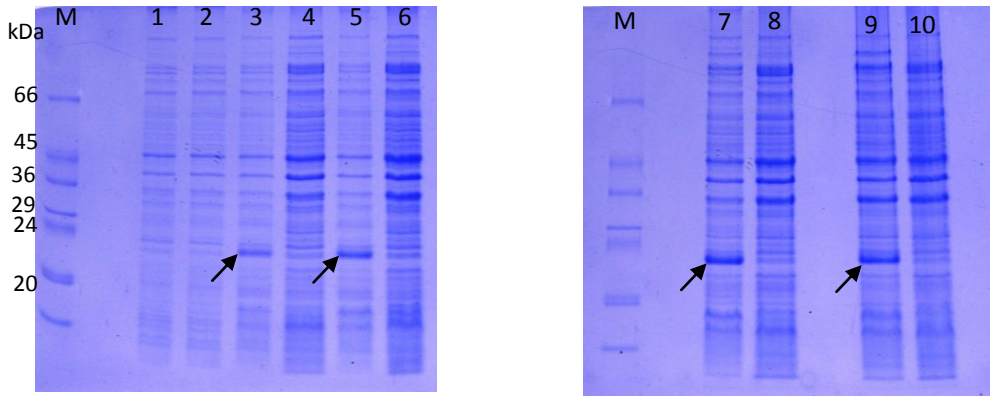

Figure II.3: SDS-PAGE analysis. Recombinant BLAD expression at 0h with [1] and without IPTG [2]; 4h with [3] and without IPTG [4]; 6h with [5] and without IPTG [6]; 12h with [7] and without IPTG [8] and 48h with [9] and without IPTG [10]. [M] LPM marker (low molecular mass protein markers, kDa).

From the results showed in figure II.3, it is possible to conclude that the best induction time is 6 h. After that period *E.coli* begins to over-express other proteins which can lead to difficulties in further purification steps. Moreover, product expression only occurs in the presence of IPTG, as expected, because it binds to the tetrameric lac operon releasing it, and allowing the transcription of the genes regulated by the operon, including the gene that codifies for BLAD.

Upon confirmation of the optimal induction time, the next step was the Scaling-up expression in order to obtain a higher volume, and, consequently, more recombinant expressed protein.

II.3.3 Recombinant BLAD purification

After inducing the culture with 1 mM IPTG, when $OD_{600\ nm}$=0.5, for 6 h, recombinant BLAD was purified under denaturing conditions using the Ni-NTA agarose columns. The protein content of the resulting fractions was quantified by the modified Lowry's method [41] and the results are showed in

table II.1. Since each column has only capacity for 8 mL, the bacterial supernatant was passed twice and is called 1st flow-through and 2nd flow-through, respectively.

Table II.1: Protein content of each sample resulted from purification under denaturing conditions.

Sample	1st Flow-through	2nd Flow-through	1st wash	2nd wash	3rd wash	Eluted
Protein content (µg/µL)	0.959	1.38	0.896	0.502	0.345	0.692

Finally, 15 µg of each sample was run in a SDS-PAGE in order to guarantee that BLAD had been correctly eluted (Figure II.4).

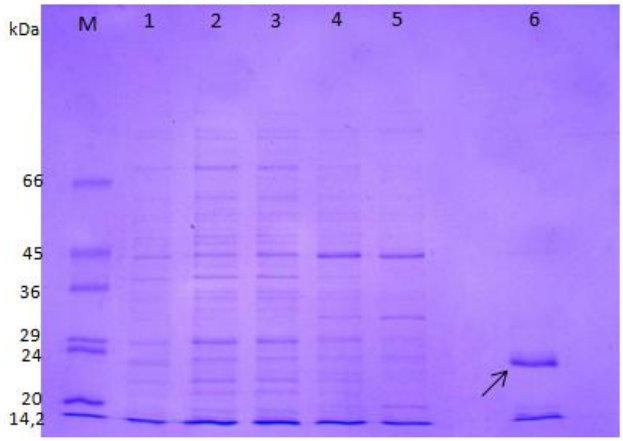

Figure II.4: SDS-PAGE analysis. Purification under denaturing condition of recombinant BLAD using the Ni-NTA agarose columns. [1] 1st flow-through; [2] 2nd flow-through; [3] 1st wash; [4] 2nd wash; [5] 3rd wash; and [6] eluted. [M] LPM marker (low molecular mass protein markers, kDa).

From the results showed in figure II.4 it is possible to conclude that the purification of recombinant BLAD was well achieved since it appears only in the eluted fraction, as expected. To remove the urea, which will interfere with the subsequent steps, but assuring that the protein will stay stable in a suitable buffer the first tested option was dialyze it directly into Tris HCl 20 mM pH 7.5. However, in the end the sample showed a great amount of precipitated material that showed to be the recombinant protein. To bypass this situation a step-a-wise dialysis was performed where the sample was dialyzed with decreasing concentrations of urea in steps until it reaches zero. Although it was not perfect it allowed recovering a great quantity of the BLAD protein in the soluble fraction.

II.3.4 Immunodetection of recombinant BLAD

In order to confirm if the expressed and purified product was, in fact, BLAD and to inquire if it retains the same biological activity than the native polypeptide, different immunoblots were performed. After being purified and quantified, the eluted fraction, containing the heterologous polypeptide, was run in a SDS-PAGE, transferred to a PVDF membrane and immunodetected using as probe the antibody anti-BLAD previously produced in rabbit [28]. The reaction was revealed using a second antibody anti-rabbit conjugated with peroxidase, produced in goat (Figure II.5).

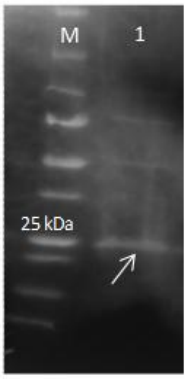

Figure II.5: Immunoblotting. [1] Immunodetection of recombinant BLAD using as probe a first antibody anti-BLAD produced in rabbit and second one, conjugated with peroxidase, anti-rabbit produced in goat. [M] Precision Plus Protein™ All Blue Standards marker (kDa).

Figure 6 shows the presence of a signal on the BLAD electrophoretical band, at 24 kDa. This proves that the expressed product was, in fact, BLAD or at least a very similar polypeptide, since the antibody used is specific for it.

One of the main properties of the native polypeptide BLAD is its lectin activity. This means that it has the capacity of recognizing glycosidic residues which results on a nonspecific binding to all the immunoglobulin glycosidic residues. To access if it also retains the capacity of recognize the glycosidic conserved region of any antibody, like its native version, the recombinant BLAD was incubated with an antibody anti-wine proteins (choose randomly). The native BLAD was used as control (Figure II.6). It is important to note that, as referred in the material and methods section, the recombinant BLAD has more 4 kDa than the native polypeptide which corresponds to the addition of the histidine residues necessary for further purification steps.

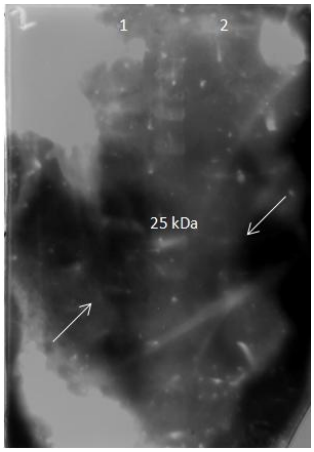

Figure II.6: Immunodetection of native [1] and recombinant BLAD [2] using as probe a first antibody anti-wine proteins produced in rabbit and second one, conjugated with peroxidase, anti-rabbit produced in goat.

As shown in figure II.6, the presence of a signal on the recombinant BLAD electrophoretical band confirms the maintenance of the lectin activity since, like the native BLAD (20 kDa), it has the ability of recognize nonspecific antibodies.

II.3.5 Antifungal susceptibility tests with recombinant BLAD

Once the expression of recombinant BLAD was confirmed and with promising results that it could retain the biological activity of its native form, the next step was to test its main property – the antifungal activity. Thereby, the antifungal activity of recombinant BLAD was tested on *Candida albicans* with the aim of comparing these results with the previously obtained with the native BLAD. This microorganism was also chosen because it is a major human pathogen and it is one of the prime targets for the recombinant production of BLAD. For this purpose, both minimum inhibitory and

fungicidal concentration, MIC and MFC respectively, were determined and compared with the previously obtained for the native polypeptide (Table II.2).

Table II.2: MIC and MFC of native and recombinant BLAD.

Sample	MIC (µg/mL)	MFC (µg/mL)
native BLAD	125	250
recombinant BLAD	78	78

After analyzing table II.2 it is possible to conclude that recombinant BLAD could possess a strong antifungal activity since it is required in a smaller concentration to induce the same degree of inhibition when compared with the values obtained for the native polypeptide. Moreover, and regarding the MFC results, there is a decrease in the number of fungal colonies remained, after exposition to BLAD, as the concentration of recombinant BLAD increases (Figure II.7) which indicates the occurrence of cell death.

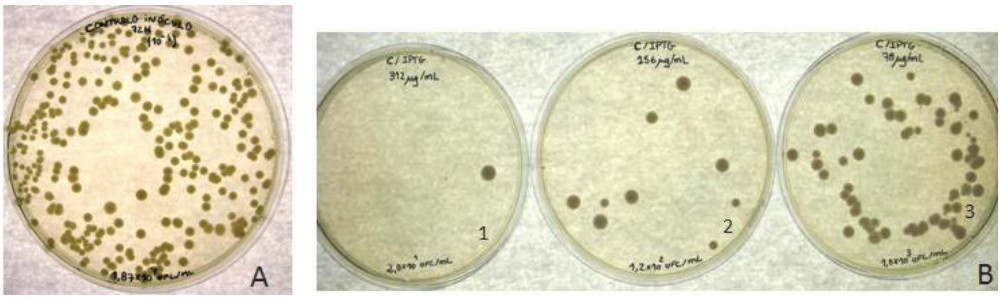

Figure II.7: *Candida albicans* colonies cultivated in GYP medium. **(A)** Drug-free control (10^{-4} dilution). **(B)** Resulted colonies after 72 h exposure to recombinant BLAD at [1] 312 µg/mL, [2] 156 µg/mL and [3] 78 µg/mL.

Unlike native BLAD, in the recombinant form, the minimum inhibitory concentration is the same as the fungicidal (Table II.2). This means that from the moment that recombinant BLAD inhibits the growth of yeasts, it kills 99.99% of the microorganisms.

II.4 Conclusion

The results obtained in this work clearly demonstrated that the production of the polypeptide BLAD in a recombinant form is possible which opens new commercial applications. It has been demonstrated that recombinant BLAD probably has the same biological activities as its native form, such as, a strong antifungal activity. In fact it is even possible to assume that the recombinant form of the polypeptide could probably possess a higher effectiveness since it is required in a less concentration to cause death of the microorganisms. However, this difference can have a simple explanation by the fact that the native BLAD is a single subunit in a major 210 kDa protein and, even though that it is the majority of the protein it is underestimated in what concerns the evaluation of the concentration. In contrast, through the recombinant way, the only thing that is being expressed and purified is the BLAD fraction.

This work is only preliminary and in the future it is necessary to confirm all these data. Further work needs to be done in order to optimize the expression system and to maximize the yield of recombinant production and, consequently, allowing some potential commercial application.

II.5 References

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Chapter III

Physiological and morphological effects of BLAD on *Candida albicans*

III.1 Introduction

Candida species are the most common fungal pathogens of humans and the causative agents of oral, gastrointestinal, and vaginal candidiasis, giving rise to severe morbidity in millions of individuals worldwide. Vaginal candidiasis alone affects ~75% of women, worldwide, at least once during fertile age, equating to ~30 million infection episodes/year [1]. In the USA, candidemia is the fourth most common cause of hospital-acquired infections, with annual Medicare costs estimated to exceed $1 billion. A simple calculation, based on an incidence rate of 8 out of 100 000 per annum, 40% mortality and 300 million population size, suggests that in the USA alone there are ~10 000 deaths a year due to *Candida* infections [2], making *Candida* species as medically important as many mainstream bacterial infections including Enterococci (*E. coli*) and Pseudomonas spp [1].

Candida species commonly reside as commensal organisms, being part of the normal microbiome in the gut, oral cavity, or vagina in approximately 50% of the population. Although normally these fungi cause no pathology, if there are changes in the local environment, such as alterations in normal microbiota or compromised local immune defences, then these fungi can become pathogenic [1].

Among the various species of *Candida* capable of causing human infection, *Candida albicans* predominates. Superficial infections of genital, oral and cutaneous sites almost always (>90% of cases) involve *C. albicans* [3] and its virulence is multi-faceted, as it depends on factors such as the secretion of proteases, the expression of cell surface adhesions and the overall fitness within the host [4].

The most commonly cited *C. albicans* virulence factors include adhesins and the Als family, extracellular enzymes, and most importantly, the ability to alternate between unicellular yeast and filamentous hyphal forms of growth [5]. In between these two extremes, the fungus can exhibit a variety of growth forms that are collectively referred to as pseudohyphae. In these forms, daughter bud elongates and, after semptum formation, the daughter cell remains attached to the mother cell. As a result, filaments composed of elongated cells with constrictions at the septa are formed [2]. Hyphae have been proposed to play a major role in adhesion, invasion, and biofilme formation while yeast cells are likely to be important for dissemination and initial colonization of host surfaces [5].

For these reasons, and giving the substantial mortality due to candidemia and the difficulties encountered in administering early and effective antifungal therapy [3], it is essential to better understand the physiological and morphological effects of BLAD on fungal cells, using *C. albicans* as a model.

III.2 Materials and methods

III.2.1 Biological materials and growth conditions

III.2.1.1 *Candida albicans*

Candida albicans var. *albicans* (CBS 562) was grown at 35 °C for 24 h in Glucose Yeast Peptone (GYP) medium (1% (w/v) peptone, 0.5% (w/v) yeast extract, 2% (w/v) glucose, 1.5% (w/v) agar). For the antifungal susceptibility tests, the media used were RPMI 1640 (20.8 g/L glucose and 69.06 g/L MOPS [3- (N-morpholino) propanesulfonic acid]), YNB (0.67 g/L YNB and 1.05 g/L MOPS) supplemented with 2% (w/v) glucose, and PDB (24 g/L Potato Dextrose Broth), all buffered at pH 7.5. For the protoplasts formation, the yeast was grown in YPD medium (20 g/L yeast extract, 20 g/L peptone and 10 g/L dextrose).

III.2.1.2 *Lupinus albus*

The seeds of *Lupinus albus* were germinated and grown in growth chambers with a photoperiod of 16 h light/8 h dark at 18 °C, for periods up to 10 days and the seed coats were removed and the intact cotyledons dissected from the axes and stored frozen at -80 °C until needed.

III.2.2 BLAD purification

III.2.2.1 Total soluble protein extraction

The extraction of total soluble protein was made according to [6]. The cotyledons from germinated seedlings were macerated in a mortar and pestle in a globulin solubilizing buffer (2 mL/g fresh weight; 100 mM Tris HCl buffer, pH 7.5, containing 10% (w/v) NaCl, 10 mM EDTA and 10 mM EGTA) .The extract was gently stirred during 30 min at 4 °C and then filtered through three layers of cheesecloth. The globulin containing solution was centrifuged at 30.000 *g*, during 1 h at 4 °C and filtered again. The resulting supernatant was desalted on PD-10 columns, according to the manufacturer instructions [7], previously equilibrated in 50 mM Tris-HCl buffer, pH 7.5.

III.2.2.2 Purification of BLAD from *Lupinus albus*

After obtaining the total globulin fraction as explained above, the individual globulins were fractionated and purified by FPLC anion exchange chromatography on a Q-Sepharose column (GE Healthcare Life Sciences; Ø = 1 cm; h = 8 cm; flow rate = 1.5 mL/min) essentially as described in [8]. The bound proteins were eluted with a gradient of NaCl (0 to 1 M) and desalted on PD-10 columns according to the manufacturer instructions [7], previously equilibrated in distilled water, pH 7.5 and then lyophilized.

III.2.3 Antifungal susceptibility tests

All the antifungal susceptibility tests were performed as described in Chapter II, section II.2.8.

III.2.4 *Candida albicans* protoplast formation

Cells from a fresh culture were grown overnight at 30 °C, 150 rpm, in 200 mL of YPD medium in a 500 mL Erlenmeyer. The $OD_{600\ nm}$ was then measured and adjusted to 0.1, in 200 mL of YPD

medium. After approximately three hours, the $OD_{600\ nm}$ reached 0.4 and the culture was then centrifuged at 1.500 *g* for 7 min, at room temperature. The pellet was gently resuspended in 20 mL of sterile distilled water and then centrifuged at 1.500 *g* for 5 min at room temperature. The cells were then washed with 20 mL SED (1 M Sorbitol, 25 mM EDTA, 1 M DTT, pH 8.0), followed by washing with 20 mL Sorbitol 1 M. After that the cells were centrifuged for 7 min at 1.500 *g* at room temperature and then ressuspended by manual agitation in 20 mL SCE buffer (1 M Sorbitol, 1 mM EDTA, 10 mM Sodium citrate pH 5.8). At this time the suspension was divided in 2x10 mL. Ten mL were used to assess the time required for protoplast formation: after addition 15 µL of Zymolyase (3 mg/mL in miliQ water), the cells were kept at 30 °C, without agitation. At regular intervals, 200 µL of the suspension were collected, added to 800 µL SDS 5% (w/v) and the absorbance was measured at 800 nm. When the $OD_{800\ nm}$ reached values near zero, 15 µL of Zymolyase (3 mg/mL) were added to the other 10 mL and kept at 30 °C for the same period of time determined earlier. After that, the cells were centrifuged at 750 *g* for 10 min at room temperature and washed with 10 mL of Sorbitol 1 M and then with 10 mL CaS (1 M Sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$). The suspension was centrifuged at 750 *g* for 10 min at room temperature and resuspended in 0.6 mL CaS. The protoplasts were kept frozen at -80 °C until needed.

III.2.5 Growth curves

The effect of BLAD on the growth of *C. albicans* was evaluated in PDB pH 7.5. The assays were conducted in the presence of different BLAD concentrations, 125 µg/mL and 250 µg/mL, respectively the Minimum inhibitory (MIC) and Minimum Fungicidal (MFC) concentration. A cell suspension was grown overnight in 20 mL of PDB pH 7.5, at 30 ºC, 150 rpm and refreshed in 20 mL of PDB pH 7.5, approximately five hours before being added to the culture medium. In order to obtain an initial concentration of approximately $1x10^5$ CFU/mL, the $OD_{640\ nm}$ was adjusted to 0.15 and then 10-fold diluted with PDB pH 7.5, to a final volume of 100 mL, in 500 mL erlenmeyers. The cultures were incubated at 35 °C without shaking. At regular intervals, samples were collected for absorbance measurements, viable cell counts and morphological evaluation. For viable cell counts, 0.1 mL aliquots of the culture were taken, diluted if needed, and plated on GYP agar plates (incubation at 35 °C for 24 h).

III.2.6 Morphology and viability assessments

The LIVE/DEAD® Yeast Viability Kit [9] was used to evaluate fungal viability. A FUN1 100 µM working solution was prepared in 10 mM MOPS buffer, pH 7.2, with 2% (w/v) glucose and a 50 µM calcofluor white working solution was prepared in distilled water. 40 µL of fungal culture and 5 µL of FUN1 working solution were mixed thoroughly and incubated at 30 °C in the dark. After 30 min, 5 µL of calcofluor white working solution were added to the culture and mixed thoroughly. For microscopical observations, 5 µL of cell culture were trapped between a microscope slide and a coverslip.

III.2.7 BLAD localization studies

The interaction of BLAD with *C. albicans* was studied by using fluorescently labeled BLAD, according to previous studies. The protein was labeled with Alexafluor 488, using the Alexafluor 488 protein labeling kit, according to the manufacturer instructions [10], except for the final step of purification, due to the small size of BLAD. In this study, the labeled protein was purified in NAP-5 columns, according to the manufacturer instructions [11], and stored at 4 °C in the dark.

A lethal concentration of labeled BLAD (250 µg/mL) was used to determine its localization in the cell, under lethal conditions. For this purpose, PDB pH 7.5 was inoculated with approximately $1x10^5$ CFU/mL, as described previously, to a final volume of 4 mL. The culture was kept in the dark at 35 °C, without agitation. For control purposes, the fraction containing the unbounded dye was also added to the cell suspension and kept under the same conditions. Five µL of cell culture were trapped between a microscope slide and a coverslip, for microscope visualization.

III.2.8 Staining with propidium iodide

After the desired incubation period, cells incubated with labeled BLAD and with the free dye were incubated with propidium iodide to a final concentration of 7.5 µM, for 10 min at 4 °C. Five µL of cell culture were trapped between a microscope slide and a coverslip, for microscope visualization.

III.2.9 Immunofluorescency studies

Immunolocalization of BLAD was accomplished according to [12]. The culture was prepared and kept under the same conditions as previously described in the Growth curves section. After 24 h incubation with BLAD, the culture was fixed in the slides with a solution of 0.1% (w/v) Poly-L-lysine (SIGMA) followed by a fixation in 4% (v/v) formaldehyde, for 10 min, at room temperature, followed by two washes with PBS (137 mM NaCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4 and 2.7 mM KCl) and PBS 0.1% (v/v) Triton 100x. The slides were blocked for 30 min with BSA 5% (w/v) in PBS 0.1% (v/v) Triton 100x, in a moist chamber. After a quick wash with PBS, the cells were incubated with a first antibody anti-BLAD, produced in rabbit, diluted 1:500 in PBS 0.1% (v/v) Triton 100x with 0.1% (w/v) BSA, for 16 h at 4 ºC, in a moist chamber. The cells were then washed in PBS and incubated with a second antibody anti-rabbit, produced in goat, conjugated with FITC, diluted 1:80 in PBS with 1% (w/v) BSA, for 1 h at 37 ºC, in a moist chamber. After being washed two times with PBS for 15 min, the slides were prepared in a solution containing DAPI and antifade.

III.2.10 Fluorescence microscopy

All the samples were observed under a fluorescence microscope (Axioscope A1 with phase contrast and epi-fluorescence, Zeiss) equipped with a camera (AxioCam ICm1, Zeiss), using three different filters: Filter Set 49 DAPI (Excitation G 365, Emission BP 420/470); Filter Set 10 FITC/GFP (Excitation BP 450-490, Emission BP 515-565) and Filter Set 15 Rodhamine (Excitation BP 540-552, Emission LP 590).

III.2.11 General procedures

III.2.11.1 Polyacrylamide gel electrophoresis in SDS-PAGE

Polyacrylamide gel electrophoresis in SDS-PAGE was performed as described in Chapter II, section II.2.9.1.2. .

III.2.11.2 Protein Staining

For the silver staining, the gel was incubated in a 50% (v/v) methanol, 12% (v/v) acetic acid and 0.05% (v/v) formaldehyde solution, for 20 min or overnight; after three 10 min washes in 50% (v/v) ethanol, the gel was incubated 1 min in a pre-treatment solution (0.02% (w/v) $Na_2S_2O_3.5H_2O$), washed three times with Milli-Q water, and incubated in staining solution (2 g/L $AgNO_3$ containing 0.75 mL/L formaldehyde) for 10 min; to remove excess $AgNO_3$, the gel was washed twice with Milli-Q water and then the development solution (60 g/L Na_2CO_3, 0.5 mL/L formaldehyde, 4 mg/L $Na_2S_2O_3.5H_2O$) was applied until achieving the desired color intensity; to stop the reaction, the gel was incubated with a stopping solution (50% (v/v) methanol, 12% (v/v) acetic acid), for at least 5 min.

III.3 Results and discussion

III.3.1 Determination of Minimum Inhibitory and Fungicidal Concentrations (MIC and MFC respectively)

Two different inoculum sizes were used for the determination of MIC: 10^3 CFU/mL (as described in [13]) and 10^4 CFU/mL (to further determine MFCs), which was accomplished by two different methodologies (described in [14] and [15]). The antifungal activity of BLAD was tested in three different media, RPMI, YNB and PDB, in order to determine in which medium BLAD is more lethal. Once this was clarified, and since the optimal inoculum density for the subsequent tests was found to be 10^5 CFU/mL, both minimum inhibitory and minimum fungicidal concentrations, BLAD MIC and BLAD MFC, respectively, were determined with this inoculum size. The results are presented in Table III.1.

Table III.1: BLAD MICs and MFCs endpoints for three different *C. albicans* inoculum size, in three different media.

Inoculum Density	Growth Medium	MIC (µg/mL)	MFC (µg/mL)
10^3	RPMI	312.5	500
	YNB	625	500
	PDB	15.6	62.5
10^4	RPMI	625	500
	YNB	625	500
	PDB	15.6	62.5
10^5	PDB	125	250

Table III.1 shows that the medium where BLAD was more lethal, both for the 10^3 and 10^4 CFU/mL inoculum density, was PDB. In both cases the BLAD MFC was 62.5 µg/mL, which was much lower than the endpoint obtained for both RPMI and YNB media (500 µg/mL). For the 10^5 inoculum

density, only PDB was tested, and the results showed that the concentration needed to induce death (as measured by the loss of the ability to grow) was only twice the minimum inhibitory concentration (250 and 125 µg/mL, respectively).

III.3.2 Growth Curves

In order to study the effect of BLAD in the growth of *C. albicans* a growth curve was performed in PDB medium. Several samples were taken during the experiment in order to assess the evolution of the number of viable cells ($OD_{640\ nm}$ and CFU counts). In these experiments two concentrations of BLAD were used, 125 µg/mL and 250 µg/mL, corresponding to the minimum inhibitory and fungicidal concentration, respectively, as determined previously. A fraction of the culture, grown under the same conditions, was kept without BLAD, for control purposes. The results are shown in Figure III.1.

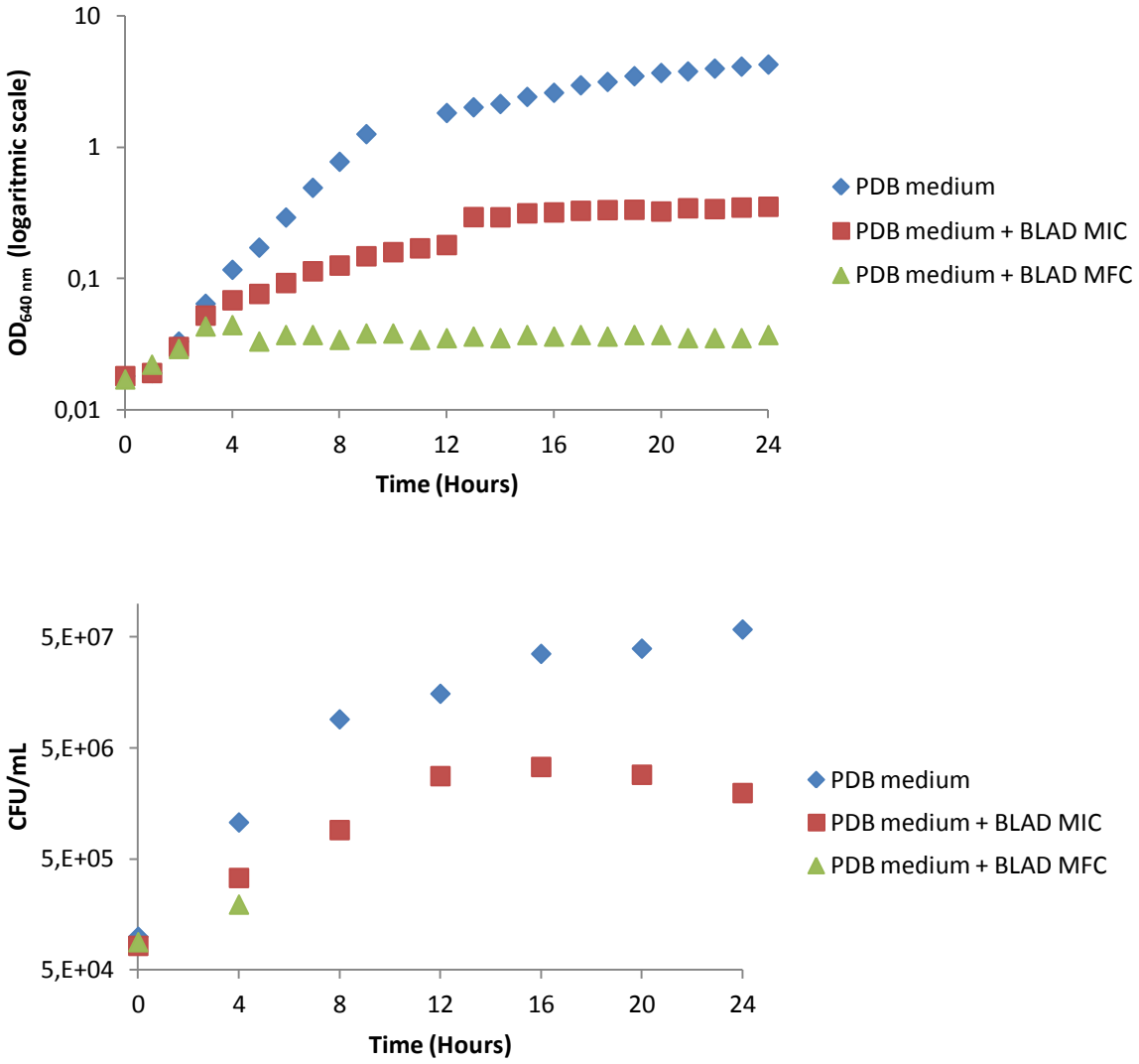

Figure III.1: Effect of BLAD on the growth of *C. albicans* in PDB medium, pH 7.5, 35 ºC, without agitation. **A** – $OD_{640\ nm}$ **B** – CFU/mL. BLAD concentration in the culture medium: 0 µg/mL (◆), 125 µg/mL (■) and 250 µg/mL (▲).

Figure III.1 shows that the addition of BLAD to the culture medium had a strong effect in the growth of *C. albicans.* It is possible to observe that the fraction of the culture grown without BLAD presents a normal growth curve, being in the exponential phase for approximately 8 h, entering then in the stationary phase. This was observed for both $OD_{640\ nm}$ readings and CFU/mL counts.

Cells grown in the presence of the minimum inhibitory concentration of BLAD, showed a decrease in the growth rate, when compared to the control fraction, which resulted in a lower optical density (Figure III.1A) and a lower CFU/mL counts (Figure III.1B). This means that the concentration of BLAD tested had, indeed, the ability to inhibit growth of microorganisms.

Cells grown in the presence of the minimum fungicidal concentration became non-viable after only 4 h of growth. This was observed by both stabilization of $OD_{640\ nm}$ (Figure III.1A) and absence of CFU counts (Figure III.1B).

III.3.3 Viability and cellular integrity assessments

The effect of BLAD on the viability and cellular integrity of *C. albicans* was evaluated using samples collected along the growth curve, in PDB medium. Each sample was stained with FUN-1 and calcofluor white and visualized on a fluorescence microscope. FUN-1 binds to nucleic acids producing a yellowish green fluorescence in death cells with a damage membrane. Cells without metabolic activity but with an intact plasma membrane also present a diffuse green coloration in the cytoplasm. On the other hand, in metabolically active cells, the formation of orange cylindrical structures designated CIVS (Cylindrical IntraVacuolar Structures), is observed inside the vacuoles. CIVS formation only occurs in metabolic active cells with an intact plasma membrane, which means that these are not observed in dead cells [16]. Calcofluor white is a compound with high affinity to chitin and is normally used as a marker of the cell wall in fungi.

Figures III.2 and III.3 suggest that in the first 4 h of incubation with BLAD there are no changes in the viability of the cells, in all conditions tested, since the presence of CIVS indicates metabolic activity. These results are consistent with the growth curve obtained in figure III.1.

Figure III.4, corresponding to 8 h of incubation with BLAD, reveals a turning point in the cell viability. The control fraction and the one incubated with the minimum inhibitory concentration of BLAD (figures III.4- 1a and 1b, respectively) exhibits CIVS in the majority of the cells, which means that they are metabolically active. This is consistent with figure III.1, where is visible that cells are still viable and culturable. On the other hand, though the culture incubated with a lethal concentration of BLAD presents a few number of cells with CIVS (figure III.4-3a) there are no records of viable or culturable cells (figure III.1). This means that after 8 h of incubation with a lethal concentration of BLAD, and despite having a few metabolically active cells, *C. albicans* no longer has the ability of being cultivated in a free-BLAD medium, or the number of culturable cells was so low that was beneath the detection limit of the method (< 10 CFU/mL).

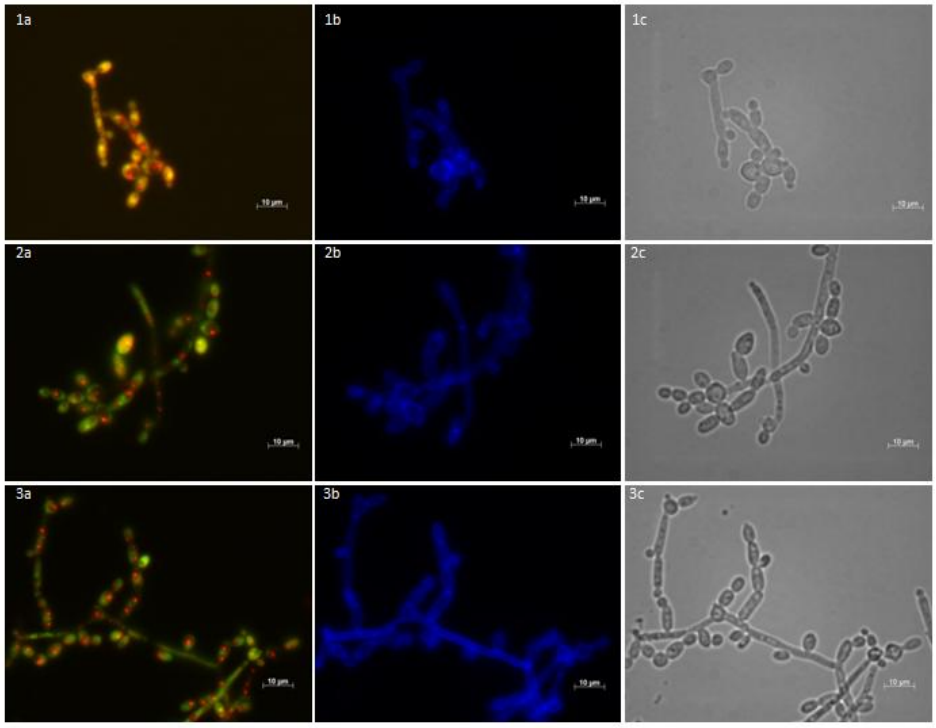

Figure III.2: Effect of BLAD on the metabolic activity and cellular integrity of *C. albicans* cultivated in PDB medium, pH 7.5, at 35 ºC, without agitation. Samples taken after 0 h of incubation. Concentration of BLAD in the culture medium: **1** – 0 µg/mL, **2** – 125 µg/mL, **3** – 250 µg/mL. Labeling with FUN-1 (**a**), calcofluor white (**b**) and bright field microscopy (**c**). Bar corresponding to 10 µm.

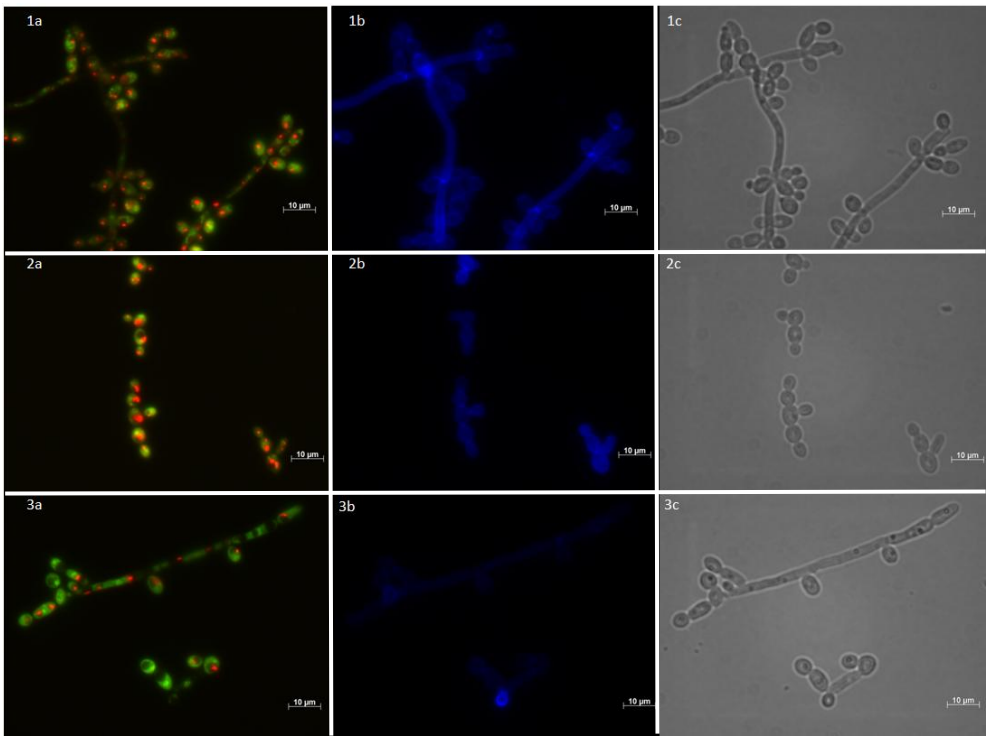

Figure III.3: Effect of BLAD on the metabolic activity and cellular integrity of *C. albicans* cultivated in PDB medium, pH 7.5, at 35 ºC, without agitation. Samples taken after 4 h of incubation. Concentration of BLAD in the

culture medium: **1** – 0 µg/mL, **2** – 125 µg/mL, **3** – 250 µg/mL. Labeling with FUN-1 (**a**), calcofluor white (**b**) and bright field microscopy (**c**). Bar corresponding to 10 µm.

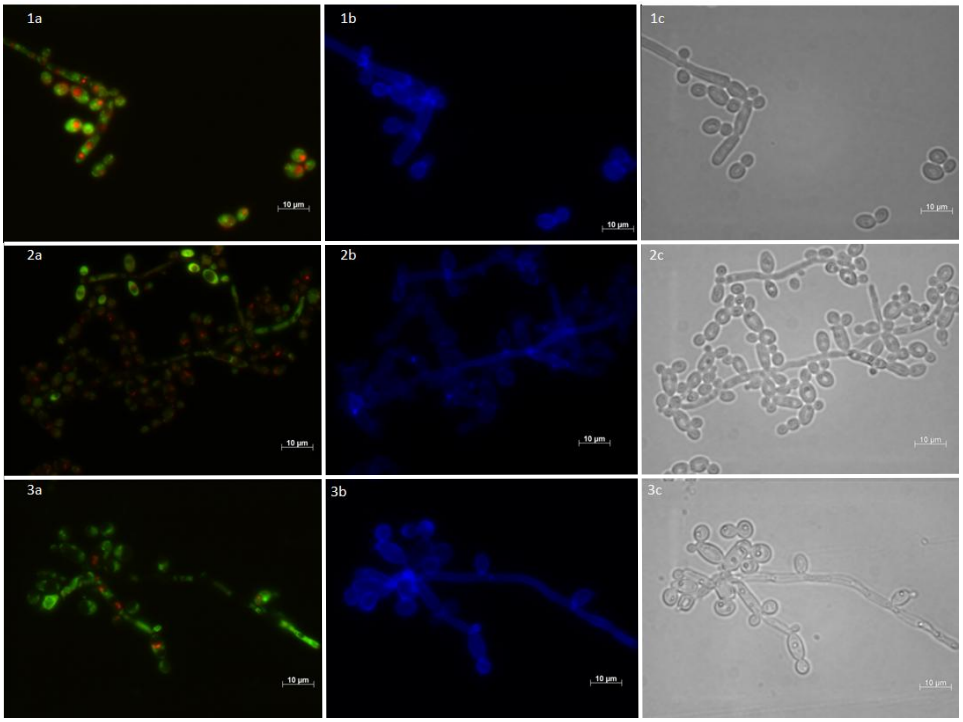

Figure III.4: Effect of BLAD on the metabolic activity and cellular integrity of *C. albicans* cultivated in PDB medium, pH 7.5, at 35 ºC, without agitation. Samples taken after 8 h of incubation. Concentration of BLAD in the culture medium: **1** – 0 µg/mL, **2** – 125 µg/mL, **3** – 250 µg/mL. Labeling with FUN-1 (**a**), calcofluor white (**b**) and bright field microscopy (**c**). Bar corresponding to 10 µm.

After 12 h of incubation with an inhibitory concentration of BLAD (Figure III.5-2a), there are a few number of cells presenting CIVS. This means that some cells are metabolically active and, therefore viable and culturable, which led to a small increase in the $OD_{640\ nm}$ and CFU counts (Figure III.1). Cells incubated with the lethal concentration of BLAD, no longer present CIVS (Figure III.5-3a), only a diffuse green coloration in the cytoplasm, corresponding to the absence of metabolic activity and cell membrane integrity.

At 24 h of incubation, the last time point studied, the control fraction presents some cells without CIVS (Figure III.6-1a), which means that the culture is old and stressed. After 24 h of incubation with the BLAD MIC, there are even fewer metabolically active cells (Figure III.6-2a), than in the previous time point. This is in accordance with the stabilization of $OD_{640\ nm}$ and with the smaller number of culturable cells observed in the growth curves (Figure III.1). The results obtained with the BLAD MFC for 24 h, show no changes with the 12 h results.

The integrity of the cell wall remains unchanged throughout the growth curve, regardless of the concentration of BLAD tested, as showed by calcofluor white stainings.

These results suggest that after 12 h of incubation with a lethal concentration of BLAD, cells are metabolically inactive (Figure III.5,III.6 -3a), non-viable and nonculturable (Figure III.1) but there are no visible changes in the integrity of *C. albicans* cell wall.

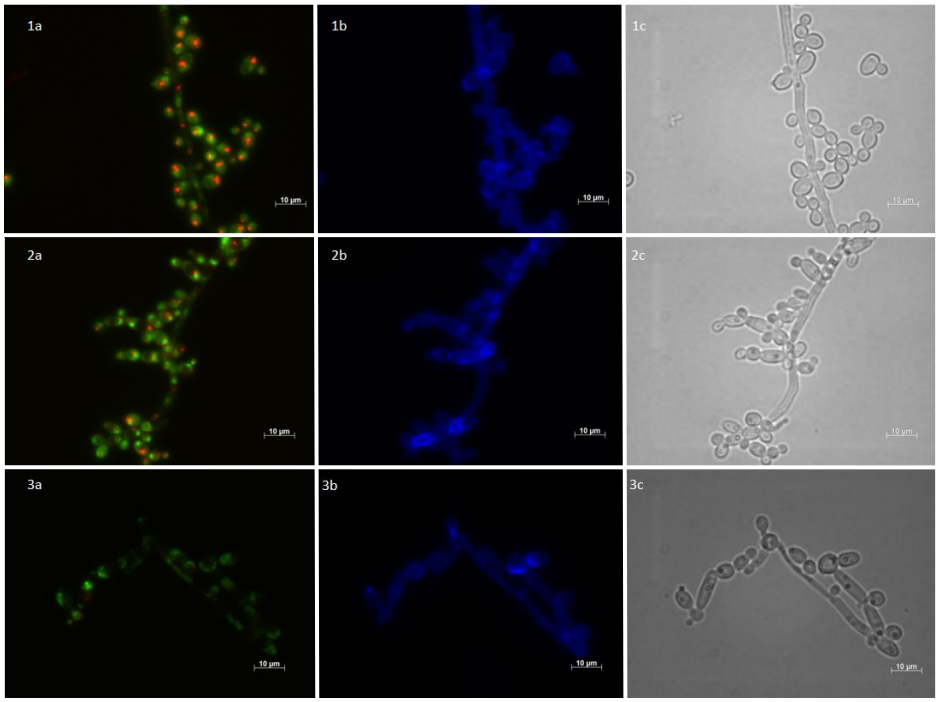

Figure III.5: Effect of BLAD on the metabolic activity and cellular integrity of *C. albicans* cultivated in PDB medium, pH 7.5, at 35 ºC, without agitation. Samples taken after 12 h of incubation. Concentration of BLAD in the culture medium: **1** – 0 µg/mL**, 2** – 125 µg/mL, **3** – 250 µg/mL. Labeling with FUN-1 (**a**), calcofluor white (**b**) and bright field microscopy (**c**). Bar corresponding to 10 µm.

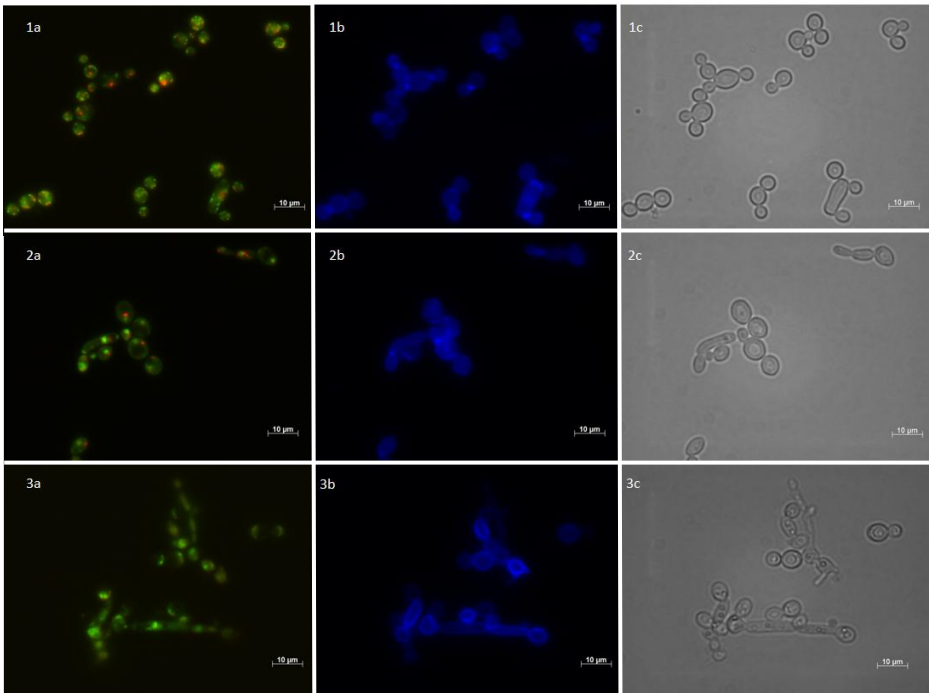

Figure III.6: Effect of BLAD on the metabolic activity and cellular integrity of *C. albicans* cultivated in PDB medium, pH 7.5, at 35 ºC, without agitation. Samples taken after 24 h of incubation. Concentration of BLAD in the culture medium: **1** – 0 µg/mL, **2** – 125 µg/mL, **3** – 250 µg/mL. Labeling with FUN-1 (**a**), calcofluor white (**b**) and bright field microscopy (**c**). Bar corresponding to 10 µm.

III.3.4 BLAD localization studies

After determining the lethal concentration of BLAD (250 µg/mL) and the time needed for the occurrence of death, the next step was to identify the location of the cellular targets of BLAD. For this purpose, BLAD was labeled with Alexa Fluor® 488 dye using the Molecular Probes Labeling kit (Invitrogen), according to the manufacturer instructions [10]. Alexa Fluor® 488, which is similar to fluorescein, is a fluorescent compound capable of binding to amine groups of proteins, in an alkaline medium, giving rise to stable conjugates that emit green fluorescence (maxima fluorescence of 519 nm) when excited in the blue region (maxima absorption of 494 nm). After labeled and purified, BLAD was added to the cell culture. For control purposes, the fraction obtained during the purification of labeled BLAD that contained only unbounded dye was also added to the cell suspension and kept under the same conditions.

Cells incubated with labeled BLAD and with the fraction that contains the excess of dye were also incubated with propidium iodide. This compound binds to DNA and RNA producing a red fluorescence when intercalated with nucleic acids. However, due to its positive charge, it cannot cross an intact cell membrane and, therefore, only dead cells or cells with a damaged membrane are stained.

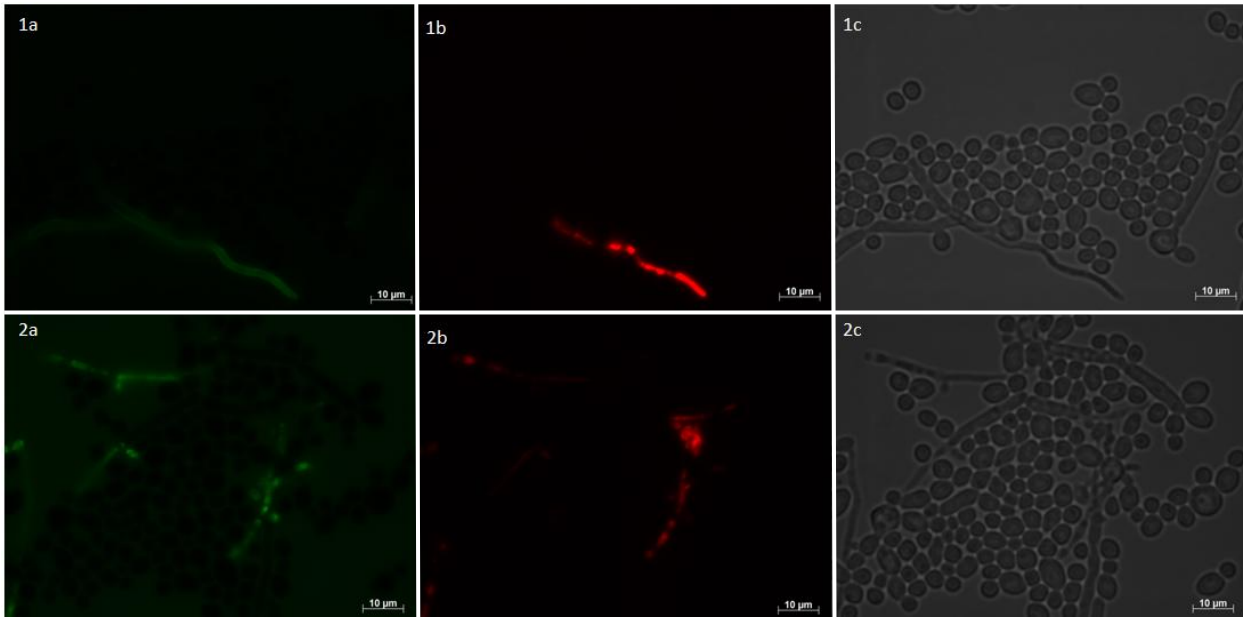

Figure III.7: Determination of the cellular localization of BLAD in *C. albicans* cultivated in PDB medium, pH 7.5, at 35 ºC, without agitation. Samples taken after 24 h of incubation with: **1** - 250 µg/mL of BLAD-Alexa Fluor® 488, **2** – marker without BLAD. Labeling with Alexa Fluor® 488 (**a**), propidium iodide (**b**) and bright field microscopy (**c**). Bar corresponding to 10 µm.

Previous studies showed that yeasts incubated with labeled BLAD presented an intracellular green fluorescence, suggesting that BLAD is capable of crossing the cell wall and membrane. In fact, this result was also confirmed in figure III.7-1a. However, when *C. albicans* was incubated only with the fraction containing the unbounded dye, without BLAD, the pattern of fluorescence was exactly the same (Figure III.7-2a). These results suggest that the intracellular green colour is only an artifact of the method since the fraction of dye without BLAD had the same labeling pattern than the fraction

containing labeled BLAD. Moreover, this pattern is only visible in cells with a damaged membrane (Figure III.7-1b, 2b). This can be explained by the fact that Alexa Fluor® 488 is not a dye specific for BLAD and, therefore, if the membrane is not intact, the unbounded dye can enter into the cell and it may freely binds to citoplasmatic proteins.

To clarify these results, both fractions obtained during the labeling of BLAD were analyzed through SDS-PAGE and then the gel was submitted to silver staining because it offers a higher accuracy in the detection of proteins (Figure III.8).

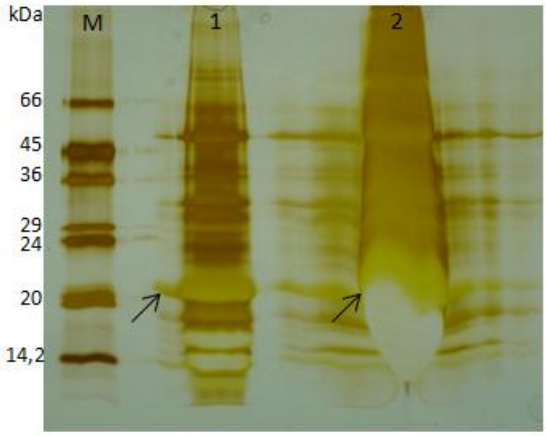

Figure III.8: SDS-PAGE analysis. BLAD labeled with Alexa Fluor® 488 dye. [1] Fraction containing only excess of dye. [2] Labeled BLAD. [M] LPM marker (low protein marker, kDa).

As figure III.8 shows, the fraction that should contained only excess of dye (Figue III.8, lane [1]) also contains BLAD, which means that the labeling was not efficient. In light of these results, and considering the previous results showed in figure III.7, two hypothesis are possible: or BLAD somehow destabilizes the plasma membrane, which justifies the entry of propidium iodide (Figure III.7-1b, 2b), and the green fluorescence observed (corresponding to the labeled BLAD), or the labeling failed and BLAD destabilizes the cell membrane allowing the entrance of the unbounded dye (once inside the cell, it binds to the citoplasmatic proteins, resulting also in a intracellular green fluorescence).

III.3.5 Immunofluorescence

Since the previous method used to determine the location and mode of action of BLAD turned out to be inconclusive, another methodology was used in order to clarify this. Immunofluorescence is a technique that allows the visualization of antigen-antibody interaction in cell suspensions. In this particularly case, *C. albicans* was incubated with the lethal concentration of BLAD for 24 h and then the cell suspension was fixed on glass slides. BLAD functions as an antigen since a first antibody anti-BLAD produced in rabbit was added, followed by a second antibody anti-rabbit produced in goat, conjugated with FITC. Calcofluor white was also added in order to investigate the cell wall integrity. The results are shown in figure III.9.

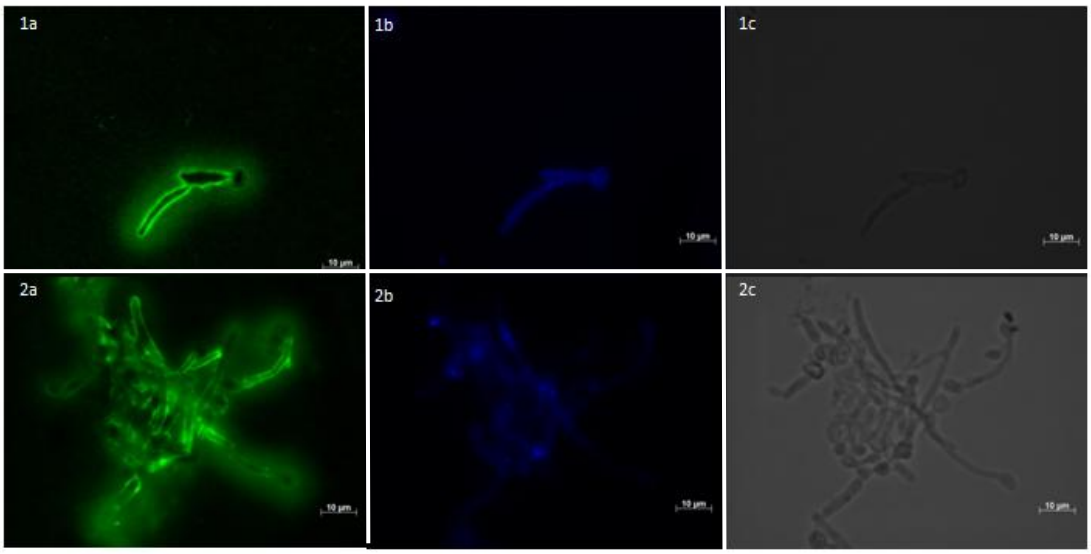

Figure III.9: Immunofluorescence in *C. albicans* incubated with BLAD for 24 h. BLAD functions as an antigen; first antibody anti-BLAD produced in rabbit; second antibody anti-rabbit produced in goat, conjugated with FITC. FITC filter (**a**), DAPI filter (**b**) and bright field microscopy (**c**). Bar corresponding to 10 µm.

In figure III.9-1a,2a it is possible to observe a green fluorescence around the cells. This result clearly shows that after 24 h of incubation, BLAD is bounded to the *C. albicans* cell envelope but it does not enter into the cell. Moreover, the staining with calcofluor white reveals that the cell wall remains intact (Figure III.9-1b,2b).

For the purpose of ascertaining if BLAD binds to the plasma membrane or to the cell wall, protoplasts of *C. albicans* were produced and incubated with BLAD for 24 h. A fraction of the culture was kept without BLAD, for control purposes. The metabolic activity of the protoplasts was evaluated using FUN-1 (Figure III.10).

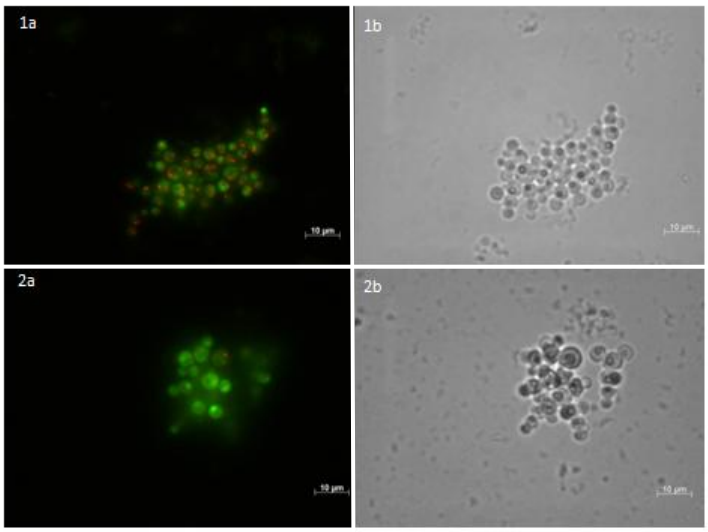

Figure III.10: Effect of BLAD on the metabolic activity of protoplasts of *C. albicans* cultivated in CaS (1 M Sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$) at 35 ºC. Protoplasts incubated for 24 h: **1**- Without BLAD, **2**- With 250 µg/mL of BLAD. Labeling with FUN-1 (**a**) and bright field microscopy (**b**). Bar corresponding to 10 µm.

As expected, and comparing the results with those obtained in figure III.6, when exposed to BLAD for 24 h, protoplasts became metabolically inactive, since there were no visible CIVS (Figure III.10-2a).

The next step was to perform immunofluorescence using *C. albicans* protoplasts, as previously done with the cells (Figure III.11). Calcofluor white was also added to make sure that there were no remains of cell wall in the protoplasts. From the results showed in figure III.11 it is possible to assume that BLAD binds to the plasma membrane because the staining with calcofluor white shows no indication of cell wall (no visible blue staining) (Figure III.11-b). Moreover, BLAD is clearly located in the periphery of the protoplast, with no traces of the protein in its interior. The procedure also resulted in an extensive destruction of protoplasts, resulting in a very "dirty" preparation. Interestingly, BLAD seemed to bind to many of the cell fragments.

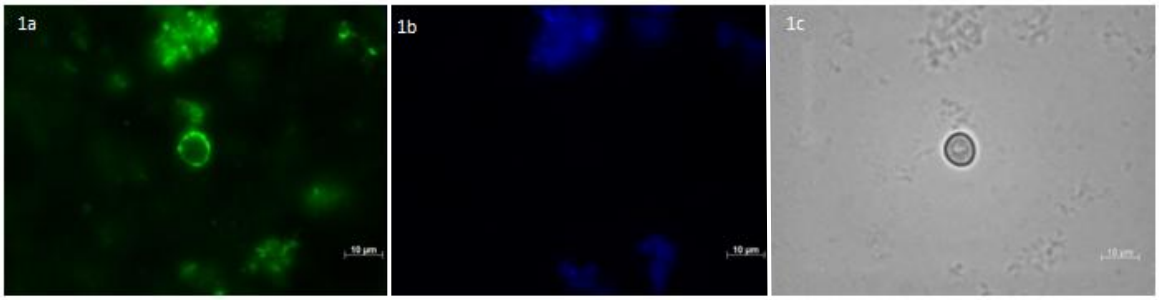

Figure III.11: Immunofluorescence in protoplasts of *C. albicans* incubated with BLAD for 24 h. BLAD functions as an antigen; first antibody anti-BLAD produced in rabbit; second antibody anti-rabbit produced in goat, conjugated with FITC. FITC filter (**a**), DAPI filter (**b**) and bright field microscopy (**c**). Bar corresponding to 10 µm.

III.4 Conclusion

The results obtained in this task demonstrate that BLAD binds to the cell envelope of *C. albicans,* without causing any damage to the cell wall, but it does not enter into the cell. Moreover, the results obtained with the protoplasts suggest that BLAD passes through the cell wall and then binds to the plasma membrane. After being exposed for more than 12 h to the lethal concentration of BLAD (250 µg/mL), some *C. albicans* cells show loss of membrane integrity, revealed by the propidium iodide staining, but, nevertheless, there are no changes in the cell wall (Calcofluor white staining). In addition, *C. albicans* became metabolically inactive, which results in the disappearance of CIVS, non-viable, resulting in stabilization of the optical density, and nonculturable, resulting in decrease of CFU counts.

III.5 References

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Chapter IV

Search for specific targets for BLAD in the pathogen cell envelope

IV.1 Introduction

The cell membrane is typically a key site on host-pathogen interactions. It is where many receptors are located and the onset of a large number of cascade mechanisms. The plasma membrane contains many proteins, a considerable proportion of which are glycoproteins. The oligosaccharides projected outwards from the cell membrane are collectively termed the exoglycome.

Given that the exoglycome extends beyond the cell wall, it plays a fundamental role in cell-cell recognition and in cell-molecule interactions, in discriminating self from non-self, in the warfare between host and pathogen before infection is established, and in certain diseases such as cancer. Protein-carbohydrate interactions control salient aspects of intra- and intercellular communication and trafficking, and are at the basis of a variety of essential biological phenomena. They are involved, for example, in adhesion of infectious agents to host cells, and cell adhesion in the immune system, malignancy and metastasis. For these reasons, and following nucleic acids and proteins, carbohydrates, or more specifically oligosaccharides, have been recently recognized as the third code/alphabet of life, with a coding capacity which far exceeds those of the other two polymers [1].

Yeasts secrete and process glycoproteins in much the same way as mammalian cells do. Proteins are passed into the endoplasmic reticulum (ER) cotranslationally, glycosylated, sent to the Golgi for further processing, and then are either targeted to various organelles, become plasma membrane components, or are secreted into the periplasm [2].

The common classes of glycoproteins found in eukaryotic cells are primarily defined according to the nature of the linkage (core) regions to the aglycone (protein or lipid): *N*- and *O*- glycoproteins [3].

An *N*-linked oligosaccharide is a sugar chain covalently linked to an asparagine residue of a polypeptide chain [3], as part of a three-amino-acid residue consensus sequence NXSer/Thr, where X is any amino acid residue except proline and the third amino acid residue can be serine (Ser) or threonine (Thr) [4], being synthesized as lipid-linked intermediates anchored to the ER membrane [2]. *N*-linked oligosaccharide share a common pentasaccharide core region and may be generally subdivided into three main classes: high-mannose-type, complex-type, and hybrid-type [3].

An *O*-linked oligosaccharide is typically linked to the polypeptide via *N*-acetylgalactosamine (GalNAc) to a serine or threonine residue and can be extended into a variety of different structural core classes [3]. There is no consensus sequence for *O*-linked oligosaccharides, but they are generally found in serine and threonine-rich regions of proteins. *O*-linked oligosaccharides are generally smaller (compared to *N*-linked oligosaccharides) consisting of 3 to 10 monosaccharide residues [4]. Other types of *O*-linked oligosaccharides do exist (e.g., *O*-linked mannose). However, since the *O*-GalNAc linkage is the best known, it is often described by the generic term *O*-linked oligosaccharide [3].

Other classes of oligosaccharides can also be found in eukaryotic cells, such as glycophospholipid, mucins (large glycoprotein that carries many *O*-linked oligosaccharides that are

often closely spaced), glycolipid and gangliosides (an anionic glycolipid containing one or more residues of sialic acid) [3].

Given that membrane proteins play critical roles in many biological functions and are frequently the molecular targets for drug discovery, they were the main subject of study in this task. Since *C. albicans* was the unicellular fungal model in this study, its membrane glycoproteins were screened for specific targets for BLAD polypeptide.

IV.2 Materials and methods

IV.2.1 Biological materials and growth conditions

IV.2.1.1 *Candida albicans*

Candida albicans var. *albicans* (CBS 562) was grown at 35 °C for 24 h in Glucose Yeast Peptone (GYP) medium (1% (w/v) peptone, 0.5% (w/v) yeast extract, 2% (w/v) glucose, 1.5% (w/v) agar). For protoplasts formation, the yeast was grown in YPD medium (20 g/L yeast extract, 20 g/L peptone and 10 g/L dextrose).

C. albicans protoplasts were made as described in Chapter III, section II.2.4.

IV.2.1.2 *Lupinus albus*

The seeds of *Lupinus albus* were germinated and grown in growth chambers with a photoperiod of 16 h light/8 h dark at 18 °C, for periods up to 10 days. The seed coats were removed and the intact cotyledons dissected from the axes and stored frozen at -80 °C until needed.

IV.2.2 BLAD purification

BLAD extraction and purification from *Lupinus albus* cotyledons was performed as described in Chapter III, section II.2.2.

IV.2.3 Isolation of *Candida albican*s protoplast cell membrane

After centrifugation of *C. albicans* protoplasts at 1.100 *g* during 5 min, the pellet was incubated at 0-4 °C during 15 min in 10 volumes of 5 mM phosphate buffer, pH 8.0, for cell lysis. After incubation, the membranes were precipitated by centrifugation at 20.000 *g*, 10 min, 4 °C. The membranes were then washed four times with the same volume of 5 mM phosphate buffer, pH 8.0, and centrifuged at 21.500 *g*, 5 min at 4 °C. The pellet was resuspended in saline containing 2 mM Ca^{2+} and 2 mM Mg^{2+}, and stored at -80 °C until use.

IV.2.4 Protein deglycosylation of *C. albicans* cell membranes

Partial deglycosylation of the glycoproteins from *C. albicans* cell membranes was performed according to the protocols described in [5]. Five hundred µg/mL of protein from *C. albicans* cell membranes were used in each deglycosylation assay.

O-linked oligosaccharides were released using reductive elimination by the addition of a sodium hydroxide solution containing 1 M sodium borohydride to the cell membranes and incubation at 45 °C for 16 h with gentle stirring. The reaction was stopped by the drop-wise addition of glacial

acetic acid until no fizzing was detected. The sample was then dialysed against distilled water, pH adjusted to 7.5, and lyophilized.

The release of *N*-linked oligosaccharides was achieved by adding 5 µL of PNGase F (0.5 U/µL) to the cell membranes followed by incubation at 37 °C for 17 h with gentle stirring. The reaction was stopped by adding four volumes of cold ethanol, kept on ice for 30 min, and then centrifuged at 15.000 *g*, 10 min at 4 °C.

To remove both *N*- and *O*-linked oligosaccharides, chemical deglycosylation with trifluoromethanesulfonic acid (TFMS) was used based on the protocol from PROzyme/Glyko Glycofree Chemical deglycosylation kit, according to the manufacturer instructions [6]. Briefly, TFMS was added to toluene to a final concentration of 10% (v/v). The sample, previously lyophilized, was placed in an ethanol/dry ice bath for 20 s and then 50 µL of the TFMS/toluene mix was added. The vial was placed at -20 °C for 4 h. Neutralisation of TFMS was achieved by adding 150 µL of a solution of pyridine, methanol and water (3:1:1) to the sample, previously placed in an ethanol/dry ice bath for 20 s. The vial was kept there for another 20 s and then was transferred to dry ice for 5 min and finally to wet ice for 15 min. In the end, 400 µL of the neutralisation solution (0.5 % (w/v) ammonium bicarbonate) were added to the solution. Recovery of the deglycosylated polypeptides was achieved by dialysis against 10 mM ammonium carbonate. The sample was then lyophilized.

IV.2.5 Binding of purified BLAD to the *Candida albicans* cell membrane

After *C. albicans* protoplast lysis, the subsequently isolated cell membranes were used as targets to bind purified BLAD. The polypeptide was lyophilized and solubilised in saline containing 2 mM Ca^{2+} and 2 mM Mg^{2+}. Five hundred µg of BLAD was incubated with 200 µL of *C. albicans* membranes, with gentle stirring (approximately 80 rpm) for 30 min at 25 °C. After incubation, the homogenate was washed three times with 500 µL of saline and centrifuged for 5 min at 7.800 *g,* 8 min at 14.000 *g,* and 8 min at 14.000 *g*. The pellet was then resuspended in 500 µL of saline and stored frozen at -80 °C until use.

IV.2.6 Immunoblotting

The immunoblotting procedure was carried out as described in Chapter II, section II.2.7. BLAD-specific polyclonal antibodies were produced in rabbit, as described in [7], and used as the primary antibody. A goat anti-rabbit antibody linked to a peroxidase, specific for the primary antibody (SIGMA) was used as the second antibody.

IV.2.7 General procedures

IV.2.7.1 Electrophoresis

IV.2.7.1.1 Polyacrylamide gel electrophoresis in SDS-PAGE

Polyacrylamide gel electrophoresis in SDS-PAGE was performed as described in Chapter II, section II.2.9.1.2.

IV.2.7.1.2 2D-electrophoresis

Protein samples were precipitated with iced cooled 80% (v/v) acetone, at -20 °C during 30 min and then centrifuged at 15.000 *g*, 10 min at 4 °C. The pellet was ressuspended in a rehydration buffer containing 7 M urea, 2 M thiourea, 2% (v/v) nonylphenoxypolyethoxylethanol (NP-40) and 1% (w/v) dithiothreitol (DTT). IPG-buffer solution (0.5% (v/v)) was also added.

The samples were then placed on a 7 cm-long strip of immobilized pH gel (Bio-Rad) with a pH gradient 3-10. The strips were placed on the Protein IEF Cell equipment (Bio-Rad), where isoelectric focusing takes place. The isoelectric focusing program comprised the following steps: rehydration: 50 V, 12 h; 1st step: 250 V/h; 2nd step: 500 V/h; 3rd step: 8.000 V, 2.5 h; 4th step: 8.000 V, 3.000V/h. In the end, the strips were incubated in a solution containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 1% (w/v) DTT, for 15 min with stirring. This solution was removed and the strips were re-incubated in a solution containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 2.5% (w/v) iodoacetamide. After these incubations, each strip was placed at the top of a SDS-PAGE polyacrylamide mini gel and sealed with 0.5% (w/v) agarose. Electrophoresis ran at 5 mA for 15 min and then 10 mA, 220 V.

IV.2.7.2 Protein Quantification

Protein content was determined according to a modification of the Bradford's method, as described in [8]. The samples were read in a spectrophotometer, at 595 nm, and bovine serum albumin (BSA) was used as the standard.

IV.2.7.3 Protein Staining

The gels were stained with Coomassie Brilliant Blue R250 (CBB R-250) or Coomassie Brilliant Blue G250 (CBB G-250). CBB R-250 staining was made as described in Chapter II, section II.2.9.3.

In the CBB G-250 staining, polypeptides were fixed in a solution containing 2% (v/v) phosphoric acid and 50% (v/v) methanol, overnight, followed by three washes with distilled water, 30 min each. The incubation process was made with a solution of 34% (v/v) methanol, 17% (w/v) ammonium sulphate and 2% (v/v) phosphoric acid, during 1 h. After the incubation, a staining solution containing 1.1% (w/v) Coomassie G in 34% (v/v) methanol was added. This step can occur overnight and be extended up to 5 days. The final washes were made with bidistilled water.

IV.3 Results and discussion

IV.3.1 Analysis of the *C. albicans* cell membrane proteome

To study the proteins located at the *C. albicans* cell membrane, the cell wall was removed by preparation of protoplasts. After *C. albicans* protoplast lysis and the subsequent isolation of their cell membranes, the protein content was quantified by the Bradford's method [8]. Protein separation was performed using both one and two-dimensional electrophoresis (Figure IV.1).

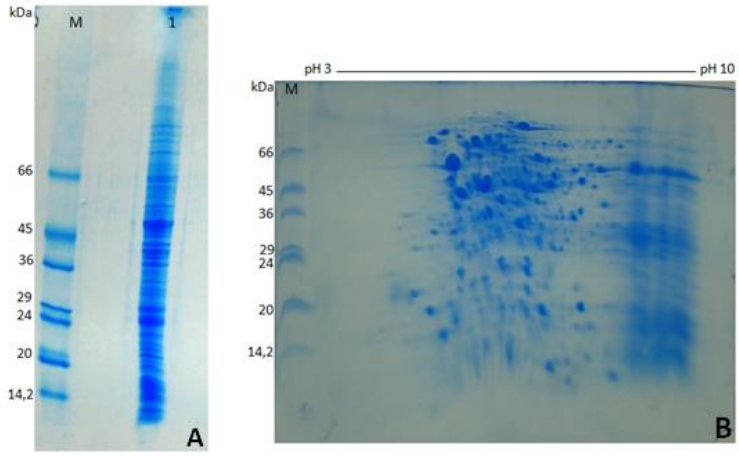

Figure IV.1: The proteome of *C. albicans* cell membrane.**(A)** SDS-PAGE analysis of *C. albicans* cell membrane proteins. [1] 100 µg of *C. albicans* cell membrane protein. **(B)** 2D-electrophoresis, 3-10 pH gradient strip; 500 µg of *C. albicans* cell membrane protein. [M] LPM marker (low molecular mass protein markers, kDa).

In figure IV.1A it is clear the presence of a large variety of cell membrane proteins, with a regular distribution of electrophoretic bands, mostly located below 66 kDa molecular mass. Figure IV.1B shows the distribution of the spots resulting from a 2D-electrophoresis, where polypeptides were separated according to their isoelectric point (IP) and, on a second phase, according to their molecular mass. Despite showing some background, probably due to a poorly resolved isoelectric focusing, most of the spots are well individualized and evently distributed throughout the pH range used.

IV.3.2 Protein deglycosylation of *C. albicans* cell membrane glycoproteins

With the purpose of identifying specific oligosaccharide targets for BLAD in the pathogen cell membrane, the total protein fraction from *C. albicans* cell membrane was subjected to three different deglycosylations procedures: removal of *N*-linked oligosaccharides, removal of *O*-linked oligosaccharides and removal of both *N*- and *O*-linked oligosaccharides, as described in the materials and methods section. The first step after deglycosylation was to perform a 1D-electrophoresis with the aim of analysing the resulting protein profile and ensure that the method used did not destroy the proteins. The results are shown in figure IV.2.

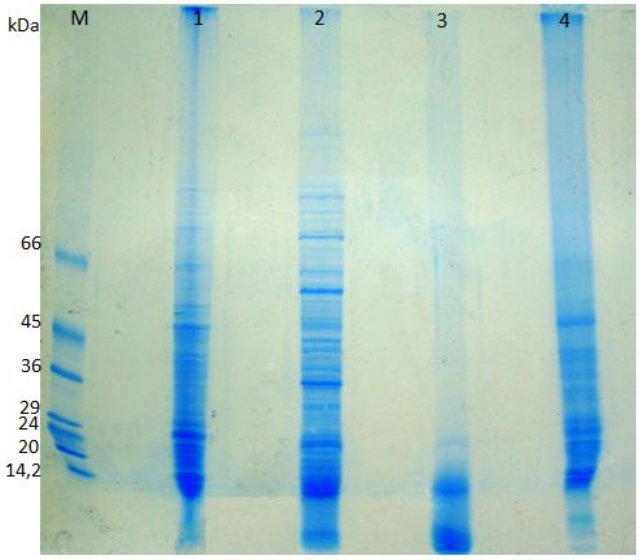

Figure IV.2: SDS-PAGE analysis of *C. albicans* cell membrane proteins, subjected to different deglycosylation processes (100 µg protein in each sample). [1] *C. albicans* total protein from the intact cell membrane; *C. albicans* cell membrane total protein after treatment for the removal of: [2] *N*-linked oligosaccharides; [3] *O*-linked oligosaccharides; [4] both *N*- and *O*-linked oligosaccharides; [M] LPM marker (low molecular mass protein markers, kDa).

The analysis of figure IV.2 suggests that the protein profile suffered an expected change when the cell membrane was subjected to removal of *N*- and both *N*- and *O*-linked oligosaccharides since the distribution of the bands is slightly different from the control. That means, basically, a change in the proteins molecular weight caused by the removal of the oligosaccharide moiety. However, and without a clear explanation, upon removal of *O*-linked oligosaccharides (Figure IV.2, lane [3]), the polypeptide profile was dramatically changed, resulting in the disappearance of the higher molecular weight polypeptides. This means that probably the method used was to aggressive and, consequently, polypeptides were destroyed resulting only in low molecular weight peptides. However, another hypothesis can be assumed supported by the results obtained in subsequent experiments: somehow the reagent used for *O*-linked oligosaccharides removal may have interfered with the correct migration of the polypeptides subjected to electrophoresis.

IV.3.3 Binding of purified BLAD to *C. albicans* cell membranes

After the isolation of *C. albicans* cell membranes and posterior deglycosylation of its total protein fraction, each type of membrane was incubated with BLAD with the aim of observing if the oligosaccharides removal affected the binding of this antifungal agent and/or to identify precisely the specific type of oligosaccharide which is targeted by BLAD. This was visualized firstly through SDS-PAGE, then by 2D-electrophoresis, and finally by immunoblotting analysis in an attempt to determine exactly BLAD binding site on *C. albicans* cell membranes. In the latter case, *C. albicans* total protein from the cell membrane was electroblotted onto a PVDF membrane that was posteriorly incubated with BLAD.

As a control, the incubation of BLAD with the intact cell membrane was performed to guaranteed, and to confirm previously obtained results from the immunoflorescency studies (see Chapter III), that those are in fact targets for BLAD (Figure IV.3).

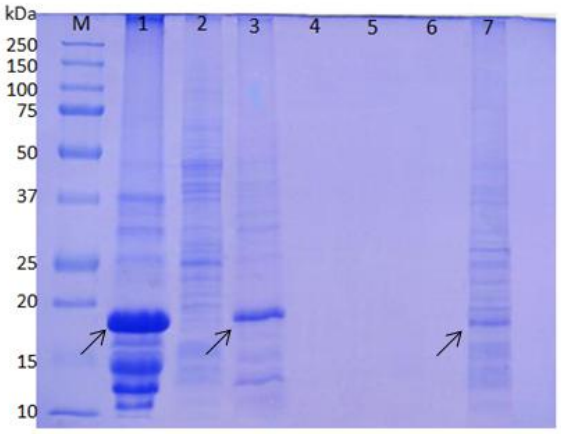

Figure IV.3: SDS-PAGE analysis of *C. albicans* total protein from the cell membranes incubated with BLAD. [1] Purified BLAD (200 µg); [2] *C. albicans* cell membrane total protein fraction (50 µg). Washing buffer after incubation of *C. albicans* membranes with BLAD:[3] 1st wash; [4] 2nd wash; [5] 3rd wash; [6] 4th wash; [7] *C. albicans* cell membrane total protein fraction resulted after incubation with BLAD and after washes; [M] Precision Plus Protein™ All Blue Standards marker (kDa).

As figure IV.3 shows, a 20 kDa (BLAD molecular weight) polypeptide is visible in lane [1], corresponding to BLAD, as well as in lane [3], corresponding to the 1st wash with saline, and in lane [7], corresponding to the result of the incubation, after the washes. This result shows that BLAD binds specifically to *C. albicans* cell membrane proteins, as it remains attached after four washes with saline.

After confirming that BLAD binds to *C. albicans* proteins from the cell membrane, the next step was to identify the precise target for BLAD (Figure IV.4).

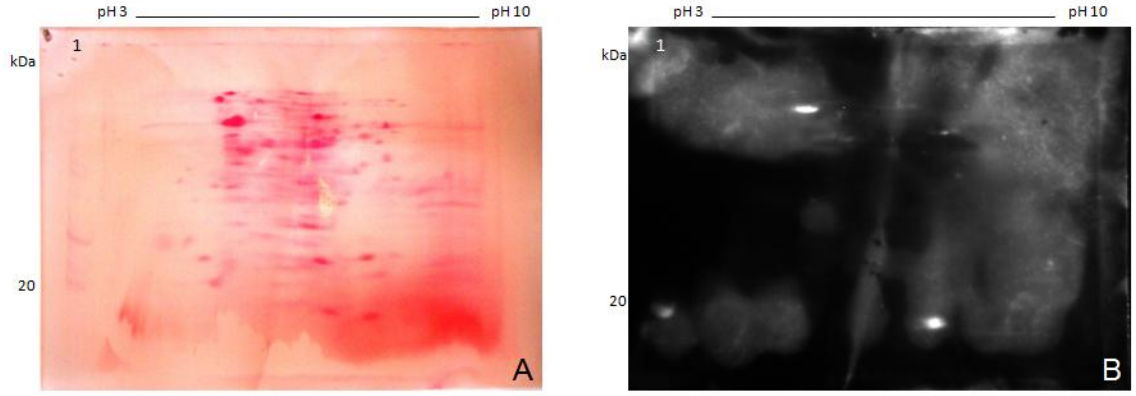

Figure IV.4: 2D-electrophoresis and immunoblotting analysis. **(A)** *C. albicans* total cell membrane proteins (500 µg) were transferred onto a PVDF membrane and stained with Ponceau S. **(B)** Incubation of the total extracted proteins from the cell membrane with BLAD and posterior immunoblotting. 3-10 pH gradient strip. [1] Purified BLAD (200 µg).

In figure IV.4A, the total 2D protein profile of the cell membrane can be observed. Lane 1 corresponds to BLAD polypeptide and was included as a control of the study. In figure IV.4B, after incubating the total protein fraction from the cell membrane of *C. albicans* with BLAD, the anti-BLAD polyclonal antibodies produced in rabbit was used as the probe to identify and bind to the fractionated *C. albicans* polypeptide spots. A second antibody specific to rabbit IgGs was used to reveal the targets of BLAD. As can be observed two major polypeptide spots are revealed in figure IV.4B which correspond to two polypeptides with isoelctric points of *ca.* 4 and 8 and molecular masses of aproximately 70 and 14 kDa, respectively. The control BLAD is also revealed (lane 1) showing the specificity of the antibody and that in fact the only polypetide being detected was BLAD.

To identify the glycoprotein targeted by BLAD, two polypeptide spots were sliced from the membrane, submitted to deglycosylation and, separately, both oligosaccharide and polypeptide residues sent to be sequenced. Such information will be rather importat because will provide an idea of the type of resideus that are involved in BLAD mode of action, i.e. the precise specificity of BLAD as lectin. However, we are still waiting for the sequencing results.

After the knowledge that BLAD binds to the *C. albicans* cell membrane proteins and with the hypothesis that the target is an oligosaccharide residue, the same procedure was repeated but after the *N*-deglycosylation procedure. Figure IV.5 shows the SDS-PAGE gel obtained after *N*-deglycosylation and incubation with BLAD. As it can be observed, again, it was possible to identify the

binding of BLAD to the cell membrane proteins after thorough washing procedures (Figure IV.5, lane [7]).

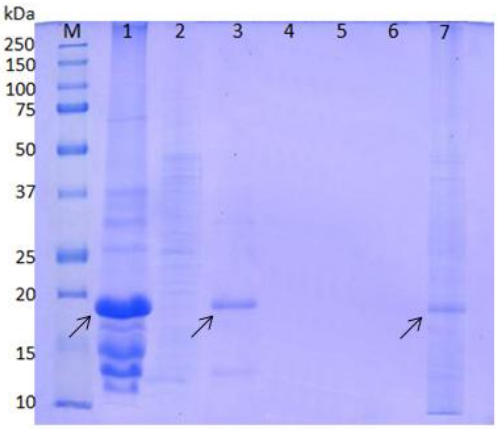

Figure IV.5: SDS-PAGE analysis of *C. albicans* cell membrane proteins incubated with BLAD upon removal of *N*-linked oligosaccharides. [1] Purified BLAD (200 µg); [2] *C. albicans* cell membrane total protein fraction (50 µg). Washing buffer after incubation with BLAD: [3] 1st wash; [4] 2nd wash; [5] 3rd wash; [6] 4th wash; [7] *C. albicans* cell membrane proteins resulted after incubation with BLAD, upon removal of *N*-linked oligosaccharides, and after washes; [M] Precision Plus Protein™ All Blue Standards marker (kDa).

The same immunoblotting study after BLAD incubation was performed to understand if there were any changes in the binding of BLAD to targeted polypeptides after each step of partial deglycosylation, beginning with those which have suffered removal of *N*- linked oligosaccharides (Figure IV.6).

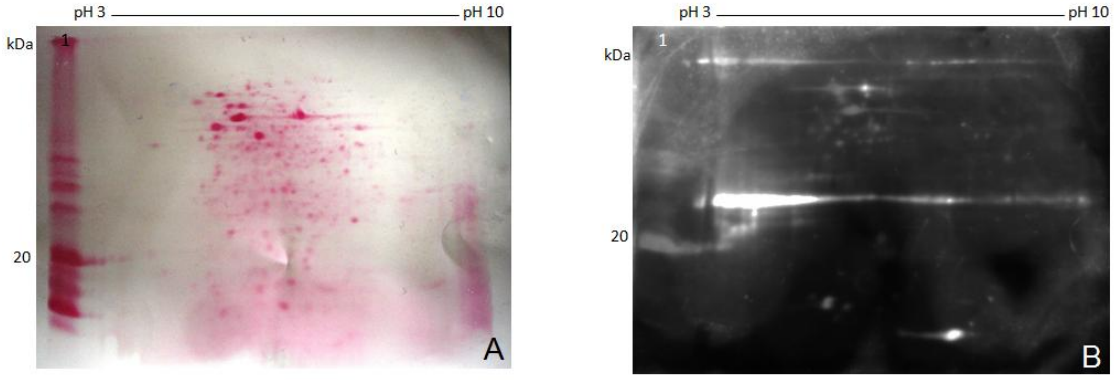

Figure IV.6: 2D-electrophoresis and immunoblotting analysis. **(A)** Upon removal of *N*- linked oligosaccharides, *C. albicans* cell membrane proteins (500 µg) were transferred onto a PVDF membrane and stained with Ponceau S. **(B)** Incubation of the *C. albicans* cell membrane proteins, upon removal of *N*-linked oligosaccharides, with BLAD and posterior immunoblotting. 3-10 pH gradient strip. [1] Purified BLAD (200 µg).

As figure IV.6 shows, there are some spots being indentified in the immunoblot which is traduced by BLAD targeting. Although with some background, two spots with isoelectric points of *ca.* 6 and 8 and molecular masses of 60 and 14 kDa, respectively, are being revealed. From these results it

is possible to assume that even upon removal of the *N*-linked oligosaccharides, BLAD still binds to membrane proteins, probably using other type of glycoproteins as target. To investigate it further, we mantained the *N*-glycoproteins and removed the *O*-linked oligosaccharides. Although the method for removal of *O*-linked oligosaccharides has shown to be very aggressive and probably has led to destruction of the proteins (Figure IV.2, lane [3]), the incubation of these deglycosylated cell membrane proteins with BLAD was performed and, as previously described, the polypeptide also binds to the *C. albicans* membranes upon treatment for removal of *O*-linked oligosaccharides, after thorough washing procedures (Figure IV.7, lane [7]).

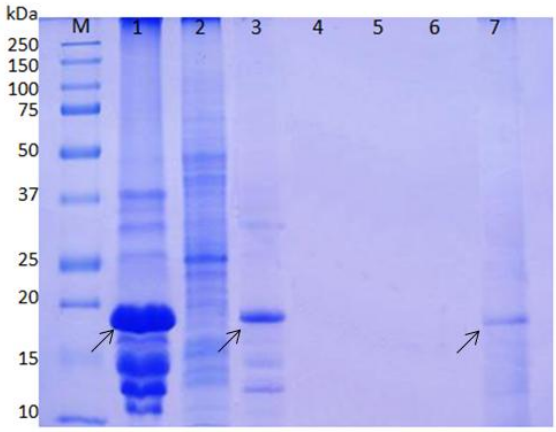

Figure IV.7: SDS-PAGE polyacrylamide analysis of *C. albicans* cell membrane proteins incubated with BLAD upon removal of *O*-linked oligosaccharides. [1] Purified BLAD (200 µg); [2] *C. albicans* cell membrane total protein fraction (50 µg). Washing buffer after incubation:[3] 1st wash; [4] 2nd wash; [5] 3rd wash; [6] 4th wash; [7] *C. albicans* cell membrane proteins resulted after incubation with BLAD, upon removal of *O*-linked oligosaccharides, and after washes; [M] Precision Plus Protein™ All Blue Standards marker (kDa).

The data shown above suggest that BLAD binds to both types of glycoproteins present in *C. albicans* cell membrane proteins. As performed to the intact *C. albicans* cell membrane and to those which have suffered *N*-deglycosylation, these ones were also submitted to 2D-electrophoresis followed by immunoblot analysis. However, after being transferred onto the PVDF membrane, no proteins of C. *albicans* cell membranes could be detected. This unexpected result probably due to the method used to remove the *O*-linked oligosaccharides since, as shown in figure IV.2 lane [3], the proteins seem to have been deteriorated, making it impossible to perform the immunoblot analysis.

The last incubation tests with BLAD were performed using *C. albicans* cell membrane proteins treated to remove both *N*- and *O*-linked oligosaccharides (Figure IV.8). Surprisingly, BLAD also binds to this type of membranes (Figure IV.8, lane [7]).

This last result raised the suspicion that the methods used for the oligosaccharides removal, in these conditions, do not fully removed either *N*-linked and/or *O*-linked oligosaccharides from *C. albicans* cell membrane glycoproteins. In this case if only a partial proportion of oligosaccharides was indeed removed it is not possible to take valuable conclusions regarding the nature of the possible target for BLAD polypeptide.

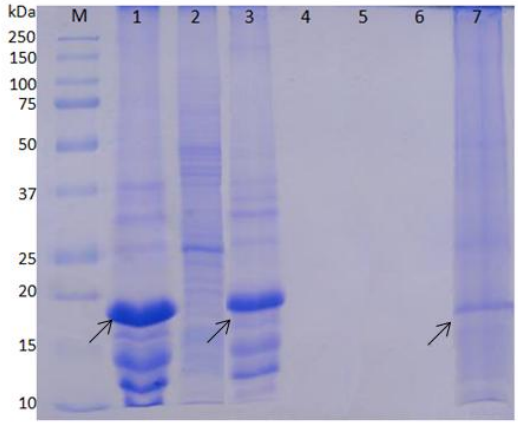

Figure IV.8: SDS-PAGE polyacrylamide analysis of *C. albicans* cell membranes proteins incubated with BLAD, upon removal of both *N*- and *O*-linked oligosaccharides. [1] Purified BLAD (200 µg); [2] *C. albicans* cell membrane total protein fraction (50 µg). Washing buffer after incubation with BLAD: [3] 1st wash; [4] 2nd wash; [5] 3rd wash; [6] 4th wash; [7] *C. albicans* cell membrane proteins resulted after incubation with BLAD, upon removal of both *N*- and *O*-linked oligosaccharides, and after washes; [M] Precision Plus Protein™ All Blue Standards marker (kDa).

After observing that even after being treated for the removal of both *N*- and *O*-linked oligosaccharides, BLAD continued to bind to *C. albicans* cell membrane, the same immunobloting study after BLAD incubation was made to understand if there are some changes in the binding target polypeptide. The results are shown in figure IV.9.

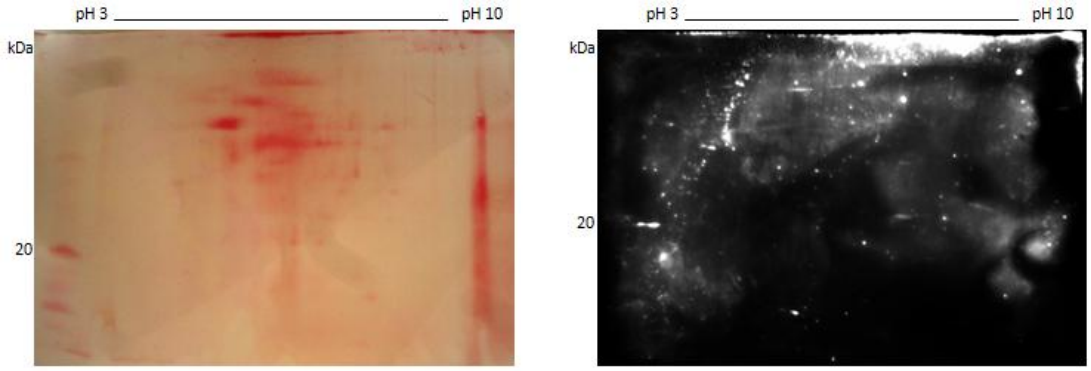

Figure IV.9: 2D-electrophoresis and immunoblotting analysis. **(A)** Upon removal of both N- and O-linked oligosaccharides, *C. albicans* cell membrane proteins (500 µg) were transferred onto a PVDF membrane and stained with Ponceau S. **(B)** Incubation of the *C. albicans* cell membrane proteins, upon removal of both *N*- and *O*-linked oligosaccharides, with BLAD and posterior immunoblotting. 3-10 pH gradient strip. [1] Purified BLAD (200 µg).

As figure IV.9 shows, there are some spots being identified in the immunoblot that is traduced by BLAD targeting. Although with some background, two spots with isoelectric points of *ca.* 5 and 7 and molecular masses of 60 and 20 kDa, respectively, are being revealed. From these results it is likely to assume that even after being treated for the removal of both *N*- and *O*-linked oligosaccharides, BLAD still binds to *C. albicans* cell membrane proteins, probably using other type of oligosaccharides as target.

IV.4 Conclusion

The data obtained in the present chapter confirm the results previously achieved from the immunoflorescent studies, which showed that *C. albicans* cell membrane is in fact a target for BLAD. Regarding the specific type(s) of oligosaccharide(s) that is(are) the specific target(s) for this polypeptide, a definitive conclusion could not be drawn, since the study gave positive results with all types of deglycosylated glycoproteins from the cell membrane tested. This probably means that or BLAD has more than one target and/or that the removal of each type of oligosaccharide have not been accomplished due to the complexity of the available methodologies for their removal and unfortunately, the time available was not enough to complete this task**.** In the future this must be one of the primary studies to fully understand BLAD mode of action.

III.5 References

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Chapter V

General discussion

This workplan is placed in a major project which aims at using BLAD in the clinical area. In this respect, the first step was the production of this polypeptide through its heterologous expression, in a recombinant form. This was achieved by using *Escherichia coli* as host cell because it remains the most promising system, considering the absence of glycosylated residues in BLAD. The production and purification of the polypeptide BLAD in a recombinant form was possible and is likely to assume that it possess the same biological activities as its native form. Moreover, probably has a higher effectiveness since it is required in a less concentration to cause the microorganisms death. However, this system needs to be optimized in order to increase the rate of the polypeptide production. Despite being equally effective, the small amount of the recombinant protein obtained, precluded their use in the remaining work. This led the use of the native form in the subsequent steps of the work plan.

Another objective was assessing the physiological and morphological effects of BLAD in fungi, using *Candida albicans* as the unicellular pathogenic fungal model. After determining the lethal concentration of BLAD in PDB medium (250 µg/mL) and exposing the cells to this concentration for more than 12 h, *C. albicans* became metabolically inactive, non viable and nonculturable. Moreover, some cells showed loss of membrane integrity, nevertheless, there were no visible changes in the cell wall. Finally, the immunofluorescence data suggest that BLAD passes through the cell wall and then binds to the plasma membrane, although not entering into the cell.

The last main goal was searching for specific targets for BLAD in the pathogen cell envelope, using *C. albicans* protoplasts. The results obtained confirm the previously achieved from the immunofluorescent studies, which means that most likely BLAD passes through the cell wall and binds to the cell membrane, destabilizing it. Keeping in mind one of the main properties of BLAD, the lectin activity, the study moved on trying to access the specific oligosaccharide target for BLAD. However, any conclusion was reached, since the study gave positive results with all type of deglycosylated glycoproteins from the cell membrane tested. This probably means that or BLAD has more than one target and/or that the removal of each type of oligosaccharide have not been accomplished, in these experiments conditions, due to the complexity of the available methodologies for their removal, and unfortunately, the time available was not enough to complete this task.

In the future more studies have to be done in order to fully understand BLAD mode of action, including understand the effectiveness of the methods used to remove *N*-linked and *O*-linked oligosaccharides. In addition, as explained in the previous section, two polypeptide spots targeted by BLAD were already sliced from the membrane, submitted to deglycosylation and, separately, both oligosaccharide and polypeptide residues sent to be sequenced. Such information will be rather importat because will provide an idea of the type of resideus that are involved in BLAD mode of action, i.e. the precise specificity of BLAD as lectin.However, we are still waiting for the sequencing results.

Finally, the production of recombinant BLAD in a great scale will allow the accomplishment of all these studies with the recombinant form of BLAD which in turn will lead to some potential commercial application.