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Unraveling the antifungal activity of a newly discovered oligomer produced by an *in vitro* proteolytic pathway

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Resumo

Os fungos patogénicos representam, à escala mundial, uma séria ameaça para a saúde humana, tendo-se vindo a registar um aumento significativo de infeções fúngicas devido a fungos patogénicos oportunistas, o que as torna num fator importante de morbidade e mortalidade.

Apesar de *Candida albicans* continuar a liderar as espécies de *Candida* causadoras de infeções da corrente sanguínea (ICS), nas últimas duas décadas tem ocorrido um desvio epidemiológico para um aumento de espécies de *Candida* "não-*albicans"*. Infeções causadas por outras espécies de *Candida* têm vindo a aumentar, sendo responsáveis por mais de 90% das infeções fúngicas invasivas. De entre essas espécies, apenas *C. glabrata* pode ser verdadeiramente reconhecida como uma espécie emergente causadora de ICS, devido à sua resistência intrínseca e adquirida aos azóis e a outros agentes antifúngicos.

Nenhuma das classes de antifúngicos atualmente disponíveis possui todas as características ideais de um agente antifúngico, o que, em última análise, conduz a falhas no tratamento. Como consequência, novas formulações de antifúngicos, combinações terapêuticas e o desenvolvimento de novos compostos bioativos podem ser úteis na obtenção de melhores resultados terapêuticos.

As sementes de leguminosas são fontes bastante abundantes de proteínas, sendo o género *Lupinus* um dos mais ricos neste tipo de proteínas de reserva. As proteínas de reserva das sementes de leguminosas, classificadas como globulinas, compreendem duas famílias principais de proteínas: as vicilinas e as leguminas, que constituem aproximadamente 80% das proteínas totais das suas sementes, atuando não só como reservas de nutrientes durante a germinação, como também como proteínas de defesa para as plantas.

A β-conglutina é a principal proteína de reserva nas sementes das espécies de *Lupinus*, sendo as suas subunidades sintetizadas durante o desenvolvimento cotiledonar de *L. albus*, a partir de um único polipéptido precursor glicosilado. Após a germinação, as subunidades da β-conglutina sofrem uma grande alteração na sua estrutura e concentração, levando ao aparecimento de um novo conjunto de polipéptidos.

Recentemente, foi acidentalmente descoberto no nosso laboratório que a β-conglutina purificada de *L. albus* sofre, *in vitro*, um processo de proteólise. Após uma incubação de 7 dias a 25ºC, a β-conglutina sofre uma degradação controlada originando um oligómero estável e com atividade antifúngica, que denominámos PDβ.

Tendo em conta o conhecimento prévio da atividade antifúngica do PDβ, este plano de trabalho teve como objetivos principais caracterizar o oligómero e determinar a sua estabilidade e a sua atividade antifúngica, bem como tentar perceber o seu mecanismo de ação como agente antifúngico, avaliando os efeitos fisiológicos e morfológicos em *C. glabrata.*

A primeira fase do trabalho passou por isolar as globulinas totais dos cotilédones de *L. albus* seguida de purificação da fração correspondente à β-conglutina. Após purificação, a β-conglutina foi incubada a 25°C durante 7 dias, de modo a ocorrer a degradação para PDβ. Começou por se tentar perceber se o resultado desta degradação é devido a uma destruição da estrutura original da proteína dando origem a subunidades independentes. Os resultados permitiram concluir que embora a proteína sofra um processo de degradação, a sua massa molecular aumenta ligeiramente, o que

permite concluir que o processo *in vitro* não se traduz num catabolismo com desaparecimento de sub-unidades mas sim de um arranjo estrutural diferente. O passo seguinte passou por avaliar a evolução do perfil polipeptídico e da atividade antifúngica durante o processo de degradação. Foi possível determinar que a degradação da β-conglutina começa a ocorrer a partir do terceiro dia de incubação a 25°C, sendo o pico máximo de degradação ao fim dos 7 dias. Esta degradação leva a uma acumulação progressiva de um polipéptido com 20 kDa, o que parece semelhante ao catabolismo que ocorre naturalmente nos cotilédones de *L. albus*. No entanto no processo *in vivo* a degradação origina exclusivamente um polipéptido de 20 kDa denominado Blad, que acaba por ser completamente degradado. Neste caso, o processo é interrompido sugerindo que falta uma peça chave para posterior degradação. Em relação à atividade antifúngica, o seu aparecimento é gradual: vai aumentando atingindo o pico máximo de atividade aos 7 dias de incubação.

De seguida tentou caracterizar-se e identificar o mecanismo envolvido na degradação da β-conglutina em PDβ, começando por se analisar se poderia ser causada por uma reação de oxidação-redução, interferindo com o oxigénio disponível na reação. Os resultados obtidos indicam que o aumento de oxigénio disponível na reação não tem qualquer influência sobre a reação de degradação mas quando se adiciona dithiothreitol (DTT) a degradação não ocorre, ou é interrompida no momento em que este é adicionado. Uma possível explicação pode ser pelo facto de o DTT ser um eficaz desnaturante de proteínas quebrando as ligações dissulfídicas nos grupos de cisteína.

Tendo em conta estes resultados e admitindo a hipótese de haver uma protease envolvida no processo de conversão a qual poderia estar a ser inibida por um agente redutor, foram testados vários inibidores de diferentes classes de proteases, adicionando-os separadamente à β-conglutina antes do período de incubação *in vitro* de 7 dias. Dos inibidores de proteases testados, apenas o ácido etilenodiaminatetraacético (EDTA) teve influência na β-conglutina, impedindo a sua degradação. De acordo com este resultado é possível concluir que a degradação da β-conglutina é dependente de uma metaloprotease, inibida aquando da adição de EDTA, resultado confirmado por recurso a uma zimografia. Esta metaloprotease encontra-se sob a forma inativa, encontrando-se apenas ativa ao fim de 3 dias de incubação da β-conglutina a 25°C, altura em que se inicia o processo de degradação. Para esclarecer a origem da molécula responsável pela degradação da β-conglutina, levantou-se a hipótese de se tratar de uma contaminação externa. No entanto, esta hipótese foi abandonada uma vez que tanto a solução de β-conglutina filtrada através de filtros de fluoreto de polivinilideno (PVDF) como a β-conglutina submetida a um tratamento com antibiótico sofreram o processo normal de degradação.

Embora nesta fase do trabalho existissem resultados bastante interessantes, nenhum permitiu identificar a molécula responsável pela degradação da β-conglutina. Uma vez que os ensaios de zimografia foram os que permitiram chegar mais perto da sua identidade, foi efetuada uma nova zimografia utilizando as vicilinas totais como substrato, visto ser o substrato natural da metaloprotease em questão. As bandas com atividade proteolítica foram excisadas do gel e enviadas para sequenciação. No entanto, após receção dos resultados verificou-se que não foi possível identificar a possível metaloprotease.

Em simultâneo com a caracterização bioquímica do processo de degradação, foi avaliada a atividade antimicrobiana de PDβ, o que revelou que apesar de PDβ não apresentar atividade bactericida, esta apresenta atividade fungicida em várias espécies de leveduras e de fungos filamentosos. Após o rastreio efetuado, foram avaliados os efeitos morfológicos e fisiológicos da PDβ, usando *C. glabrata* ISA 2163 como modelo. Começou por se comparar a actividade antifúngica de PDβ com a do itraconazole e anfotericina B, o que revelou que PDβ parece apresentar maior atividade inibitória e fungicida numa base molecular do que tanto o itraconazole como a anfotericina B.

De seguida, realizaram-se curvas de crescimento de *C. glabrata* ISA 2163 exposta às concentrações inibitórias e letais de PDβ e de anfotericina B, de modo a estudar e comparar os seus efeitos no crescimento da levedura. As curvas obtidas permitiram concluir que a adição tanto de PDβ como de anfotericina B teve um forte efeito inibitório no crescimento de *C. glabrata* ISA 2163, visto que as células expostas a ambas as concentrações testadas de ambas as drogas apresentam uma diminuição na taxa de crescimento, quando comparado com a situação controlo (sem droga). Em ambos os casos, os efeitos de inibição começam a ocorrer ao fim de 4 h de incubação, verificando-se uma estabilização da DO_{640nm} e das contagens de unidades formadoras de colónias (UFC), mais acentuada a partir das 12 h de incubação, na fração incubada com a concentração letal de PDβ. Simultaneamente foi avaliado o efeito de PDβ na atividade metabólica, com recurso ao fluorocromo FUN-1, e na integridade da parede celular, com recurso ao *calcofluor white*, de *C. glabrata* ISA2163, recolhendo amostras ao longo da curva de crescimento. Os resultados obtidos sugerem que, mesmo ao fim de 24 h de incubação com uma concentração letal de PDβ, não ocorre perda de viabilidade celular sugerindo que as células apenas perdem a capacidade de se multiplicar, dada a estabilização do número de células. Relativamente à integridade da parede celular, existe uma diferença notória entre a fração incubada com PDβ e a fração controlo. Enquanto a integridade da parede celular da fração controlo permanece inalterada ao longo da curva, a fração exposta à PDβ apresenta falhas na marcação com *calcofluor white,* a partir das 8 h de incubação, tornando-se mais evidentes após as 12 h. Estes resultados sugerem perda de integridade da parede celular e podem ser a razão pela qual as células perdem a capacidade para se multiplicar.

Por último, determinou-se a localização celular da PDβ ao fim de 24 h de incubação em *C. glabrata* ISA 2163. Os resultados obtidos sugerem que PDβ se liga à parede celular da levedura, destabilizando-a. No entanto, não há vestígios da proteína no interior da célula.

Este trabalho permite concluir que a degradação *in vitro* da β-conglutina para PDβ ocorre de forma controlada, possivelmente sob a ação de uma metaloprotease. A atividade desta metaloprotease só é visível após o início da conversão da β-conglutina, aumentando de atividade à medida que o oligómero é convertido. Isto sugere que na semente seca, quando se purifica a β-conglutina, a metaloprotease se encontra inativa, sendo necessária a sua ativação para que a conversão da β-conglutina tenha início. No que respeita a atividade antimicrobiana de PDβ, é possível concluir que a mesma apresenta uma forte atividade em diferentes fungos, tanto leveduras, patogénicas e alimentares, como fungos filamentos. Relativamente ao modo de ação do oligómero, verificou-se que este tem capacidade para se ligar à parede celular da levedura em estudo, criando

danos significativos e afetando a sua integridade celular. Os danos causados ao nível da parede celular parecem ser suficientes para impedir a multiplicação do microrganismo, levando à inibição do seu crescimento.

Palavras chave: PDβ, β-conglutina, metaloprotease, *C. glabrata*, agente antifúngico.

Abstract

Pathogenic fungi represent, worldwide, a serious threat for human's health. *Candida* species are among the top ten pathogens causing bloodstream infections (BSI). *C. glabrata* can be described as a truly emerging pathogen that cause BSI due, in part, to its intrinsic and acquired resistance to azoles and other commonly used antifungal agents. All antifungal agents available display several disadvantages. It is therefore essential to identify new, potent and safe antifungal drugs with novel modes of action. PDβ is an *in vitro* breakdown oligomer resultant from the degradation of β-conglutin, with proved antifungal activity.

Based on this information and in an attempt to go deeper in knowledge, the first objective of this work was the biochemical characterization of PDβ oligomer and the understanding of the metabolic route involved in the degradation process. This process was found to occur gradually and controlled by a metalloproteinase that needs to be activated, converting an inactive protein into an oligomer with antifungal activity.

The second goal was the characterization of PDβ stability and antimicrobial activity against an array of different species. The antimicrobial activity of PDβ was shown to be quite diverse among yeasts and filamentous fungi; however no bactericidal activity was detected.

Finally, an attempt to understand PDβ mode of action by assessing its physiological and morphological effects on *C. glabrata* was explored. Upon exposure of *C. glabrata* to PDβ, the yeast is still metabolically active but loses its cell wall integrity. Furthermore, the activity of PDβ suggests binding to the cell wall, without entering into the cell. The binding of PDβ leads to damages and destabilization of the cell wall which are severe enough to prevent *C. glabrata* ISA 2163 multiplication.

Key words: PDβ, β-conglutina, metaloproteinase, *C. glabrata*, antifungal agent.

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I. Introduction

Increase of the incidence and severity of fungal infections

Fungi are an extremely diverse group of organisms widely distributed in essentially every ecosystem with capability to grow on almost any surface, causing disease in plants, humans and animals (Ferrreira, *et al.*, 2012). Pathogenic fungi represent, worldwide, a serious threat for human's health with significant increase of severe fungal infections due to opportunistic fungal pathogens (Hayes, *et al*., 2013) being responsible for the infection of billions of people every year. Some fungal diseases have a mortality rate similar to tuberculosis or malaria (Brown, *et al.*, 2012), and since 1980s is being reported that fungi were becoming common pathogens in nosocomial infections (Fridkin & Jarvis, 1996). Because the vast majority of life-threatening fungal infections affects people with altered immune function, the increased incidence of invasive fungal infections can be correlated with an expansion in the number of people living with conditions or treatments that affect immune function (Roemer & Krysan, 2014), like AIDS or diabetes, and/or those hospitalized with serious underlying diseases (Sardi, *et al.*, 2013). In addition, advances in medicine that allow more patients to undergo transplantation procedures and receive aggressive immunosuppressive therapies in general create a more susceptible patient population (Klepser, 2011). Fungal infections have thus become an important factor of morbidity and mortality and represent an increasing burden on medical systems (Del Poeta, 2010), especially in seriously ill patients (Tani, *et al*., 2012), giving that they are difficult to treat and control because of rising problems of antifungal drug resistance and the lack of diagnostics, novel antifungal drugs, and vaccines (Drummond, *et al.*, 2014). Knowing the complexity of the population of patients who are at risk for infection and the diversity and increasing array of fungal pathogens, opportunistic mycoses pose considerable diagnostic and therapeutic challenges (Pfaller & Diekema, 2004).

In order to effectively eliminate these infections, early diagnosis and species identification are of paramount importance. Isolation and identification of the infecting organisms are extremely important for the proper management of infections due to the less common opportunistic fungi (Pfaller & Diekema, 2004). Diagnosis depends upon clinical suspicion and the retrieval of appropriate material for culture and histopathology. Unfortunately, the current standard diagnostic methods are far from adequate. The diagnosis of invasive candidiasis, for example, requires biopsy of the involved tissue, followed by staining, culture, and histopathology. Blood cultures remain the gold standard for the diagnosis of candidemia and should be the initial diagnostic test when candidemia is suspected. However, cultures take 1 to 3 days to grow and an additional 1 to 2 days for identification of the organism, which often leads to considerable delays in initiation of targeted treatment. The impact of such delays in the case of invasive fungal infections (IFIs) is vast, with studies showing significant daily increases in mortality and hospitalization costs for every day without appropriate antifungal agents (Arvanitis, *et al.*, 2014).

Apart from these problems, it has also been a significant increase in the emergence of resistance by some pathogenic fungi. Some microorganisms are naturally resistant to certain types of antifungal medications when other species although may be susceptible to a particular type of

medication, may develop resistance over time as a result of improper antifungal use (Lortholary, *et al*., 2011; Shah, *et al.*, 2012).

Antifungal drug resistance can be characterized as microbiological or clinical. Microbiological resistance is defined as the non-susceptibility of a fungal pathogen to an antifungal agent as determined by in vitro susceptibility testing and compared with other isolates of the same species, and can be further categorized as intrinsic or acquired. Whilst intrinsic resistance is found naturally among certain fungal strains without prior exposure to drugs, acquired resistance develops in previously susceptible fungal strains following drug exposure and can often occur as a result of altered gene expression. Clinical resistance refers to the persistence of a fungal infection despite treatment with adequate therapy. Although microbiological resistance can contribute to the development of clinical resistance, other factors may also be involved, such as impaired immune function, underlying disease, reduced drug bioavailability and increased drug metabolism (Sanguinetti, *et al.*, 2015). Some studies have indicated that antibacterial medications may also contribute to antifungal resistance which could occur for a variety of reasons, one of which is that antibiotics reduce bacteria in the gut and create favorable conditions for *Candida* growth.

Most common human fungal pathogens

Candida species are among the top ten pathogens causing bloodstream infections (BSIs), and are responsible for candidaemia, an invasive fungal infection associated with substantial morbidity, mortality and healthcare costs (Orasch, *et al.*, 2013). *Candida albicans* and *Candida glabrata*, for example, are ubiquitous commensals of humans, and can be found especially in the oral cavity and the gastrointestinal tract of most healthy humans (Cole, *et al.*, 1996; Fidel, *et al.*, 1999). On the other hand, they are also considered the most important pathogenic yeasts (Brunke & Hube, 2013). Although *C. albicans* remains the leading *Candida* species that causes BSIs, a shift in the epidemiology towards greater isolation of non-*albicans Candida* species has been a global concern in the past two decades (Won, *et al.*, 2015). *C. albicans* is implicated in approximately 50% of patients with candidiasis. However, infections caused by other species, such as *Candida glabrata, Candida tropicalis, Candida krusei, Candida parapsilosis*, and *Candida lusitaniae*, are rising (Klepser, 2011), and being already responsible for more than 90% of invasive infections (Spampinato & Leonardi, 2013). Among these common species, only *C. glabrata* can be said to be truly emerging as a cause of BSIs, due in part to its intrinsic and acquired resistance to azoles and other commonly used antifungal agents (Pfaller, 2004). According to the ARTEMIS Global Antifungal Surveillance Program, *C. glabrata* is the candida cause responsible for 44% of invasive fungal infections, being only defeated by *C. albicans* (63–70%) (Miceli, *et al.*, 2011). *C. glabrata* has emerged as an important and potentially antifungal-resistant pathogen, especially since the introduction of fluconazole in 1990 for the treatment of candidiasis, including invasive candidiasis (IC) (Pfaller, *et al.*, 2012). *C. glabrata* is innately less susceptible to fluconazole and amphotericin B than most other species of *Candida* and displays the capacity to rapidly develop resistance to all azoles via induction of CgCDR1 and PDH1 efflux pumps. Exposure of *C. glabrata* to sub-therapeutic concentrations of fluconazole may result in resistance, and

clinical studies have shown that the frequency of colonization and infection of patients with C. glabrata may be increased in populations subjected to fluconazole prophylaxis (Pfaller & Diekema, 2004).

C. glabrata is capable of colonizing host tissues as well as abiotic surfaces, where it develops as a multilayered biofilm structure, despite not being able to form filaments. In opposition to the rest of the *Candida* species, *C. glabrata* do not produce proteases. It produces phospholipases which hydrolyze phospholipids into fatty acids, contributing to host cell membrane damage, promoting cell damage or exposition of receptors that facilitate adherence. This promotes a greater and powerful interface with the host mucosae, destroying it and endorsing an effective invasion of the tissues involved. Also *C. glabrata* is able to degrade hemoglobin using hemolysins in order to obtain iron from host cells for metabolic processes (Rodrigues, *et al.*, 2014). Apart from that, *C. glabrata* also displays several virulence factors (adherence, biofilm formation, and secretion of hydrolytic enzymes), swelling their persistence within the host, triggering host cell damage, and, finally, resulting in clinical and microbiological failure (Rodrigues *et al*., 2014). As a consequence, candidemia due to *C. glabrata* is becoming increasingly common (Pfaller, 2012), being the systemic infections due to yeast characterized by a high mortality rate giving its reduced susceptibility to azole drugs, especially to fluconazole (Spreghini, *et al.*, 2012). Leclerc *et al.*, (2010), reported a case of IC due to *C. glabrata* in which the infecting strain acquired resistance to flucytosine, fluconazole, voriconazole, and caspofungin through successive independent events following prolonged exposure to each class of antifungal agent.

Most common used antifungal agents

Four main classes of antifungal agents are presently available: polyenes, azoles, echinocandins and nucleoside analogues (Tani, *et al.*, 2012). Polyenes, like amphotericin B, are broad-spectrum antifungal agents (Denning & Hope, 2010). Although the development of many antifungal agents in the last years, amphotericin B is still considered standard therapy for serious fungal infections (Cornely, et al., 2012; Mesa-Arango et al., 2014), even though 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 (Patterson, 2006). Polyenes bind ergosterol and disrupt the major lipidic component of the fungal cell membrane resulting in the production of aqueous pores. Consequently, the cellular permeability is altered and leads to the leakage of cytosolic components and, therefore, fungal death (Spampinato & Leonardi, 2013). Although, 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, this selectivity is low and suggests potential toxicity for mammalian cells (Odds, *et al.*, 2003).

The antifungal activity of azoles, like polyenes is by targeting ergosterol in the fungal cell membrane and were first approved in 1980. Their main mode of action is to disrupt the cell membrane by inhibiting the activity of the lanosterol 14-α-demethylase enzyme (Odds, *et al.*, 2003) involved in the biosynthesis of ergosterol (Spampinato & Leonardi, 2013). Its activity leads ergosterol to be

replaced with unusual sterols and the normal permeability and fluidity of the fungal membrane is impaired (Odds, *et al*., 2003).

The third class of antifungal agents with FDA (Food and Drug Administration) approval was the echinocandins in 2001. These agents are lipopeptidic antifungal agents that inhibit the synthesis of fungal wall by noncompetitive blockage of the (1,3)-*β*-Dglucan synthase. This inhibition leads to the formation of fungal cell walls with impaired structural integrity, resulting in cell vulnerability to osmotic lysis (Spampinato & Leonardi, 2013). Echinocandins are being use as the first-line therapy for invasive candidiasis because of their favorable safety profile and broad-spectrum anti-*Candida* activity. Nevertheless, echinocandin resistance is increasing, especially among fluconazole-resistant isolates (Alexander, *et al.*, 2013).

Azoles, unlike the other two classes of antifungal agents, present higher solubility and lower toxicity (Chapman, *et al.*, 2008), on the other hand, clinical use of azoles is limited because of the increase of resistant strains, particularly during long-term treatments (Bendaha, *et al.*, 2011).

Flucytosine, a pyrimidine analogue, it is a compound that mostly has some value as adjunctive treatment with amphotericin B in clinically difficult infections (Odds, 2003). It is transported into fungal cells by cytosine permeases, and then converted to 5-fluorouracil and phosphorylated to 5-fluorodeoxyuridine monophosphate. This fluorinated nucleotide inhibits thymidylate synthase thereby interferes with DNA synthesis (Spampinato & Leonardi, 2013), preventing cell proliferation(Odds, 2003).

The frequency of both invasive fungal infections (IFIs) and resistance to antifungal therapy continue to increase despite the introduction of new antifungal agents(Pfaller, 2012b) and the clinical outcomes for most invasive fungal infections are far from ideal (Roemer & Krysan, 2014). As a consequence, new formulations of antifungals, combination therapies and development of new bioactive compounds might be useful for a better therapeutic outcome (Spampinato & Leonardi, 2013).

Antifungal proteins

All organisms have evolved several defense systems in order to protect themselves against bacteria, fungi and viruses.

Both proteins and polypeptides with proved antifungal activity have been isolated from diverse groups of organisms, including plants, fungi, bacteria, insects, and animals (both vertebrates and invertebrates). The mechanisms of action of these proteins are as varied as their sources and include fungal cell wall polymer degradation, membrane channel and pore formation, damage to cellular ribosomes, inhibition of DNA synthesis, and inhibition of the cell cycle. There are hundreds of polypeptides described in the literature which exhibit fungicide activity, with more being discovered almost daily (Selitrennikoff, 2001).

Antifungal proteins, as their name imply, serve a protective function against fungal invasion. They are involved in both constitutive and induced resistance to fungal attack and are produced by a multitude of organisms. Antifungal proteins have been categorized according to their enzymatic

properties (e.g. β-glucanases, chitinases), their structure (e.g. cysteine rich) or their similarity to a known type of protein (e.g. thaumatin-like protein) (Theis & Stahl, 2004). Several proteins may be classified into more than one family and some are included in the group of pathogenesis-related (PR) proteins.

The most prominent group within the antifungal proteins with a close structural relationship constitutes defensins from plants, insects and mammals. Antifungal defensins and defensin-like antifungal proteins exhibit complex biological functions in addition to antimicrobial toxicity. The multitude of biological functions undoubtedly underscores the great impact of these proteins on our life (Hegedüs & Marx, 2013).

Several applications of natural occurring antifungal proteins have been discussed during the last 2 decades. They seem an attractive alternative for chemical food additives, and may also be a new source of clinically useful therapeutics. For example, heliomycin is currently examined in preclinical tests for antifungal treatment (Zasloff, 2002).

A main hurdle that has hindered the development of antimicrobial and antifungal proteins as therapeutic agents is the fact that many naturally occurring proteins with antifungal activity *in vitro* (e.g. magainin) are only effective *in vivo* at very high doses, often close to the toxic doses of the peptide (Darveau, *et al.*, 1991; Zasloff, 2002).

A prerequisite for any application of antifungal proteins is the lack of effects on the host cells. For that reason, and despite antifungal proteins may be powerful tools in clinical treatment of pathogens, several aspects have to be thoroughly examined for a possible application (Theis & Stahl, 2004).

Seed storage proteins

Legume seeds are an abundant source of proteins and, among them, lupin is one of the richest. Lupin is a non-starch leguminous seed with a high protein content, almost as high as that of soybean (about 35% of the dry weight), and a relatively low oil content (Duranti, *et al.*, 2008). The main proteins of legume seeds are located in the storage vacuoles of the cotyledonary tissues and mostly, but not exclusively, belong to the family of the storage proteins which contain a vast repertoire of proteolytic enzymes involved in the degradation of protein reserves to amino acids, which serve as nitrogen and carbon skeleton source during seed germination. Some of these enzymes are already present in the mature dry seed and are thought to be involved in early cleavage events and others are synthesized at germination and cause the massive degradation of the protein reserves (Magni, *et al.*, 2012; Müntz, *et al.*, 2001).

Lupin seeds contain two classes of proteins, which according to Osborne's classification correspond to the albumin and globulin fractions. Albumins comprise a number of molecules which represent the functional proteins of a seed. Globulins are the typical salt-soluble storage proteins of the seeds (Duranti, *et al.*, 2008).

The seed storage proteins classified as 7S globulins occur in a wide range of plants and are often called vicilins because of their presence in the Viciae group of legumes (Monteiro, *et al.*, 2010).

Together with legumins, they usually account for approximately 80% of the total protein in mature legume seeds. Vicilins are oligomeric proteins (150 to 170 kDa) with variable degrees of glycosylation, composed of three similar subunits of ~40 to 70 kDa, with no disulphide linkages and stabilized by non-covalent forces (Monteiro, *et al.*, 2015). Vicilins isolated from a variety of legume seeds have been reported to bind strongly to chitin, chitosan and fully acetylated chitin. In vicilins metabolism, synthesis and degradation are temporarily separated, occurring during different developmental stages. Thus, no degradation of mature vicilins is observed at the time of synthesis and accumulation during seed maturation. Also, no synthesis of vicilins is detected when breakdown takes place at the time of germination and seedling growth. The pattern of degradation of the vicilin-type globulins during germination and seedling growth of legume species was found to be species specific and independent of the rate of proteolysis. In some cases, a transient accumulation of stable intermediates of vicilin catabolism is detected (Monteiro *et al.*, 2010).

Degradation of storage proteins is an essential stage of metabolism in growing seedlings. Storage protein degradation is a complex process involving a successive action of several proteolytic enzymes. This degradation may be carried out by proteases from both dry seeds (i.e. synthesized at maturation) and seedlings (i.e. activated or synthesized at seedling growth). Besides, the above proteases reveal their apparent specificity to the protein. Some of them attack the initial storage protein while others hydrolyze the *in vivo* or *in vitro* modified storage protein.(Dunaevsky, *et al.*, 1993).

An understanding of proteolytic processes and the enzymes involved is paramount to a thorough knowledge of metabolism at both the cellular and tissue levels. Proteolytic enzymes are categorized into four classes based on catalytic mechanisms, serine proteases, cysteine proteases, aspartate proteases, and metalloproteases, such as matrix metalloproteinases (MMPs), based on their residue or cofactor essential in catalysis. Plant MMPs are conserved proteolytic enzymes found in a wide range of monocotyledonous and dicotyledonous plant species. MMPs are distinguished from other endopeptidases by their dependence on metal ions as cofactors, their ability to degrade extracellular matrix (ECM) and their involvement in many aspects of plant physiology including growth, development, and the response to stress such as pathogen attack (Sekton, 2010).

Magni, *et al.*, (2012) described a metallo-endopeptidase activity from lupin seeds which by acting on twin-arginine residues, is capable of producing a limited and controlled breakdown of endogenous storage protein substrates, in both lupin 7S and 11S globulins, corresponding to the vicilin and legumin-like storage proteins, respectively. The presence of specific double arginine motifs in the seed storage proteins may represent a pre-defined cleavage site for the generation of polypeptide chains bearing a new biological activity, since by the action of lupin twin-arginine endopeptidase a stable 20 kDa polypeptide with lectin and antifungal activities is generated during seed germination (Magni, *et al.*, 2012).

Whether these events of limited proteolysis occurring in the storage proteins are merely preparatory to a facilitated degradation at germination or they concur to liberate polypeptide fragments with specific biological activity, including defense, protective, or regulatory ones, is still to be understood.

From β-conglutin to PDβ

β-conglutin is the major seed storage protein in Lupinus species, with subunits appear to be synthesized in *Lupinus albus* developing cotyledons as a single glycosylated precursor polypeptide. β-conglutin is composed of 10 to 12 major types of subunits with molecular masses ranging from 15 to 72 kDa. Between days 3 and 5 following the onset of germination, β-conglutin undergoes a dramatic change in its structure and concentration, involving the appearance of a new set of polypeptides, including a higher molecular mass group, whose concentration steadily declines until complete disappearance after 11 days, and a lighter molecular mass group, whose concentration surprisingly increases from 5 to 11 days. Particularly evident is the abrupt accumulation of an abundant 20 kDa polypeptide, named Blad-oligomer, which accumulates abundantly in *Lupinus albus* cotyledons between days 4 and 12 after the onset of germination. This polypeptide is a stable breakdown product of β-conglutin catabolism and has been shown to display lectin activity, an observation that highlights the potential physiological roles played by this protein. In this respect, vicilins have long been considered important seed storage proteins that not only play a role as a nutrient reserve for the germinating seedling but also appear to serve a dual role as plant defense-related proteins (Monteiro, *et al.*, 2010).

Recently, in our lab, was accidentally discovered that β-conglutin from *L. albus* seeds undertakes a proteolytic degradation process in an *in vitro* reaction. After seven days at 25°C, the β-conglutin oligomer undergoes a controlled degradation process involving the appearance of a stable breakdown oligomer, fully functional, with antifungal activity, named PDβ. After the formation of PDβ the degradation process does not proceed, may be due to possible missing elements that would lead to further degradation as the *in vivo* process.

Objectives

In a previous work has been observed that PDβ displays a potent antifungal activity against important human pathogens. This observation along with the total absence of anti-fungal activity of β-conglutin raised the foundations for this workplan. The major goals of this work were:

(i)The biochemical characterization of PDβ oligomer and the understanding of the metabolic route involved in the degradation process;

(ii) Characterization of PDβ stability and antifungal activity against an array of different species, both pathogenic and spoilage yeasts;

(iii) Finally, an attempt to understand PDβ mode of action by assessing their physiological and morfoplogical effects on *C. glabrata* was made.

II. Materials and Methods

II.1. Biological materials and growth conditions

II.1.1 *Lupinus albus* seeds

Dry seeds of white lupin (*Lupinus albus* L.) cv. amiga from France were utilized for the extraction of total vicilins.

II.1.2. Bacteria

All bacteria (Table 1) were grown at 34°C for 24 h in Tryptone Soy Agar (TSA) medium (3% (w/v) Tryptone Soy Broth, 1.5% (w/v) Agar). For the antifungal susceptibility tests bacteria was grown in Mueller-Hinton medium.

Table 1. Collection of bacteria used as biological material. *Strains courtesy from Instituto Superior de Agronomia. CBISA - Collection Bacteria Instituto Superior de Agronomia.

Specie	Strain *
Bacillus subtilis	CBISA 4050
Listeria monocytogenes	CBISA 3001
	CBISA 3998
Pseudomonas aeruginosa	CBISA 4076
Staphylococcus aureus	CBISA 3015

II.1.3. Fungi

All fungi (Table 2) were grown at 25°C for 1 week in Sabouraud Dextrose Agar (SDA) medium, except for *Colletotrichum acutatum* (CBS 294.67) and *Fusarium oxysporum* (CBS 114750) which were grown at 25°C for 1 week in Potato Dextrose Agar (PDA) medium (2.4% (w/v) Potato Dextrose Broth (PDB), 1.5% (w/v) Agar). For the antifugal susceptibility tests the medium used was PDB medium (2.4% (w/v) Potato Dextrose Broth), buffered at pH 7.5.

Table 2. Collection of filamental fungi used as biological material. *Strains courtesy from Converde. lda. CEV- Converde.

Specie	Strain *
Alternaria alternata	CEV 1
Botrytis cinerea	CEV 6
Colletotrichum acutatum	CBS 294.67
Colletotrichum gloeosporioides	CBS 119204
Fusarium graminearum	CBS 184.32
Fusarium oxysporum	CBS 114750

II.1.4. Yeast

All yeasts (Table 3) were grown at 34°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 medium used was PDB medium (2.4% (w/v) Potato Dextrose Broth), buffered at pH 7.5.

Table 3. Collection of yeast used as biological material. *Strains courtesy from Instituto Superior de Agronomia. ISA - Collection of Yeasts Instituto Superior de Agronomia.

Specie	Strain*	Specie	Strain*
Candida albicans	ISA 2151	Hanseniasporaguillermondi	ISA 2438
	ISA 2218	*Hanseniaspora uvarum*	ISA 2392
	ISA 1862		ISA 2393
Candida apicola	ISA 2424	*Issatchenkia occidentalis*	ISA 2384
Candida cantarellii	ISA 2391	*Issatchenkia orientalis*	ISA 2382
Candida deformans	ISA 2415		ISA 2383
Candida diversa	ISA 2433	*Issatchenkia terricola*	ISA 2437
Candida famata	ISA 1830	*Kluyveromyces marxianus*	ISA 2444
	ISA 1831	*Lachancea thermotolerans*	ISA 2164
Candida frutus	ISA 2390		ISA 2379
Candida dubliniensis	ISA 2228		ISA 2380
Candida guilliermondii	ISA 2258	*Pichia burtonii*	ISA 2377
	ISA 2167	*Pichia fermentans*	ISA 2435
Candida glabrata	ISA 2163		ISA 2445
	ISA 2242	*Pichia guilliermondii*	ISA 2375
	ISA 2243		ISA 2376
	ISA 2244	*Pichia kluyveri*	ISA 2434
	ISA 2245	*Pichia manshuriea*	ISA 2426
	ISA 2246		ISA 2427
Candida krusei	ISA 2223	*Pichia sporocuriosa*	ISA 2374
	ISA 2290	*Saccharomyces cerevisiae*	ISA 2418
Candida neoformans	ISA 2293		ISA 2425
Candida parapsilosis	ISA 2238		ISA 1000
	ISA 2237	*Saccheromycopsis crataegensis*	ISA 2373
Candida pomicola	ISA 2389	*Saccharomycopsis vini*	ISA 2372
Candida stellimalicola	ISA 2387	*Yarrowia lipolytica*	ISA 1718
	ISA 2388	*Zygoascus hellenicus*	ISA 2284
Candida tropicalis	ISA 2292		ISA 2367
	ISA 2232	*Zygoascus meyerae*	ISA 2365
Candida zemplinina	ISA 2432		ISA 2366
Debaryomyces hansenii	ISA 2416	*Zygosaccharomyces bailli*	ISA 2283
Hanseniasporaguillermondi	ISA 2417		ISA 2419
	ISA 2385	*Zygosaccharomyces bisporus*	ISA 1868

II.2. Isolation of total globulins

A previously optimized methodology was used to isolate the total globulin fraction from the dry seeds of *Lupinus albus* (Ramos, *et al.*, 1997; Freitas, *et al.*, 2000). The dry seeds, after embryo removal, were milled and the resulting meal was defatted with *n*-hexane (34 mL/g of flour) at 4°C, for 4 h with agitation, decanted and air-dried. The albumin fraction of the proteins was extracted by stirring with 10 mM $CaCl_2$, 10 mM $MgCl_2$ (pH 8.0) (34 mL/g of flour) at 4°C, for 4 h with agitation. The suspension was centrifuged at 4°C 30,000 *g* for 1 h. The supernatant was discarded and the total globulin fraction of the proteins was extracted by stirring the pellet with 100 mM Tris-HCl buffer, pH 7.5, containing 10% (w/v) NaCl, 10 mM (w/v) Ethylenediaminetetraacetic acid (EDTA) and 10 mM (w/v) Ethylene glycol tetraacetic acid (EGTA) (34 ml/g of flour), at 4°C for 4 h. The solution was then centrifuged at 4°C 30,000 *g* for 1 h. The supernatant containing the globulins was precipitated by the addition of ammonium sulphate (561 g/L) in order to induce the proteins salting-out. The precipitated globulins were centrifuged at 30,000 *g* at 4°C for 20 min, resuspended in 50 mM Tris-HCl, pH 7.5 (5.7 mL/g of flour) and desalted on PD-10 columns previously equilibrated with the same buffer.

II.3. Purification of β–conglutin

After obtained the total globulin fraction, the individual globulins were fractionated and purified by AKTA anion exchange chromatography on a Q-Sepharose column (GE Healthcare Life Sciences; ∅ = 1 cm; h = 8 cm; flow rate = 1.0 mL/min) essentially as described in (Monteiro *et al.*, 2010). The bound proteins were eluted with a gradient of NaCl (0 to 1M) and desalted on PD-10 into 50 mM Tris-HCl, pH 7.5. To obtain the PDβ, the fraction correspondent to β-conglutin, hereby named β-conglutin 0 days, was then incubated at 25°C during a period of seven days.

For size exclusion chromatography, β-conglutin 0 days and PDβ were analyzed on AKTA pure chromatography on a Superose 12 HR 10/30 column (GE Healthcare Life Sciences). The column was equilibrated with 50 mM Tris-HCl, pH 7.5, and the data were analyzed with a calibration curve utilized in our laboratory ($log_{10} MW = 6.23405 - 4.06619Kav; Kav = \frac{Ve-V0}{Vt-V0}; r^2 = 0.9814$). The molecules utilized to obtain the calibration curve were ferritin (440 kDa), cytochrome C (12.4 kDa), malate dehydrogenase (73 kDa), alcohol dehydrogenase (150 kDa), aldolase (158 kDa), RUBPcase (532 kDa), trypsin (23.8 kDa) , catalase (232 kDa) and blue dextran (2000 kDa).

II.4. Polyacrylamide gel electrophoresis in SDS-PAGE

For the observation of polypeptides a polyacrylamide gel in denaturing conditions (SDS-PAGE, “sodium dodecyl sulfate polyacrylamide gel electrophoresis”) was used in a discontinuous system with a concentration and a separation gel, according to the method described by Laemmli (1970). 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 then 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. The electrophoresis was performed on a vertical system using mini gels, with the addition of a cathode buffer (25mM Tris-HCl pH 8.8, 192 mM glycine and 0.1% (w/v) SDS). The Precision Plus Protein™ All Blue Standards marker (kDa), a marker from BioRad, that range from 10 to 250 kDa was used as a standard. The electrophoresis ran at 30 mA constant current and variable voltage up to 200 V by 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 gel.

II.5. Protein Quantification

Protein content was determined according to a modification of the Lowry's method (Bensadoun & Weinstein, 1976) using bovine serum albumin as standard. The samples absorbance was read in a spectrophotometer, at 750 nm.

II.6. 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 2 to 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 visualized.

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 $Na_2S2O_3.5H_2O$) 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.

II.7. Zymography

Zymography was performed as suggested by Lantz & Ciborowski (1994) with small modifications. The samples were precipitated with ice cooled 80% (v/v) acetone, as described previously, and treated with non-denaturing buffer (62.6 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 30% (v/v) glicerol, 0.01% (w/v) bromophenol blue). A solution containing 0.5% (w/v) gelatin was added to the separation gel and the electrophoresis occurred as described in the Polyacrilamide gel electrophoresis in SDS-PAGE section.

Two different substrates were utilized, separately, a solution containing 0.5% (w/v) gelatin and a solution containing 0.5% (w/v) of total vicilins, was added to the separation gel and the electrophoresis occurred as described in the Polyacrilamide gel electrophoresis in SDS-PAGE section.

After electrophoresis the zymogram was washed three times with 2.5% (v/v) Triton X-100 solution for 30 min with gentle stirring. This step is critical in order to remove the SDS from the gel to allow the refolding of the enzyme. After that, the zymogram was incubated in a buffer solution (50 mM Tris-HCl pH 7.4, 20% (w/v) NaCl) for 10 min at 37°C, followed by an overnight incubation, in the same buffer also at 37°C, with gentle stirring. The gel was stained in a solution of 0.5% (w/v) Comassie Brilliant Blue G250, 30% (v/v) ethanol and 10% (v/v) acetic acid, with stirring for 30 min, at room temperature. After being slightly destained, in a solution containing 30% (v/v) ethanol and 10% (v/v) acetic acid, areas of digestion should appear as clear bands against a darkly stained background, where the gelatin has been degraded by the enzyme.

II.8. Protein identification by mass spectra (MS) peptide mapping and sequencing analysis

The protein samples were reduced and alkylated with iodoacetamide, i.e. carbamidomethylated, and subsequently digested with trypsin that cleaves after lysine and arginine residues. The resulting peptides were eluted onto an anchorchip target for analysis on a Bruker Autoflex Speed MALDI TOF/TOF instrument. The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination. MALDI MS/MS was performed on 15 peptides for peptide fragmentation analysis, i.e partial sequencing. The MS and MS/MS spectra were combined and used for database searching using the Mascot software. The data are searched against in-house protein databases downloaded from UniProt and NCBI containing more than 47 million known non-redundant protein sequences.

II.9. Assessment of the optimum conditions for the stability of PDβ

Once the oligomer was extracted and purified, several conditions were tested including the preservation at various temperatures (4°C, -20°C and -80°C), in two different physical status (solution *versus* lyophilized), in different solutions (H_2O pH 7.5; 50 mM Tris-HCl, pH 7.5; and 50 mM Tris-HCl pH 7.5 with 400 mM NaCl). The stability of the oligomer was assessed by determining the Minimum Inhibitory Concentration (MIC) in each condition throughout time, using a reference yeast specie, as described in section *Yeast inhibition tests*. In summary, this task determined the best set of conditions to preserve PDβ for the remaining tasks of this work.

II.10. Evolution of the antifungal activity of PDβ – from inactive to fully functional

The conversion of the inactive oligomer into a fully functional oligomer with antifungal activity was assessed by determining its MIC daily, from the day of the extraction until it reaches the highest

antifungal activity value. The polypeptide profile of PDβ was also evaluated daily, by SDS-PAGE, in order to identify the newly produced breakdown products responsible for the increased antifungal activity.

II.11. Degradation assays

II.11.1. Temperature

β-conglutin 0 days in 50 mM Tris-HCl pH 7.5 was incubated at different temperatures, 25°C, 40°C and 60°C, during a period of seven days. The results obtained were observed by SDS-PAGE.

II.11.2. Oxidation-Reduction

After the assay with different temperatures, β -conglutin 0 days in 50 mM Tris-HCl pH 7.5 was incubated at 25°C for seven days, in two different forms. One with more oxygen by letting the tubes opened, and another with 100 mM Dithiothreitol (DTT). The results obtained were observed by SDS-PAGE.

II.11.3. Protease inhibitor

Various protease inhibitors, from different classes, were added, separately, to β-conglutin 0 days and incubated at 25°C during a period of seven days. The protease inhibitors used were 10 μM E-64 (target cysteine proteases), 1 mM Pepstatin (target aspartic proteases), 5 mM Ethylenediaminetetraacetic acid (EDTA) (target metalloproteases), and 1 mM Pefabloc (target serine protease). The results obtained were observed by SDS-PAGE.

II.11.4. Microorganism

β-conglutin 0 days was filtered in polyethersulfone (PES), polyvinylidene fluoride (PVDF), GD/X Polyethersulfone and acetate cellulose filters, with both pore sizes of 0.22 μm and 0.45 μm. After that, the filtered β-conglutin 0 days was incubated at 25°C for a period of seven days, and then evaluated by SDS-PAGE. .

II.12. Antifugal susceptibility tests

II.12.1. Antifungal agents

The antifugal agent PDβ was purified and stored lyophilized at room temperature, as described in sections "Purification of β–conglutin", "Assessment of the optimum conditions for the stability of PDβ". For the antifungal susceptibility tests, the solution was prepared in Tris-Hcl 50 mM pH 7.5 and

200 µL was added to the first line of the microplate. A twofold dilution was made, twelve times, using mili-Q sterile water, in the 96-wells microplates. The final concentration of PDβ, after the addition of the inocula, ranged from 1000 to 480 µg/mL. BLAD, amphoterinc B and itraconazole were used as standards. BLAD was obtained and prepared as described in (Monteiro *et al*., 2015) and ranged from 1000 to 480 µg/mL. Amphotericin B and itraconazole were obtained from their respective manufacturers (Discovery and Sigma respectively). Stock solutions of each antifungal agent were prepared and frozen at -20°C until used. The twofold dilution ranges were 0.03 to 16 µg/mL for amphotericin B and 0.03 to 64 µg/mL for itraconazole.

II.12.2. Yeast inhibition tests

The susceptibility tests were made according to the CLSI - Clinical and Laboratory Standards Institute (former NCCLS - National Committee for Clinical Laboratory Standards) guideline M27-A3 (Clsi, 2008a), using broth microdilution method with some modifications.

II.12.2.1. Minimum inhibitory concentrations (MICs) determination

Yeast cells were grown on GYP medium for 24 h at 34°C and the inoculum suspension was prepared by picking colonies and resuspending them in 5 mL of sterile 0.9% (w/v) saline solution (NaCl). The resulting suspension was vortexed for 15 s and the cell density was adjusted with a spectrophotometer to an OD_{640nm} =0.05, except some strains who needed an OD higher or lower in order to obtain the same inoculum concentration (1x10 cells/mL). The final inoculum suspension was made by a 1:50 dilution followed by a 1:20 dilution with double-strength Potato Dextrose broth medium (pH 7.5), which resulted in a final concentration of $1x10^3$ cells/mL. Another final inoculum concentration was tested: $1x10^5$ 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 drug 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.12.2.2. Minimum fungicidal concentrations (MFCs) determination

MFCs were determined according to Espinel-Ingroff, A. (1998) with small modifications. MFCs were determined with two inoculum sizes: 10^3 CFU/mL and 10^5 CFU/mL. After MIC determination, as previously described, 30 µl aliquots from each well that showed complete inhibition (no visual growth) were subcultured onto GYP plates. In both cases, the plates were incubated at 34°C for 24 h. The MFC was the lowest drug concentration that killed over 99.99% of the final inoculum.

II.12.3. Bacteria inhibition tests

The susceptibility tests performed in bacteria were made according to the CLSI - Clinical and Laboratory Standards Institute (former NCCLS - National Committee for Clinical Laboratory Standards) guideline M31-A2 (Clsi, 2008b) using broth microdilution method.

II.12.3.1. Minimum inhibitory concentrations (MICs) determination

Bacteria were grown on TSYA medium, overnight at 34°C and the inoculum suspension was prepared by picking colonies and resuspending them in 5 mL of sterile 0.9% (w/v) saline solution (NaCl). The resulting suspension was vortexed for 15 s and the cell density was adjusted with a spectrophotometer to an $OD_{550\ nm}$ = 0.15, in order to obtain a concentration of $1x10^8$ cells/mL. The final inoculum suspension was made by a 1:100 dilution with double-strength Mueller-Hinton medium, which resulted in a final concentration of $1x10^6$ cells/mL. The inoculum size was verified by enumeration of CFU obtained by subculturing on TSYA plates.

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

The microplate was incubated at 34°C and examined after 24 h. The MIC endpoints were the lowest drug concentration that showed absence of growth, as recorded visually.

II.12.4. Fungal inhibition tests

II.12.4.1. Minimum inhibitory concentrations (MICs) determination.

The susceptibility tests were made according to the CLSI (former NCCLS) guideline M38-2A (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; CLSI) (Clsi, 2008c), using broth microdilution method, with small modifications. The inoculum suspension was prepared by covering the colonies with approximately 5 mL of sterile 0.9% (w/v) saline (NaCl) with 0.01% (v/v) tween 20. The resulting suspension was transferred to a sterile tube, vortexed for 15 s, and the cell density adjusted to 0.4–5.0 x 10^6 CFU/mL by direct counting of spores using a neubauer chamber. The final inoculum suspension was made by a 1:50 dilution with a double-strength Potato Dextrose broth medium (pH 7.5), which resulted in a final concentration of approximately 0.4 x 10^4 to 5 x 10^4 cells/mL. The inoculum size was verified by enumeration of CFU obtained by subculturing on SDA plates.

Filamentous fungi inocula (100 µL) were added to each well of the microplate, containing 100 µL of the diluted drug solution (twofold). Final volume in each well was 200 µL. Amphotericin and itraconazole were also tested using the same inocula, but following the dilution scheme described in M38-2A for each case. The microplates were incubated at 25°C without agitation and examined after 72 h. The MIC endpoints were the lowest drug concentration that showed absence of growth, as recorded visually.

II.13. Growth curves

The effect of PDβ on the growth of *C. glabrata* ISA 2163 was evaluated in PDB pH 7.5. The assays were conducted in the presence of different PDβ concentrations, 37.5 µg/mL and 150 µg/mL, respectively the Minimum inhibitory (MIC) and Minimum Fungicidal (MFC) concentrations. A cell suspension was grown overnight in 20 mL of PDB pH 7.5, at 30°C, 150 rpm and refreshed in 20 mL of PDB pH 7.5, approximately five hours before the beginning of the assay. In order to obtain an initial concentration of approximately $1x10^5$ CFU/mL, the $OD_{640\ nm}$ was adjusted to 0.05 and then 10-fold diluted with PDB pH 7.5, to a final volume of 100 mL, in 250 mL erlenmeyers. The cultures were incubated at 34°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 34°C for 24 h).

II.14. Morphology and viability assessments

The LIVE/DEAD® Yeast Viability Kit (Molecular probes, 2001) 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 for 30 min. Five µL of calcofluor white working solution were then added to the culture and mixed thoroughly. For microscopical observations, 5 µL of the cell culture were trapped between a microscope slide and a coverslip.

II.15. PDβ localization

The interaction of PDβ with *C. glabrata* ISA 2163 was studied by using fluorescently labeled PDβ, according to previous studies (Pinheiro, 2012). The protein was labeled with Fluorescein isothiocyanate (FITC), using the The FluoroTag FITC Conjugation Kit by Sigma (Sigma-Aldrich, 2010), according to the manufacturer instructions [Sigma], except for the final step of purification. In this study, the labeled protein was purified in NAP-5 columns, according to the manufacturer instructions (GE Healthcare Life Sciences, 2006), and stored at 4°C in the dark. An inhibitory concentration of labeled PDβ (150 µg/mL) was used to determine its localization in the cell. For this purpose, PDB medium pH 7.5 was inoculated with approximately $1x10^5$ CFU/mL, as described in the Growth curves section, to a final volume of 4 mL. The culture was kept in the dark at 34°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.

II.16. Staining with propidium iodide

After the desired incubation period, cells incubated with labeled PDβ 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.

II.17. Immunofluorescency

Immunolocalization of PDβ was made according to Lawrence, *et al*., (2004). The culture was prepared and kept under the same conditions as previously described in the Growth curves section. After 24 h incubation with PDβ, the culture was heat fixed in the slides with followed by a fixation in 4% (v/v) formaldehyde, for 10 min, at room temperature. After that two washes were made with PBS (137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 and 2.7 mM KCl) and PBS with 0.1% (v/v) Triton 100x. The slides were blocked for 30 min with BSA 5% (w/v) in PBS with 0.1% (v/v) Triton 100x, in a moist chamber at room temperature. After a quick wash with PBS, the cells were incubated with a first antibody anti-*Lupinus albus* β-conglutin, produced in rabbit, diluted 1:500 in PBS with 0.1% (v/v) Triton 100x and 0.1% (w/v) BSA, for 16 h at 4°C, 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°C, in a moist chamber. After being washed two times with PBS for 15 min, the slides were prepared in a solution containing Calcofluor White and antifade (Vectashield® Mounting Medium from Vector Laboratories).

II.18. Fluorescence microscopy

All the samples were observed under a fluorescence microscope (Leica DM R) equipped with a camera (Leica DFC 420), using three different filters: Filter Set 49 DAPI (Excitation BP 340-380, Emission LP 425); Filter Set 10 FITC/GFP (Excitation BP 450-490, Emission BP 515) and Filter Set 15 Rodhamine (Excitation BP 515-560, Emission LP 590).

III. Results and discussion

III.1. Isolation and purification of β-conglutin

After total globulins isolation from the dry seeds of *Lupinus albus,* as described in materials and methods section - Isolation of total globulins, the individual globulins were fractionated and purified by AKTA anion exchange chromatography. A representative chromatogram is shown in Figure 1.

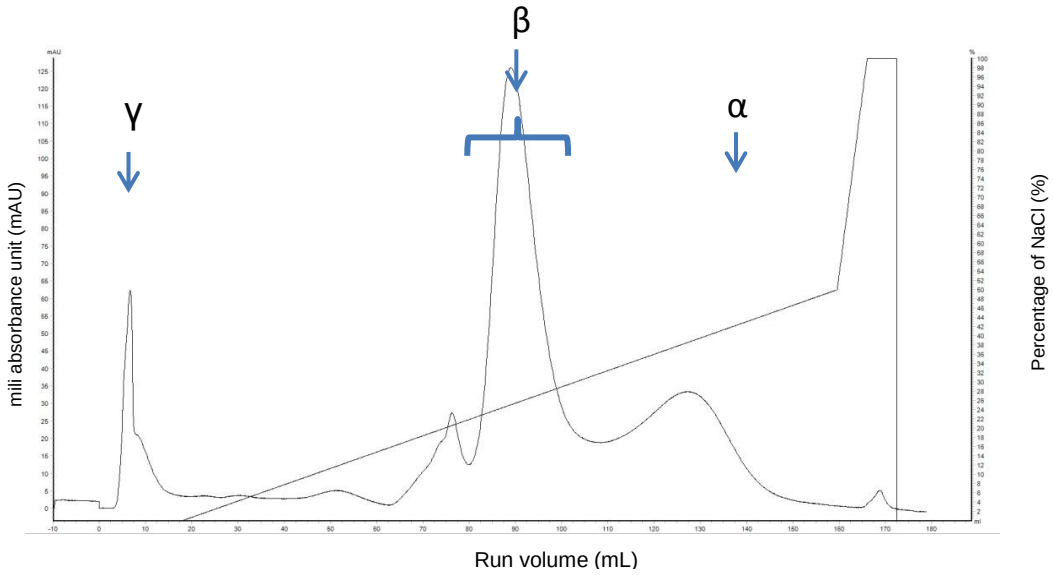

Figure 1. Chromatogram of dry seeds *L. albus* β-conglutin purification by anion exchange chromatography.

Among the three globulins that were fractioned, γ-conglutin had the lower affinity to the Q-sepharose column, followed by β-conglutin, the fraction of interest, which was eluted between 20-30% (w/v) NaCl. α-conglutin, with a higher affinity for the column matrix, was eluted with approximately 40% (w/v) NaCl.

To originate PDβ, after purification, the fraction that corresponds to β-conglutin, was incubated at 25°C during a period of 7 days. To shed a light on the degradation route and to compare the polypeptide composition and molecular masses, β-conglutin 0 days and PDβ were evaluated by SDS-PAGE and both were subjected to a size exclusion chromatography on a Superose 12 column (Figure 2).

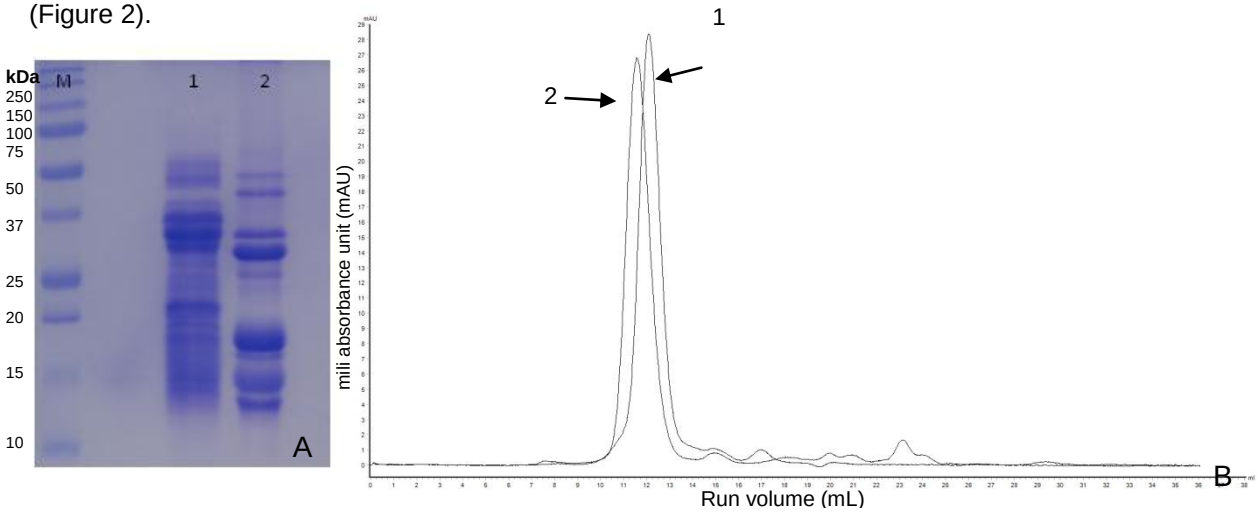

Figure 2. A - SDS-PAGE analyses. Degradation of β-conglutin day zero [1] to PDβ [2]. Precision Plus Protein™ All Blue Standards marker (kDa) [M]. B - Size exclusion chromatogram in a Superose 12 column of β-conglutin 0 days (1) and PDβ (2).

Figure 2.A shows the polypeptidic profile of β-conglutin 0 days (lane 1) and 7 days β-conglutin (PDβ) (lane 2). Comparing both profiles, it is possible to observe that after 7 days of incubation at 25°C the number of polypeptides of β-conglutin declines and an accumulation of a polypeptide with approximately 20 kDa occurs. It seems that this *in vitro* degradation process is similar to the *in vivo* catabolism of β-conglutin, responsible for the accumulation of Blad-oligomer in the cotyledons of *L. albus*. (Monteiro, *et al.*, 2010). However, Figure 2.B shows that despite the notable differences observed on the electrophoretic polypeptidic pattern of β-conglutin 0 days and PDβ, during the degradation the oligomer is not breaking in multiple parts, since it is only visible one single peak on the size exclusion chromatogram. Still, when comparing the peaks of β-conglutin 0 days and PDβ, there is a shift of the peak of PDβ from the peak of β-conglutin 0 days. This shift leads to an increase in molecular weight, from 151.6251 kDa (β-conglutin 0 days) to 174.7518 kDa (PDβ). Taking both results together, it is possible to conclude that despite the visible degradation of β-conglutin by SDS-PAGE, that leads to a much more simplified electrophoretic profile, there is a slightly increase of the molecular weight of the total oligomer.

III.2. Evolution of the antifungal activity of PDβ – from inactive to fully functional

Giving the major differences observed between the polypeptidic profiles of β-conglutin 0 days and PDβ (7 days of incubation at 25°C), the next step was to analyze the progression of the polypeptidic degradation with time and consequently the antifungal activity of PDβ. β-conglutin 0 days was incubated at 25°C and allowed to stay for 7 days. Aliquots were collected daily. All aliquots were tested for antifungal activity, by determination of the minimum inhibitory concentration (MIC) in *C.glabrata* ISA 2163, as described in the section "Antifungal susceptibility tests" of the materials and methods (Table 4) and analyzed by SDS-PAGE to visualize the evolution of the polypeptidic profile (Figure 3).

Table 4. β-conglutin MICs endpoints at different times along degradation.

Days	MIC (µg/mL)
0	> 1000
1	> 1000
2	> 1000
3	31.25
4	31.25
5	15.625
6	15.625
7	15.625

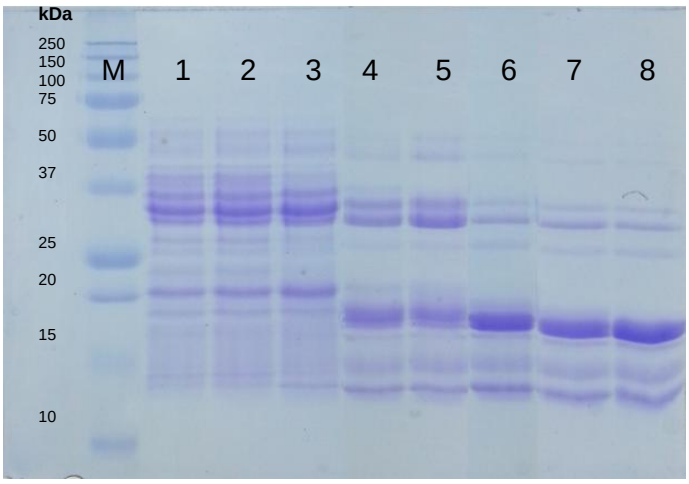

Figure 3. SDS-PAGE analysis. Degradation of β-conglutin during 7 days. Day 0 [1]; day 1 [2]; day 2 [3]; day 3 [4];day 4[5]; day 5[6]; day6 [7] day 7 *(PDβ)* [8]. Precision Plus Protein™ All Blue Standards marker (kDa) [M].

In Figure 3, it is possible to observe that the degradation of β-conglutin 0 days into PDβ occurs gradually during the period of 7 days. In the first three days of incubation the polypeptidic profile of the oligomer remains unchanged being the turn point at day 3 (Figure 3, lane 4). After this point, it is visible a progressive accumulation of a 20 kDa polypeptide and a decrease of the polypeptides with higher molecular masses. This catabolism occurs gradually until the day 7 of incubation (Figure 3, lane 8) time at which the degradation ends and remains stable for at least 1 month (Figure 4, lane 2).

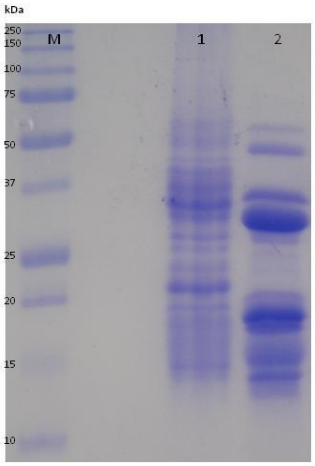

Figure 4. SDS-PAGE analysis. Polypeptide profile of β-conglutin after 1 month of degradation. β-conglutin day 0 [1]; β-conglutin 1 month [2] . Precision Plus Protein^{TM} All Blue Standards marker (kDa) [M].

Once again it is evident the resemblance between this *in vitro* degradation process and the catabolism that naturally occurs in the *L. albus* cotyledons (Dos Ramos, *et al.*, 1997). However, whereas in the *L. albus* cotyledons the catabolism continues to occur, until day 14 of germination, originating almost exclusively a 20 kDa polypeptide, named Blad by the authors that finally declines until complete disappearance, in this case, the process ends, suggesting that there is a missing piece (probably a missing enzyme) for further degradation. Regarding the antifungal activity of PDβ*,* similarly to the polypeptidic profile evolution, it appears progressively being the maximum activity at the 7 day of degradation, which is also the apparent maximum degradation state that PDβ can achieve *in vitro* (Table 3). In the first 3 days, PDβ has no antifungal activity towards *C. glabrata* ISA 2163 within the range of concentrations tested. However, as soon as the degradation process begins to occur, at day 3, PDβ has an antifungal activity measured as a MIC of 31.25 µg/mL reaching 15.625 µg/mL after 7 days of incubation at 25°C (Table 4). Taking both these results together, it is possible to conclude that the *in vitro* degradation route of β-conglutin 0 days, which promotes a simplified polypeptide profile with an accumulation of a 20 kDa polypeptide, is also contributing, somehow, to convert an inactive form of the protein in a fully functional oligomer with a remarkable antifungal activity. ,

III.3. Assessment of the optimum conditions for the stability of PDβ

After the degradation of β-conglutin 0 days into PDβ, several conditions were tested to assess the optimum storage conditions for PDβ stability:

i) PDβ was desalted on PD-10 columns into three different solutions: milli-Q water pH 7.5, 50 mM Tris-HCl, pH 7.5 (Buffer A) and 50 mM Tris-HCl, pH 7.5 with 400 mM Nacl (Buffer B);

ii) The oligomer was conserved at different temperatures (4°C, -20°C and -80°C) and also the protein lyophilized during a period up to one month was also tested.

The evaluation of the conditions tested was performed by determination of PDβ MIC (Table 5).

Table 5. PDβ MICs endpoints in three different buffers.

Buffer	**PDβ Buffer A**	**PDβ Buffer B**	**PDβ milli-Q water pH 7.5**
MIC (µg/mL)	31.25	77.5	>1000

From the results showed in Table 5 is possible to conclude that when PDβ *is* diluted in a solution of mili-Q water, pH 7.5 loses its antifungal activity. One possible explanation is the low buffering capacity of water. When PDβ is diluted in water, the solution pH decreases from pH 7.5 to approximately pH 5.5, which is near to it predictable isoelectric point. As a result the oligomer precipitates and that will cause a loss in the biological activity. When comparing the results of PDβ ressuspended in buffer B vs buffer A, PDβ has higher antifungal activity in buffer A, with a minimum inhibitory concentration of 31.25 µg/mL. Giving the above results and also due to the high salt content of buffer B which could interfere with the microorganisms growth in further experiments, our option to proceed with the work was the maintenance of PDβ in Buffer A.

For PDβ biological activity preservation 4°C, - 20°C, - 80°C storage temperatures and lyophilization were tested for a period up to one month. MIC was determined one week and one month after storage. The samples stored lyophilized were ressuspended in Buffer A before the MIC determination (Table 6).

Table 6. PDβ *samples* MICs endpoints after storage in four different preservation conditions, at different times.

Preservation condition	**4°C**		**- 20°C**		**- 80°C**		**Lyophilized**	
Time	1 week	1 month	1 week	1 month	1 week	1 month	1 week	1 month
MIC (µg/mL)	31.25	31.25	31.25	31.25	31.25	15.625	31.25	15.625

The analysis of Table 6 reveals that there are no significant differences between the conditions tested and the storage period, with MIC results between 15.625 and 31.25 µg/mL. Since the common knowledge that proteins should be stored lyophilized to preserve the biological properties, PDβ was from now on preserved lyophilized and ressuspended when needed in 50 mM Tris-HCl, pH 7.5.

III.4. Characterization and identification of the subunit with activity

To clarify the mechanism involved in the *in vitro* proteolytic degradation of β-conglutin 0 days into PDβ, the first attempt was increase the incubation temperature during the degradation process. Considering that 7 days at 25°C are necessary to obtain a complete degradation of β-conglutin *in vitro*, increasing the temperature of incubation, theoretically, would raise the degradation rate. To this end, β-conglutin 0 days was incubated at 25°C, as the standard sample, 40°C and 60°C. Aliquots were collected at 0 h, 3 h, 6 h, 1 day and 5 days after the beginning of the incubation period and analyzed by SDS-PAGE (Figure 5).

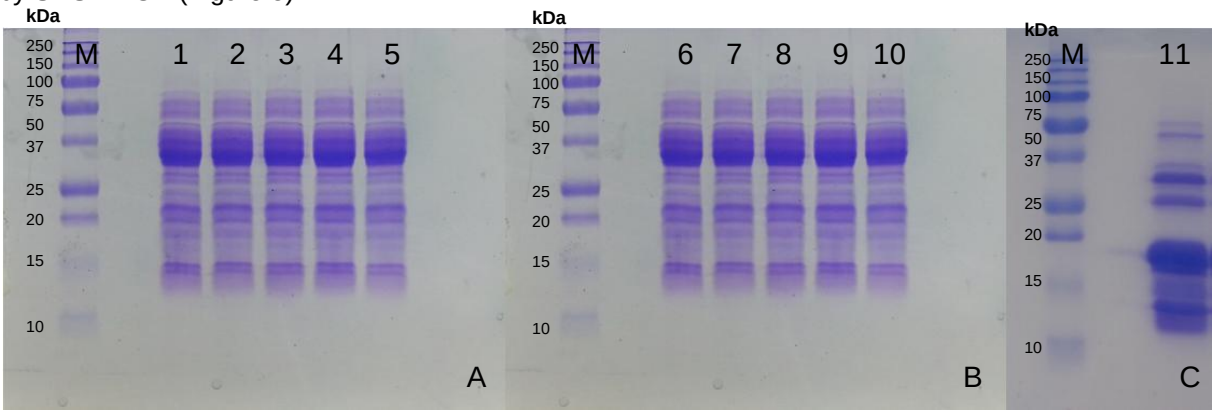

Figure 5. SDS-PAGE analysis. Degradation of β-conglutin at different temperatures, 40°C [6A], 60°C [6B] and 25°C [6C] in different times 0 h [1;6] 3 h [2;7] 6 h [3;8] 1 day [4;9] 5 days [5;10] and 7 days [11]. Precision Plus Protein™ All Blue Standards marker (kDa) [M].

Interestingly, as can be observed in Figure 5, the results were rather different than expected. Comparing the polypeptide patterns of each sample during the incubation period at 25, 40 and 60°C, increasing the temperature of incubation did not rise the time of the reaction, as expected, quite the contrary, it seems that it stopped or delayed the process of proteolytic degradation (Figure 5A; 5B). The result obtained clearly point out that the mechanism or molecule responsible for triggering the degradation process is highly affected by the temperature increase. This means that probably the trigger agent or agents becomes somehow unviable, inactive or degraded, at high temperatures, since at 25°C the profile of PDβ remains unchanged.

With the last result on mind, the next attempt was evaluate if the degradation process could be caused by an oxidation-reduction reaction blocked with the temperature increased (Figure 6). To this way, two different experiments were conducted:

(i) Increase the amount of oxygen present in the reaction, letting the tubes opened during the incubation period;

(ii) add a reducing agent, 100 mM Dithiothreitol (DTT), to decrease the oxidative environment of the reaction.

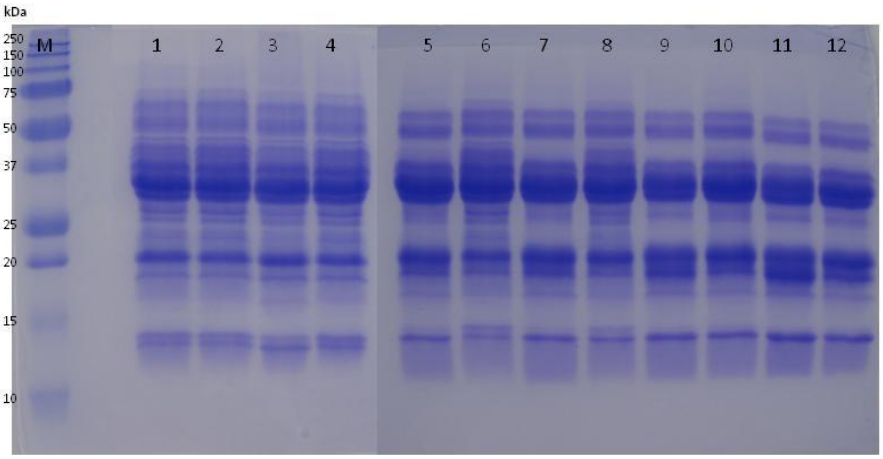

Figure 6. SDS-PAGE analysis. Degradation of β-conglutin during 32 h at 25°C. Standard [1]; with 100 mM DTT [2]; β-conglutin after 24 h without [3] and with more oxygen [4]; β-conglutin after 26 h without [5] and with more oxygen [6]; β-conglutin after 28 h without [7] and with more oxygen [8]; β-conglutin after 30 h without [9] and with more oxygen [10] and β-conglutin after 32 h without [11] and with more oxygen [12]. Precision Plus Protein™ All Blue Standards marker (kDa) [M].

In Figure 6 are depicted the results obtained. Comparing the polypeptide profile of lane 3 to 12 it seems that increasing the amount of oxygen in contact with the solution (Figure 6, lanes 4,6,8,10 and 12) had no effect in the rate of degradation. However, when DTT was added to the sample, the *in vitro* catabolic process of β-conglutin did not occur (Figure 6, lane 2). This result strongly suggests that DTT or the presence of a reduced environment inhibits the activity or the activation of the responsible molecule or molecules involved in the degradation process.

In order to better understand the involvement of DTT, a new experiment was conducted. Several aliquots of β-conglutin 0 days, were let to degrade at 25°C. At the time points of 0 h, 30 h, 40 h and 45 h after the beginning of incubation, 100 mM DTT was added and the reaction was allowed to proceed for 7 days. A control sample of β-conglutin without added DTT was maintained to see if the degradation process occurred normally (Figure 7).

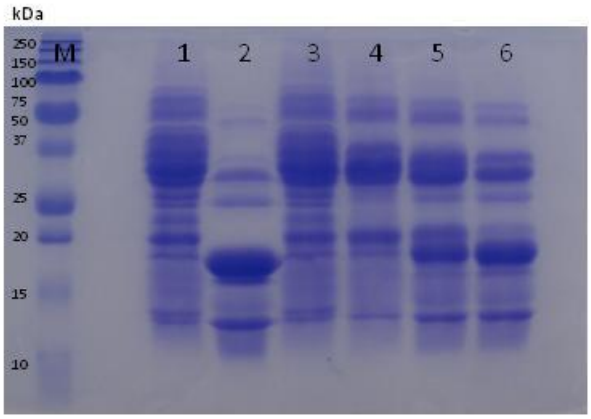

Figure 7. SDS-PAGE analysis. Degradation of β-conglutin for 7 days at 25°C. β-conglutin 0 days [1] PDβ [2] β-conglutin with 100 mM DTT added at day 0 [3] β-conglutin with 100 mM DTT added after 30 h[4] β-conglutin with 100 mM DTT added after 40 h [5] β-conglutin after with 100 mM DTT added aftter 45 h [6]. Precision Plus Protein™ All Blue Standards marker (kDa) [M].

Figure 7 shows the SDS-PAGE analysis of the samples after 7 days of incubation. In the lane 2 is shown the polypeptide profile of PDβ in the control sample (7 days without DTT). Comparing lanes 1 (β-conglutin 0 days) and 3 (DTT added in time 0 of incubation) it is clearly demonstrated that the presence of DTT blocks the degradation process even after the incubation proceeds for 7 days. However, when DTT was added after 30, 40 and 45 h of the beginning of the incubation at 25°C, the degradation stopped, and remained at the same state of degradation (please see Figure 6). Lanes 4, 5 and 6 show progressively a more advanced state of degradation. This can be easily visualized by the thickness of the 20 kDa polypeptide band that is increasing along with the time of DTT adding and comparing with 20 kDa band in lane 2 (β-conglutin incubated without DTT). Furthermore, it is also possible to observe a decrease in the complexity of the polypeptide profile with the time of DTT addition. These results clearly confirm that DTT, or a reducing environment caused by the presence of this agent, strongly interferes with the molecule or in the path involved in the degradation of β-conglutin into PDβ. Moreover, it also has the ability to block this process after it had already started, which is in accordance with several literature reviews where is clear stated that, at high concentrations DTT is an effective protein denaturant, cleaving disulfide linkages between cysteine groups, which may be preventing the catabolism process from happening (Hart, *et al.*, 1994).

These results pointed us for the possibility of having a protease involved in the process that could be inhibited by a reducing agent. To test this hypothesis various protease inhibitors were added to the reaction medium. 2 mL aliquots of β-conglutin 0 days were incubated at 25°C for 7 days (Figure 8). To each sample one of the following proteases inhibitors was added: 10 μM E-64 (target cysteine proteases), 1 mM Pepstatin (target aspartic proteases), 5 mM EDTA (target metalloproteases) and 1 mM Pefabloc (target serine protease).

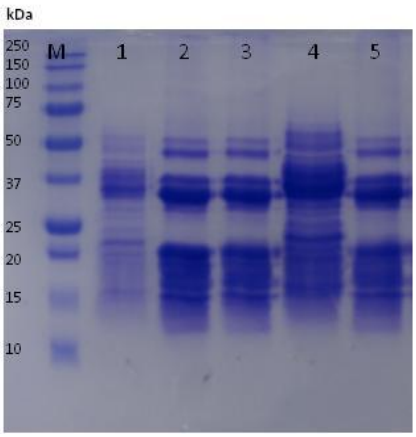

Figure 8. SDS-PAGE analysis. Degradation of β-conglutin with different protease inhibitors. β-conglutin 0 days [1] β-conglutin with different protease inhibitors, 10 μM E-64 [2], 1 mM Pepstatin [3], 5 mM EDTA [4] and 1 mM Pefabloc [5]. Precision Plus Protein™ All Blue Standards marker (kDa) [M].

As shown in Figure 8 no differences can be detected between the degradation profiles after the addition of the protease inhibitors E-64, Pepstatin and Pefabloc to β-conglutin 0 days (Figure 8, lane 2, 3 and 5). The addition of these proteases has no influence in the conversion of β-conglutin 0 days into PDβ, which indicates that none of these protease-families are involved in the process. However, in the sample with added EDTA no degradation was detected (Figure 8, lane 4), being the polypeptidic profile similar to that of β-conglutin 0 days. Taking these results together, it is likely to

conclude that at least one of the molecules involved in β-conglutin 0 days degradation into PDβ is a protease belonging to the class of metalloproteinases. The addition of EDTA causes the inhibition of this protease, enabling the degradation pathway to proceed.

With the knowledge that probably one of the molecules responsible for the degradation of β-conglutin into PDβ is a protease type metalloproteinases, the next experimental phase was run a zymography electrophoresis using as substrate either gelatin or total vicilins extracted from the dry seed of *L. albus* , as described in the section "Zymography" of the materials and methods. These substrates were selected due to the metalloproteinases specificity. As major group of metalloproteinases are the matrix metalloproteinase (MMP), a group of structurally related zinc-dependent enzymes, such as MMP-2 and MMP-9 (also known as gelatinase A and B) and mostly are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. MMPs are involved in the breakdown of extracellular matrix (ECM), in normal physiological processes, such as embryonic development, reproduction, angiogenesis, bone development, wound healing, cell migration, learning and memory, as well as in pathological processes. Since MMPS present high specific activity for denatured collagens (gelatin), degrading types IV and V colagens and other extracellular matrix proteins, gelatin B is usually used as substrate to characterize these enzymes (Vandooren, *et al.*, 2011; Vandooren, *et al.*, 2013; Vu, *et al.*, 1998; Wang & Tsirka, 2005; Xu, *et al.*, 2002). Total vicilins extrated from the dry seeds of *L. albus* were also used as substrate since, supposedly, are the natural substrate for the metalloproteinase involved in this degradation process.

If one of the β-conglutin subunits or other protein purified along with β-conglutin has a function of metalloproteinase, when submitted to a zymography electrophoresis with the appropriate substrate incorporate in the gel matrix, the active protein will appear, after staining the gel, as a white band since the substrate has been degraded in the position corresponding to it molecular mass.

To this end a zymography electrophoresis with gelatin B as substrate was performed (Figure 9).

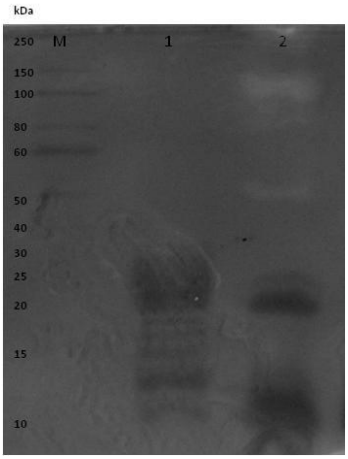

Figure 9. Zymography analysis with gelatin B as substrate. β-conglutin degradation during 7 days at 25 °C. β-conglutin at 0 days [1] and 7 days [2]. Precision Plus ProteinTM All Blue Standards marker (kDa) [M].

As seen in Figure 9, in the lane corresponding to β-conglutin 0 days no white band is visible, this could be that either the metalloproteinase activity is absent or has a very low activity. However, in the lane the correspond to β-conglutin 7 days is possible to visualize the appearance of three white bands, around 150, 100 and approximately 50/60 kDa, which can be associated with the presence of a metalloproteinase activity.

Given the last results, to observe if also the metalloproteinase activity follows the degradation process, β-conglutin samples at different stages of degradation (which correspond to different days of degradation) were ran in a zymography gel with gelatin or total vicilins as substrate. The purpose of this assay was to understand if the metalloproteinase activity is an intrinsic characteristic of native β-conglutin, active even before the degradation process, or in the contrary it needs some kind of activation and is only visible some days after the beginning of the degradation (Figure 10).

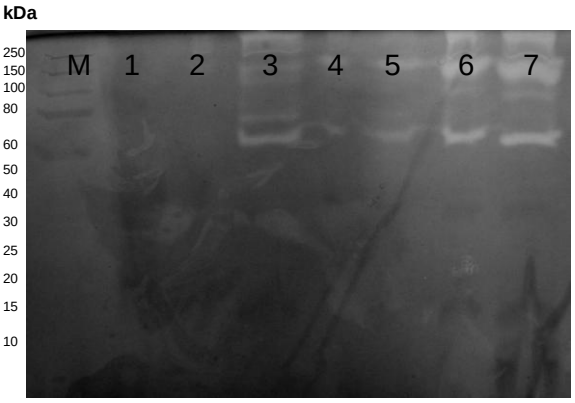

Figure 10. Zymography analysis with gelatin B as substrate. β-conglutin degradation during 7 days at 25 °C. β-conglutin at 0 days [1], 1 day [2], 2 days [3], 3 days [4], 4 days [5] 6 days [6], 7 days [7]. Precision Plus ProteinTM All Blue Standards marker (kDa) [M].

In Figure 10 the zymography electrophoresis gel using as substrate gelatin B is shown. In the lanes corresponding to β-conglutin 0 days and β-conglutin 1 day of degradation, respectively lane 1 and 2, no white band are visible. This means that at these stages the metalloproteinase activity is absent or has a very low activity. However, following the degradation process around day 2 of incubation, the metalloproteolytic activity increases and is possible to visualize the appearance of three white bands, around 150, 100 and 60 kDa. These three subunits manifest their metalloproteinase activity when β-conglutin begins to be degraded and remain active when PDβ is already formed.

In spite of all the results obtained, until now none was conclusive on the origin or the identity of the protein (or other molecule) responsible for the degradation process. In the superpose 12 analysis (Figure 2) it was clear that only one peak with 210 kDa constitutes the β-conglutin sample and later the PDβ. This means that probably a subunit of β-conglutin oligomer with proteolytic activity that needs to pass through an activation process is the molecule responsible for the catabolic process. But several other hypothesis can be raised such as another covalently bound protein, not a sub-unit of the oligomer, that could not be isolated during the superpose 12 purification or even that the molecule responsible for this process could be a contaminant from an external source such as a bacteria.

In order to eliminate the contamination hypothesis, several different types of sterile filters units were tested along with the addition of 100 µg/mL ampicillin in the filtered samples. Different filter matrix materials and pore sizes were used. After the filtration step to sterile tubes, β-conglutin was incubated at 25°C during 7 days and the results were evaluated by SDS-PAGE (Figure 11).

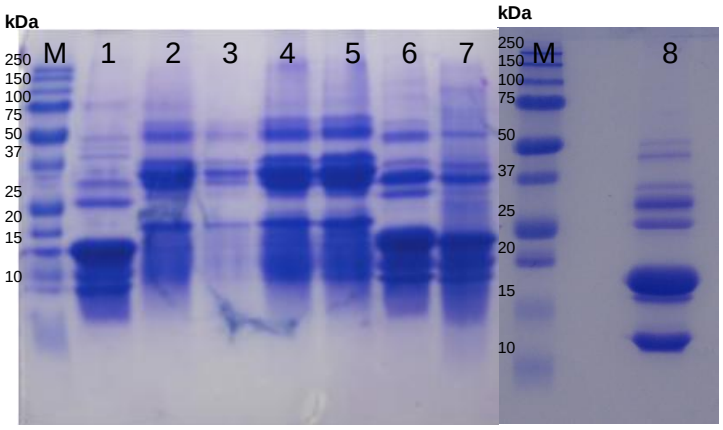

Figure 11. SDS-PAGE analysis. β-conglutin incubated for seven days at 25°C subjected to different treatments. β-conglutin without treatment (PDβ*)* [1]*;* β-conglutin filtered by PES filters 0.45 µm [2]; β-conglutin filtered by PES filters 0.22 µm [3]; β-conglutin filtered by acetate celulose filters 0.45 µm [4]; β-conglutin filtered by acetate cellulose filters 0.22 µm [5]; β-conglutin filtered by PVDF filters 0.45 µm [6]; β-conglutin filtered by PVDF filters 0.22 µm [7]; β-conglutin with 100 µg/mL ampicilin [8]. Precision Plus ProteinTM All Blue Standards marker (kDa) [M].

Observing the results shown on Figure 11, lane 8, (sample with 100 µg/mL ampicillin added), they suggest that the appearance or activation of the metalloproteinase is not due to a microbial contamination since β-conglutin was converted into PDβ even after treatment with ampicillin. Interestingly are all the other results. Although we tested different filter materials and pore sizes, only the samples that were filtered through PVDF filters, independently of the pore size (0.22 µm or 0.45 µm), were degrade into PDβ (Figure 11 lane 6 and 7, respectively). When filtered through acetate cellulose and PES filters, and regardless the pore size, β-conglutin loses the ability to be converted into PDβ (Figure 11 lanes 3, 4, 5 and 6). Several reasons could be attributed for these results. The major difference between PVDF filters and both the other two is its hydrophobic nature (both PES and cellulose acetate are hydrophilic). Probably, the starting molecule which will begin the degradation process by activating the metalloproteinase becomes trapped in a hydrophilic matrix.

In an attempt to identify the molecules that reacted with the filters matrix responsible for the activation of the metalloproteinase two experiments were conducted:

(i) After β-conglutin filtration by a PES unit, the filter membrane was removed from the filter and boiled in sample buffer for subsequent SDS-PAGE analysis of the eluted fraction. However, it was impossible to obtain eluted liquid sample, since the result from the boiled membrane was very viscous, turning impossible to load into the SDS-PAGE gel;

(ii) β-conglutin was filtered by PES filters, then the filter was inverted and 1 mL of 0.1 M HCl were filtered to detach the molecule. The eluted sample was lyophilized to extract the HCl and the lyophilized powder was ressuspended in mili-Q water, pH 7.5 for posterior spectrum analyses between

190_{nm} and 900_{nm}. As standards, Buffer A (50 mM Tris-HCl, pH 7.5) and mili-Q water, pH 7.5 were used (Figure 12).

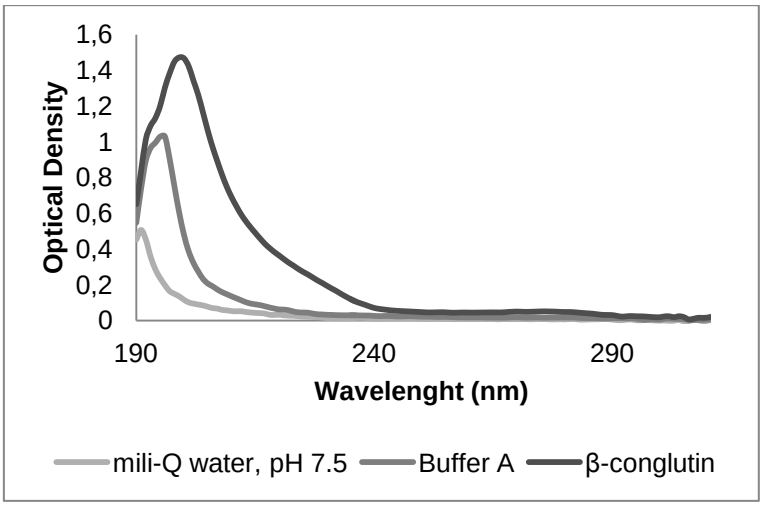

Figure 12. Spectrum of bounded molecule to PES filter.

Figure 12 shows the spectrum obtained between 190_{nm} to $310_{nm,}$ (from 310_{nm} to 900_{nm} only the baseline is visible). Although it is possible to see an increase in the optical density of the eluted peak when compared with both peaks from mili-Q water, pH 7.5 and buffer A, no concrete conclusion can be withdrawn since there is no particular changes on the peak obtained.

Upon failure of the previous experiments and still with the aim of the metalloproteinase characterization, at this point of the work it seems that only by zymography the metalloproteinase can be identified, so, the next logical step was trying to sequence the bands with positive signal isolated from the zymography gel. To this end, a new zimography was performed with PDβ using the total vicillins from the dry seeds of *L. albus* as the gel matrix substrate (Figure 13).

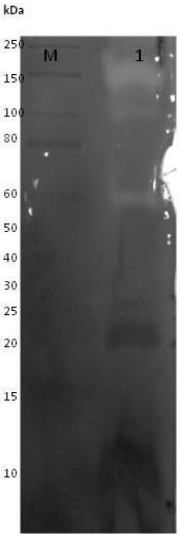

Figure 13. Zymography analysis with total vicilins as substrate. β-conglutin degradation during 7 days (PDβ) [1]; NEB Unstained Protein Ladder [M].

As seen before (Figures 9 and 10) in Figure 13 can be visualized three white bands, around 150, 100 and 60 kDa. The three bands were individually cut from the gel, milled in a mortar with a pestle until very small pieces and ressuspended in sample buffer. After a centrifugation step the supernatant was treated to be run in a SDS-PAGE followed by the gel staining using a silver stain protocol specifically for sequencing purposes. Finally, after identification of the polypeptides of interest, they were cut from the gel and sent to a sequencing service facility. For control purposes, a piece of a lane from the zymography gel with only sample buffer was cut from the gel and submitted to the same treatments as the bands of interest. This allowed the differentiation of the polypeptides that correspond to the vicilin, incorporated in the gel, from the target polypeptides. Given the difficulty to precisely separate the bands with 100 and 150 kDa, the decision was mix both samples and run together in a silver stained gel and the results are shown in Figure 14.

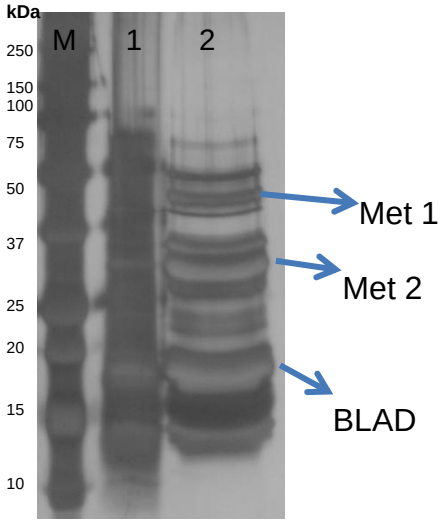

Figure 14. SDS-PAGE analysis of the bands extracted from zimography. 150 plus 100 kDa [1] and 60 kDa [2]. NEB Unstained Protein Ladder [M].

The analysis of Figure 14 reveals that after extracted and submitted again to SDS-PAGE, the bands corresponding to 150 and 100 kDa appears as a complex polypeptide profile with an incomprehensible amount of bands impossible to differentiate. Unfortunately, the band with 60 kDa also appears with an excessive amount of polypeptide bands but it was possible to distinguish three major bands with 50, 37 and 20 kDa. The bands with 50 and 37 kDa seemed to us that could be the polypeptides that constitute the metalloproteinase, not only because of the amount that appear in the gel but also due it significate increase when compared with PDβ profile. The 20 kDa band was also sent to be sequenced due to the similarity in molecular mass and catabolism route with BLAD polypeptide. These 3 bands were cut from the gel and sent to be sequenced (Table 7).

Table 7. MALDI-TOF mass spectrometric sequencing analysis.

Sample name	Protein found in database	Entry name	Confidence level (%)
MET 1	No identification		0%
MET 2	β-conglutin precursor [*Lupinus albus*]	gil46451223	95%
BLAD	BLAD [*Lupinus albus*]	gil77994351	95%

Table 7 presents the results obtained from mass spectroscopy analysis (for the complete results of the sequencing analysis please see Annex I). Unfortunately, it was only possible to identify two out of the three bands extracted from the gel. The 37 kDa band shows homology with β-conglutin and the 20 kDa band shows high homology with Blad-oligomer (Band of *Lupinus albus* doce), both with 95% confidence level. The band corresponding to 50 kDa was not possible to identify. It is considered a positive identification when the score is above the 95% confidence level (score approximately 90 for the NCBInr database), and at least one peptide has an ions score above 40, or at least 2 peptides have ion scores above 20. Both positive identifications came with a confidence level of 95% and in both cases it was possible to identified more than one peptide with an ions score above 40. The band corresponding to the 50 kDa was not possible to be sequenced, and could be the wanted answer.

Due to the timeframe of this thesis it was not possible to determine and identified the metalloprotease and/or the activator molecule. In the future new studies or new approaches must be performed in order to better understand the proteolytic mechanism involved in the transformation of β-conglutin into PDβ.

III.5. Screening of the antimicrobial activity of PDβ

Previous studies in our laboratory showed that PDβ has antifungal activity against *C. glabrata* ISA 2163, but no further data was available on its antimicrobial range. A general screening was performed in order to determine if other microbial species are also inhibited by PDβ .

For this screening PDβ, was tested in three different groups of microorganisms: bacteria, yeasts and filamentous fungi, as described in materials and methods (Tables 8,9 and 10).

Table 8. PDβ MICs and MFCs endpoints in µg/mL in PDB medium for the collection of yeasts. (NG) – Absence of growth in PDB, (ND) - Not determined.

Specie	Strain	MIC	MFC	Specie	Strain	MIC	MFC
Candida albicans	ISA 2151	>1000	ND	Hanseniasporaguillermondi	ISA 2438	NG	ND
	ISA 2218	>1000	ND	*Hanseniaspora uvarum*	ISA 2392	NG	ND
	ISA 1862	>1000	ND		ISA 2393	NG	ND
Candida apicola	ISA 2424	>1000	ND	*Issatchenkia occidentalis*	ISA 2384	31.25	31.25
Candida cantarellii	ISA 2391	>1000	ND	*Issatchenkia orientalis*	ISA 2382	125	125
Candida deformans	ISA 2415	>1000	ND		ISA 2383	125	125
Candida diversa	ISA 2433	NG	ND	*Issatchenkia terricola*	ISA 2437	NG	ND
Candida famata	ISA 1830	>1000	ND	*Kluyveromyces marxianus*	ISA 2444	NG	ND
	ISA 1831	NG	ND	*Lachancea thermotolerans*	ISA 2164	3.906	3.906
Candida frutus	ISA 2390	>1000	ND		ISA 2379	15.625	15.625
Candida dubliniensis	ISA 2228	>1000	ND		ISA 2380	31.25	31.25
Candida guilliermondii	ISA 2258	>1000	ND	*Pichia burtonii*	ISA 2377	NG	ND
	ISA 2167	>1000	ND	*Pichia fermentans*	ISA 2435	500	500
Candida glabrata	ISA 2163	31.25	31.25		ISA 2445	NG	ND
	ISA 2242	62.5	62.5	*Pichia guilliermondii*	ISA 2375	>1000	ND
	ISA 2243	15.625	15.625		ISA 2376	>1000	ND
	ISA 2244	62.5	62.5	*Pichia kluyveri*	ISA 2434	62.5	62.5
	ISA 2245	31.25	31.25	*Pichia manshuriea*	ISA 2426	NG	ND
	ISA 2246	15.625	15.625		ISA 2427	NG	ND
Candida krusei	ISA 2223	62.5	62.5	*Pichia sporocuriosa*	ISA 2374	NG	ND
	ISA 2290	125	125	*Saccharomyces cerevisiae*	ISA 2418	NG	ND
Candida neoformans	ISA 2293	31.25	31.25		ISA 2425	NG	ND
Candida parapsilosis	ISA 2238	>1000	ND		ISA 1000	39	39
	ISA 2237	>1000	ND	*Saccheromycopsis crataegensis*	ISA 2373	NG	ND
Candida pomicola	ISA 2389	15.625	15.625	*Saccharomycopsis vini*	ISA 2372	NG	ND
Candida stellimalicola	ISA 2387	NG	ND	*Yarrowia lipolytica*	ISA 1718	NG	ND
	ISA 2388	15.625	15.625	*Zygoascus hellenicus*	ISA 2284	>1000	ND
Candida tropicalis	ISA 2292	>1000	ND		ISA 2367	>1000	ND
	ISA 2232	>1000	ND	*Zygoascus meyerae*	ISA 2365	>1000	ND
Candida zemplinina	ISA 2432	>1000	ND		ISA 2366	>1000	ND
Debaryomyces hansenii	ISA 2416	>1000	ND	*Zygosaccharomyces bailli*	ISA 2283	NG	ND
Hanseniasporaguillermondi	ISA 2417	>1000	ND		ISA 2419	NG	ND
	ISA 2385	NG	ND	*Zygosaccharomyces bisporus*	ISA 1868	NG	ND

Table 9. PDβ MICs endpoints in µg/mL in PDB medium for the collection of fungi.

Specie	Strain	MIC
Alternaria alternata	CEV 1	250
Botrytis cinerea	CEV 6	125
Colletotrichum acutatum	CBS 294.67	250
Colletotrichum gloeosporioides	CBS 119204	250
Fusarium graminearum	CBS 184.32	>1000
Fusarium oxysporum	CBS 114750	>1000

Table 10. PDβ MICs endpoints in µg/mL in Mueller-Hinton medium for the collection of bacteria.

Specie	Strain	MIC
Bacillus subtilis	ISA 4050	>1000
Listeria monocytogenes	ISA 3001	>1000
	ISA 3998	>1000
Pseudomonas aeruginosa	ISA 4076	>1000
Staphylococcus aureus	ISA 3015	>1000

Two groups of yeasts species were tested in this screening: pathogenic, and food spoilage yeasts.Within the group of pathogenic species the results (Table 8) show that PDβ has the ability to inhibit the growth and to kill 99.99% of all strains of *C. glabrata, C. krusei and C. neoformans* tested. However, PDβ was unable to inhibit the growth of the others pathogenic yeasts in the range of concentrations tested (>1000 µg/mL).

The majority of the spoilage yeasts species tested are present in grapes and wine, namely, *C. pomicola, C. stellimalicola, I. occidentalis, I. orientalis ,L. thermotolerans, P. fermentans, P. kluyveri and S. cerevisiae.* The results obtained with these strains were similar to those obtained above: it was observed that when PDβ *was* capable of inhibiting growth it also induced the death of 99.99% of the cells. Although these results were not detailed here, they are extremely important, not only because PDβ could be an important and very effective way to combat food poisoning and spoilage but also a way to combat the resulting economic losses in the food industry.

In Table 9 it is observed that PDβ is also capable of inducing growth inhibition in some of the phytopathogenic filamentous fungi tested, such as *Alternaria alternata, Botrytis cinerea, Colletotrichum acutatum and Colletotrichum gloeosporioides*. A potential bactericidal activity was also tested and the results are presented in Table 10. Three species of gram-positive and one gram-negative bacteria were tested, and in all cases the concentration needed to inhibit the growth was higher than the range of concentration tested. These preliminary results indicate that probably PDβ does not seem to be bactericidal.

Given that the results obtained so far suggest a strong antifungal activity against a wide range of yeasts and that many recent studies enhance the emerge of non-albicans infections (Pfaller, *et al*., 2012; Spreghini, *et al*., 2012), the strain *C. glabrata* ISA 2163 was selected as a model for the remaining tests of this work. Furthermore, this strain is also used as reference at the Converde, SA laboratory.

III.6. Comparison of PDβ with two commonly used antifungal agents

After the initial screening, new determinations of MIC and MFC were assayed but using Itraconazole and Amphotericin B as standards, in order to compare the activity of PDβ with two of the most common antifungal drugs used in the treatment of fungal infections (Table 11). These drugs belong to two different chemical groups, with different modes of action: azoles and polyenes, respectively.

Table 11. Itraconazole, amphotericin B and PDβ MICs and MFCs endpoints in µg/mL in PDB medium for *C. glabrata* ISA 2163.

C. glabrata ISA 2163	MIC	MFC
Itraconazole	8	>16
Amphotericin B	0.25	1
PDβ	31.25	31.25

As it is possible to see in Table 11, and as described in the literature, itraconazole was unable to cause cell death, within the range of concentration tested. Azoles, are referenced as being fungistatical drugs, inducing only growth inhibition (Barros, *et al.*, 2007; Bueno, *et al*., 2010; Manavathu, *et al.*, 1998).

Regarding amphotericin B, the results are also in accordance with the literature (Barros, *et al.*, 2007; Bueno, *et al*., 2010; Manavathu, *et al*., 1998; Spreghini, *et al*., 2012) where a concentration of 0.25 µg/mL was needed to inhibit the growth of *C. glabrata* ISA 2163 and a concentration of 1 µg/mL was needed to cause 99.99% of cell death. The results obtained with PDβ show that a concentration of 31.25 µg/mL is required to perform both cellular inhibition and death of *C. glabrata* ISA 2163, under this set of conditions.

Although the doses of PDβ required to induce growth inhibition seem to be substantially higher than those from both itraconazole and amphotericin B, its molecular weight is also substantially higher (210 kDa). This means that the number of molecules necessary to induce the same inhibitory effect is quite similar between these drugs. Comparing the MIC and MFC values obtained for these three drugs, and expressing the results in number of molecules, the same inhibitory effect is obtained with: 8.960×10^{13} molecules/mL of PDβ, 1.075×10^{14} molecules/mL of amphotericin B and 6.827×10^{15} molecules/mL of itraconazole. For the fungicidal effect the number of molecules required are 8.96×10^{13} molecules/mL of PDβ and 6.5×10^{14} molecules/mL of amphotericin B. Therefore, PDβ seems to have a higher inhibition and fungicidal power by molecule than both the azole and polyene tested.

III.7. Growth curves

Growth curves were performed with strain ISA 2163 of *C. glabrata*, in order to determine the effect of PDβ on the cells during the different phases of the growth curve. In order to perform these curves, a new determination of MIC and MFC were made with an inoculum size of 10^5 CFU/mL.

As expected, when increasing the inoculum size by 100 fold, the concentration necessary to induce growth inhibition also increased (Table 12) and must be readjusted for performing the growth curves. The minimum inhibitory concentration of amphotericin B was 0.25 µg/mL and the minimum fungicidal concentration 2 µg/mL. Regarding PDβ the values were 37.5 and 150 µg/mL, respectively for the minimum inhibitory and minimum fungicidal concentration. However, when the inoculum size was increased, PDβ lost the capability to induce 99.99% cellular death, within the range of concentration tested. Therefore, for these tests, the minimum fungicidal concentration of PDβ is considered to be the concentration responsible for causing 90% cell death.

Table 12. PDβ MICs and MFCs endpoints in µg/mL in PDB for *C. glabrata* ISA 2163.

C. glabrata ISA 2163	MIC	MFC
Amphotericin B	0.25	2
PDβ	37.5	150

For understanding the effect of PDβ in the growth of *C.glabrata* ISA 2163, a growth curve was performed in PDB medium. During the experiment, at regular intervals, various samples were collected in order to evaluate the number of viable cells (OD_{640nm} and CFU counts). Two previously determined concentrations of PDβ and amphotericin B were used, corresponding to the minimum inhibitory and minimum fungicidal concentration, respectively (Table 12). A culture, grown under the same conditions but without drugs, was used as control (Figures 15 and 16).

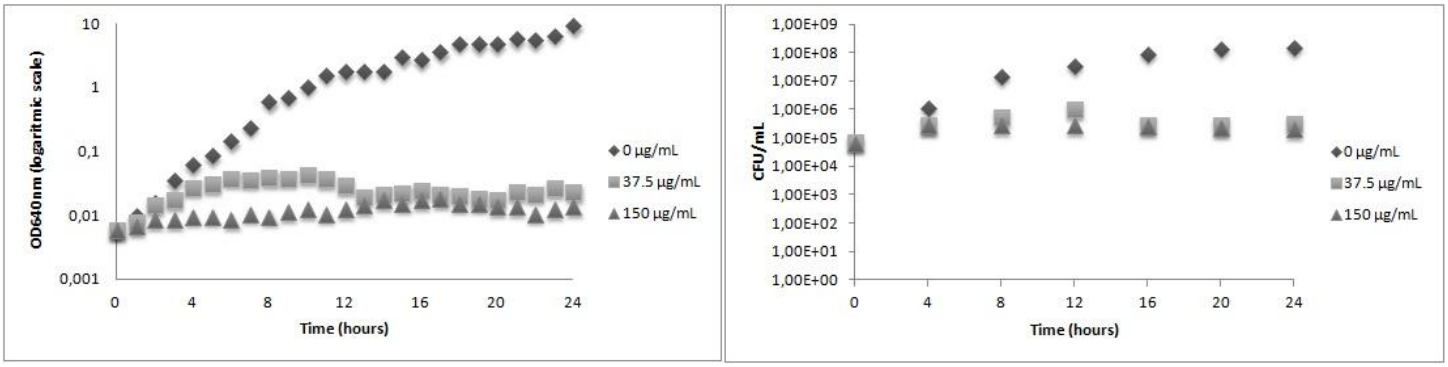

Figure 15. Effect of PDβ on the growth of *C. glabrata* ISA 2163 in PDB medium, pH 7.5, 34°C, without agitation. Concentration of PDβ in the culture: 0 µg/mL, 37.5 µg/mL and 150 µg/mL.

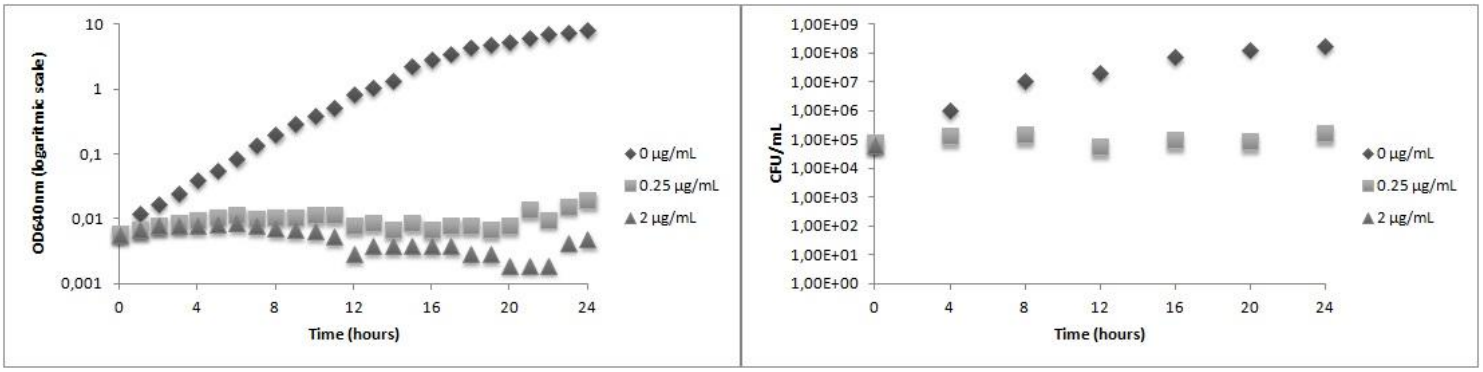

Figure 16. Effect of Amphotericin B on the growth of *C. glabrata* ISA 2163 in PDB medium, pH 7.5, 34°C, without agitation. Concentration of Amphotericin B in the culture: 0 µg/mL, 0.25 µg/mL and 2 µg/mL.

The addition of PDβ to the culture medium had an intense effect on the growth of *C. glabrata* ISA 2163, as seen in Figure 15. The fraction of the culture without PDβ shows a normal growth curve, with an exponential phase of approximately 8 to 10 h, initiating then the stationary phase. This is possible to observe with both OD_{640nm} readings and CFU/mL counts.

Cells grown in the presence of the minimum inhibitory concentration (MIC) of PDβ, 37.5 µg/mL, showed a high decrease in the growth rate, as compared to the control fraction, resulting in lower optical density readings and in lower CFU/mL counts (Figure 15). These results show that this concentration has, in fact, the capability to inhibit the cellular growth of this strain.

Regarding the minimum fungicidal concentration of PDβ, 150 µg/mL, it is possible to observe that cells showed a slightly decrease in the growth rate, when compared to the MIC fraction, resulting in lower optical density readings and lower CFU/mL counts (Figure 15). It was also possible to confirm that, as observed previously, when increasing the initial inoculum size from 10^3 CFU/mL to 10^5 CFU/mL, PDβ loses the ability to cause 99.99% cellular death, within the range of concentration tested. As seen in Figure 15, during the period of the experience, the CFU counts did not decrease. Also, and as expected, the inhibition of growth is slightly higher with 150 µg/mL than with 37.5 µg/mL.

Regarding amphotericin B, the addition of the drug to the culture medium had also a strong effect in the growth of *C.glabrata* ISA 2163, as seen in Figure 16. Similarly to the PDβ experiment, the fraction without drugs presents a normal growth curve and the MIC of amphotericin B, 0.25 µg/mL, induced a decrease in the growth rate, comparing to the control fraction, resulting in a lower optical density and CFU/mL counts. These results are consistent with the values for amphotericin B in the literature (Barros, *et al*., 2007; Bueno, *et al*., 2010; Manavathu, *et al.,* 1998; Spreghini, *et al*., 2012) and very similar to those obtained with PDβ. When added the minimum fungicidal concentration of amphotericin B to the culture medium, cells became non-viable within the first 4 h of growth. This was observed by both stabilization of OD_{640nm} and absence of CFU counts.

III.8. Viability and cellular integrity assessments

The effect of PDβ on the viability and celular integrity of *C. glabrata* ISA 2163 was evaluated through samples collected during the growth curve, in PDB medium. Each aliquot was stained with FUN-1 and calcofluor white and visualized in a fluorescence microscope. FUN-1 binds to nucleic acids producing a yellowish green fluorescence in death cells with a damaged membrane. Cells metabolically inactive but with intact plasma membrane also present a diffuse green fluorescence in cytoplasm. When cells are metabolically active, is possible to observe the formation of orange cylindrical structures designated Cylindrical IntraVascuolar Structures (CIVS) inside the vacuoles. The formation of CIVS only occurs in metabolic active cells with an intact plasma membrane, which means that these are not observed in dead cells (Henry-Stanley, *et al.*, 2004). Calcofluor white is a compound with high affinity to chitin and is normally used as a marker of the cell wall in fungi.

Figures 17 and 18 suggest that in the first 4 h of incubation with PDβ there are no changes regarding the viability and integrity of the cells, in all conditions tested, since the presence of CIVS indicates metabolic activity and the fluorescence with calcofluor white is normal and equal to the

control fraction, indicating cell wall integrity. These results are consistent with the ones obtained in the growth curves (Figure 15).

After 8 h of incubation with both concentrations of PDβ, Figure 19, it seems to occur a turning point in the cell wall integrity. The control fraction exhibits CIVS and a good labeling with calcofluor white, indicating that the cells are metabolically active and with an intact cell wall. These results are consistent with Figure 15 where it is visible that cells are still viable and culturable. Both concentrations of PDβ show CIVS in the majority of the cells indicating that cells are metabolically active. However, in terms of cell wall integrity, it is possible to see a decrease in the labeling of calcofluor (Figure 19.2b and 3b).

After 16 h of incubation with both concentrations of PDβ there are no changes regarding the viability of the cells (Figure 20. 2c and 3c), since CIVS are still visible in all fractions, but the labeling with calcofluor white became even weaker in the fraction incubated with the higher concentration of PDβ. The majority of the cells show no visible fluorescence with 150 μg/mL (Figure 20.3b).

After 24 h of incubation, the last time point studied, all the three fractions show some cells without CIVS, meaning that the cultures are becoming old (Figure 21.1c). However, although most of the cells of the fractions incubated with PDβ are still metabolically active at this point, since the cells still present CIVS as described by Henry-Stanley, *et al*. (2004) and Millard, *et al.* (1997) and contrary to what happens in the control fraction, it appears that they are no longer able to multiplicate, giving the stabilization of the OD_{640nm} and CFU counts over the last 12 h (Figure 15). Regarding the integrity of the cell wall, there is an enormous difference between the control fraction and both concentrations of PDβ. While the integrity of the cell wall in the fraction incubated without PDβ remains unalterable (Figure 21.1b), the fractions that were exposed to the drug present gaps in the calcofluor white labeling (Figures 21.2b and 3b), suggesting a loss in the cell wall integrity. These results are similiar to the ones seen by Thevissen, *et al*. (2012), where the same labeling profile was observed in calcofluor white with a an antifungal drug (defensin) that acts in the cell wall. This phenomenon is more visible with the concentration of 150 μg/mL, since the majority of the cells exposed to this concentration are no longer stained with calcofluor white.

These results indicate that although PDβ is not able to cause cellular death, with this inoculum density and for these concentrations, it is somehow able to perform a massive change in the cell wall, destabilizing it. Taking these results together with those from Figure 15 (stabilization of OD_{640nm} and CFU counts between 12 h and 24 h of incubation with PDβ), it is possible to conclude that the damages in the cellular wall, are sufficiently severe to impair cell multiplication.

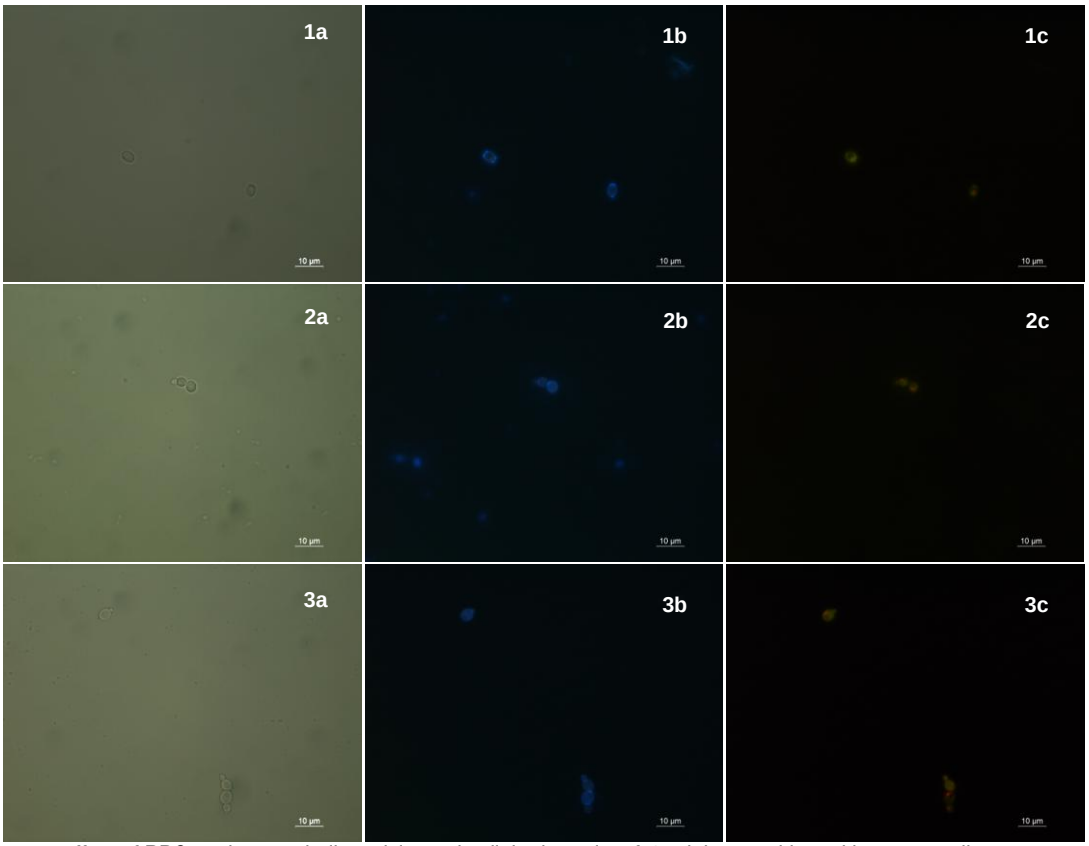

Figure 17. Effect of PDβ on the metabolic activity and cellular integrity of *C. glabrata* cultivated in PDB medium, pH 7.5, at 34 °C, without agitation. Samples taken after 0 h of incubation. Concentration of PDβ in the culture medium: 1 – 0 µg/mL, 2 - 37.5 µg/mL, 3 - 150 µg/mL. Bar corresponding to 10 µm.

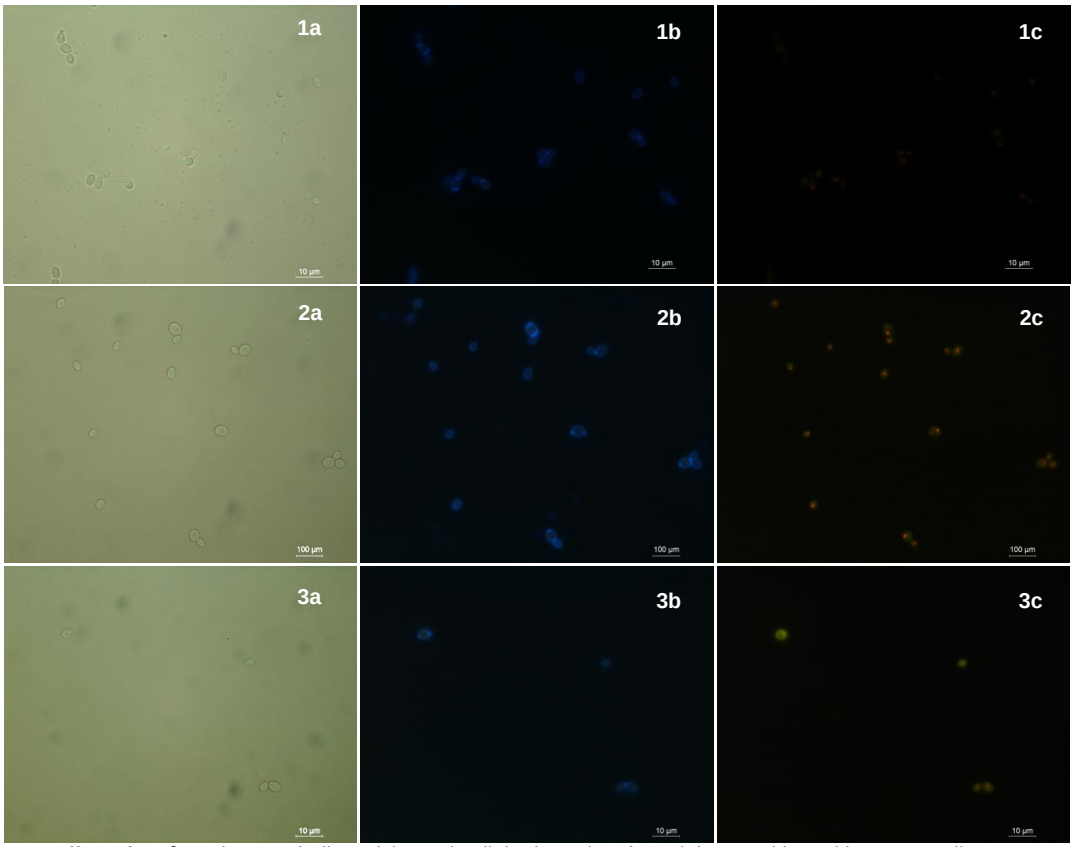

Figure 18. Effect of PDβ on the metabolic activity and cellular integrity of *C. glabrata* cultivated in PDB medium, pH 7.5, at 34 °C, without agitation. Samples taken after 4 h of incubation. Concentration of PDβ in the culture medium: 1 – 0 µg/mL, 2 - 37.5 µg/mL, 3 - 150 µg/mL. Bar corresponding to 10 µm.

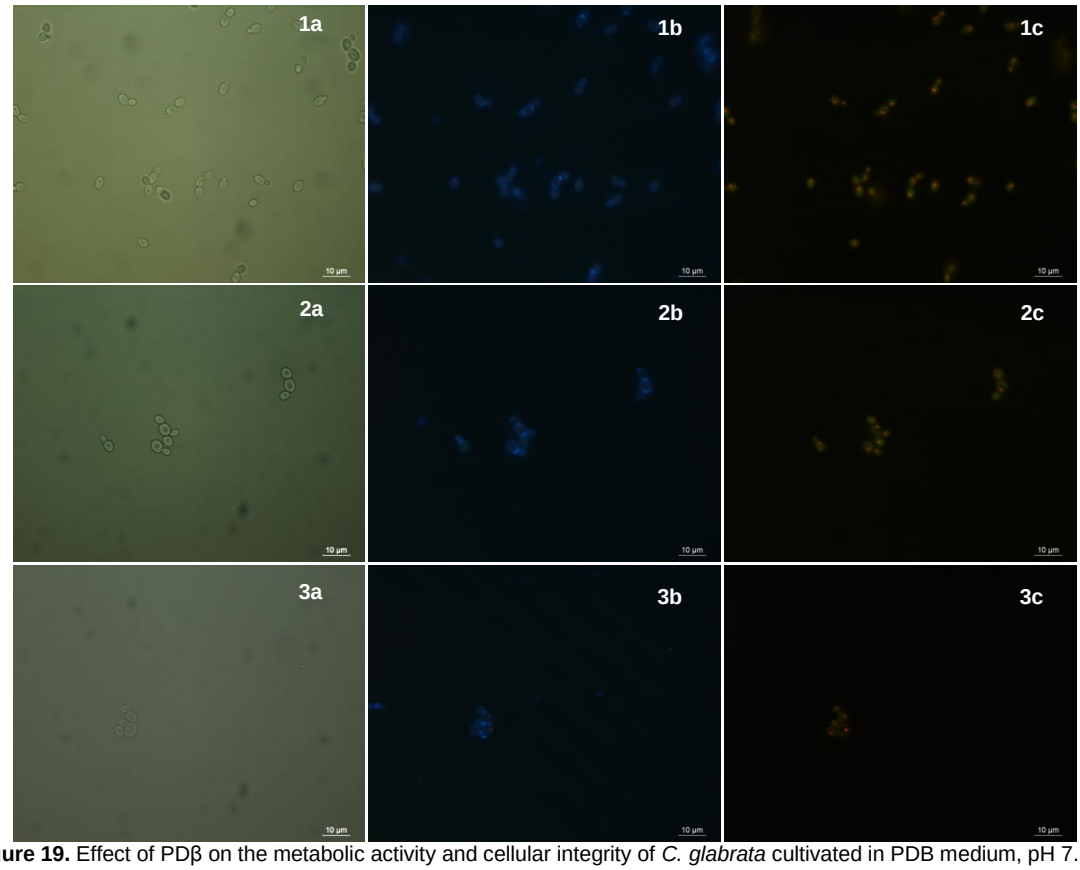

Figure 19. Effect of PDβ on the metabolic activity and cellular integrity of *C. glabrata* cultivated in PDB medium, pH 7.5, at 34°C, without agitation. Samples taken after 8 h of incubation. Concentration of PDβ in the culture medium: 1 – 0 µg/mL, 2 - 37.5 µg/mL,150 µg/mL. Bar corresponding to 10 µm.

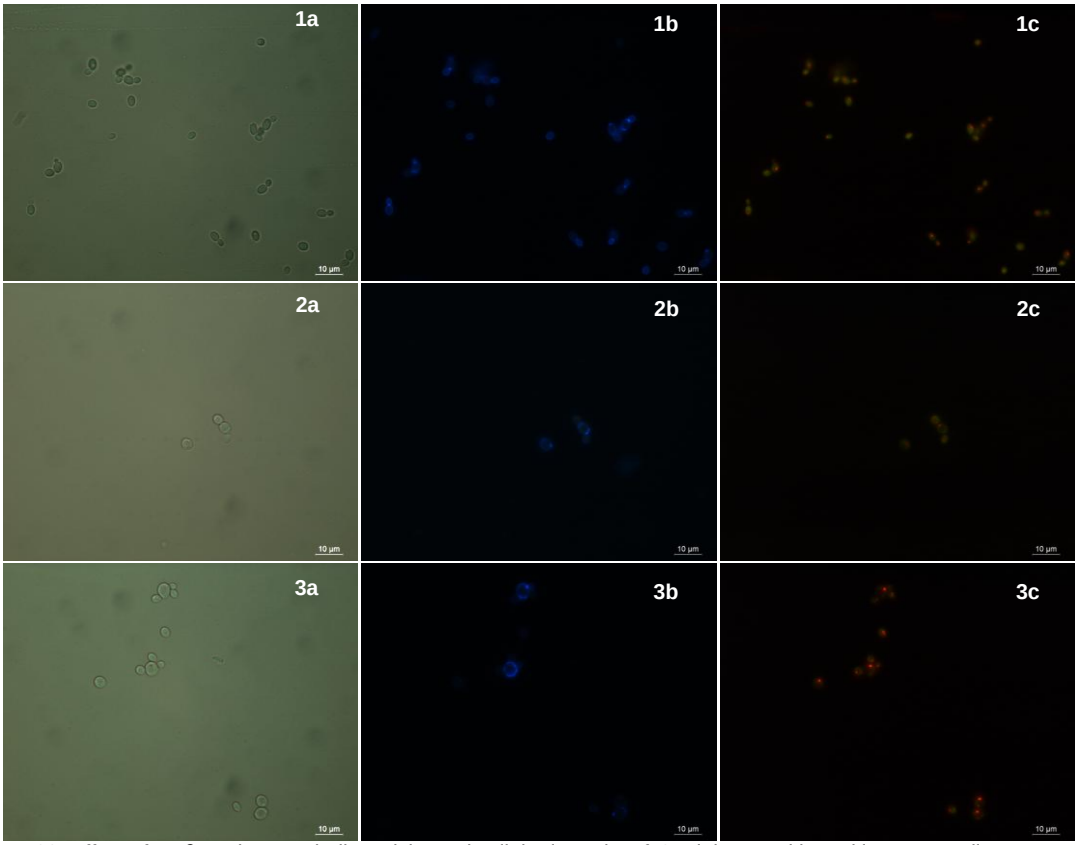

Figure 20. Effect of PDβ on the metabolic activity and cellular integrity of *C. glabrata* cultivated in PDB medium, pH 7.5, at 34°C, without agitation. Samples taken after 16 h of incubation. Concentration of PDβ in the culture medium: 1 – 0 µg/mL, 2 - 37.5 µg/mL, 150 µg/mL. Bar corresponding to 10 µm.

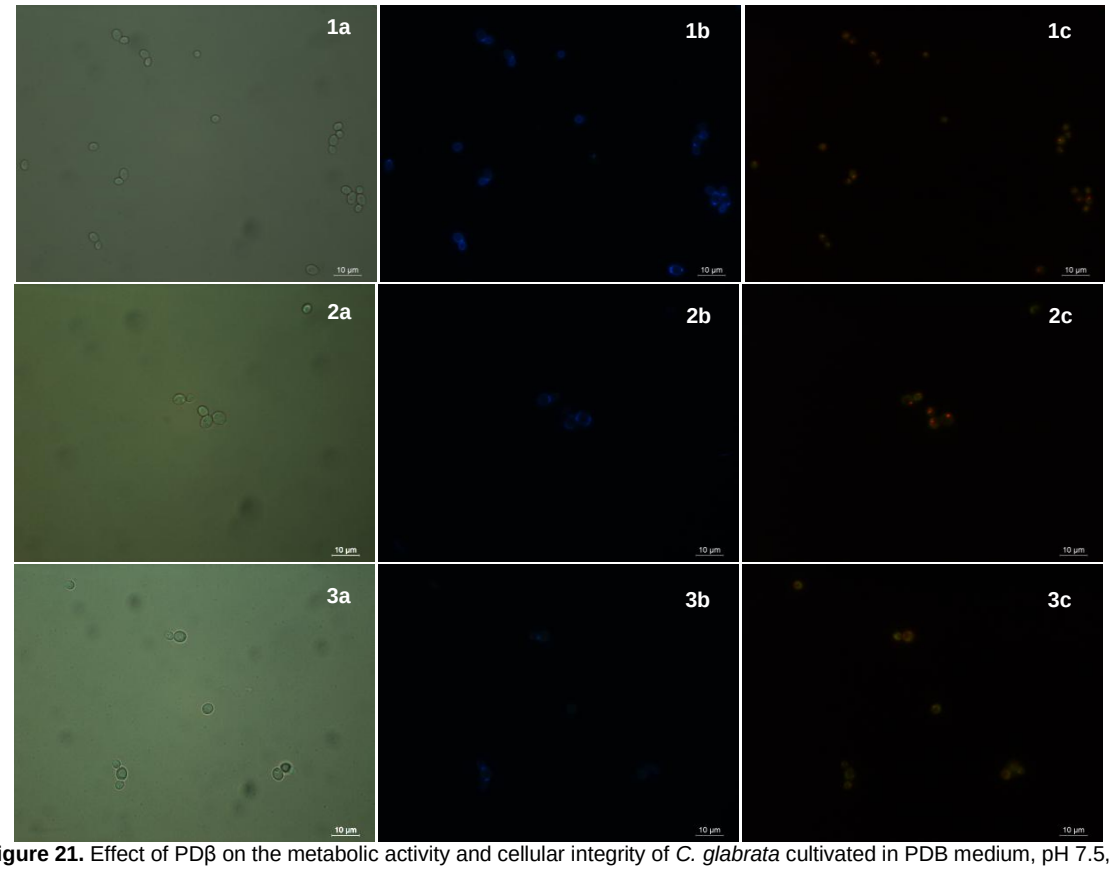

Figure 21. Effect of PDβ on the metabolic activity and cellular integrity of *C. glabrata* cultivated in PDB medium, pH 7.5, at 34°C, without agitation. Samples taken after 24 h of incubation. Concentration of PDβ in the culture medium: 1 – 0 µg/mL, 2 - 37.5 µg/mL, 150 µg/mL. Bar corresponding to 10 µm.

III.9. PDβ localization studies

PDβ was labeled with Fluorescein isothiocyanate (FITC), using the The FluoroTag FITC Conjugation Kit, according to the manufacturer instructions (Sigma-Aldrich, 2010), with the purpose of identifying the location of the cellular targets. FITC, 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 (maximum fluorescence of 519 nm) when excited in the blue region (maximum absorption of 494 nm). After labeled and purified, PDβ was added to the cell culture. For control purposes, the fraction contained the unbounded dye was also added to the cell suspension and kept under the same conditions. Cells incubated with labeled PDβ 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.

According to the protocol, an alkaline medium is required for the proper binding of FITC to PDβ, and according to the manufacturer instructions, the protein needs to be ressuspended in a sodium carbonate buffer, pH 9.0. However, when PDβ was ressuspended in this buffer, it completely lost its activity and was no longer able to cause cellular growth inhibition. There are no reports in the

literature that corroborate these results, so more studies are needed in order to better understand this result and to overcome this obstacle.

III.10. Immunofluorescence

Once the previous method to determine the location of PDβ revealed impracticable, another method was used for the same purpose: secundary (indirect) immunofluorescence. Immunofluorescence allows the visualization of an antigen-antibody interaction in cell suspensions. *C. glabrata* ISA2163 was incubated with the higher inhibitory concentration of PDβ previously tested (150 µg/mL) for 24 h and then the cell suspension was heat-fixed on glass slides. PDβ acts as an antigen whereas a first antibody anti-β-conglutin produced in rabbit was added, followed by a second antibody anti-rabbit produced in goat conjugated with FITC. In order to investigate the cell wall integrity, calcofluor white was also added to the slide.

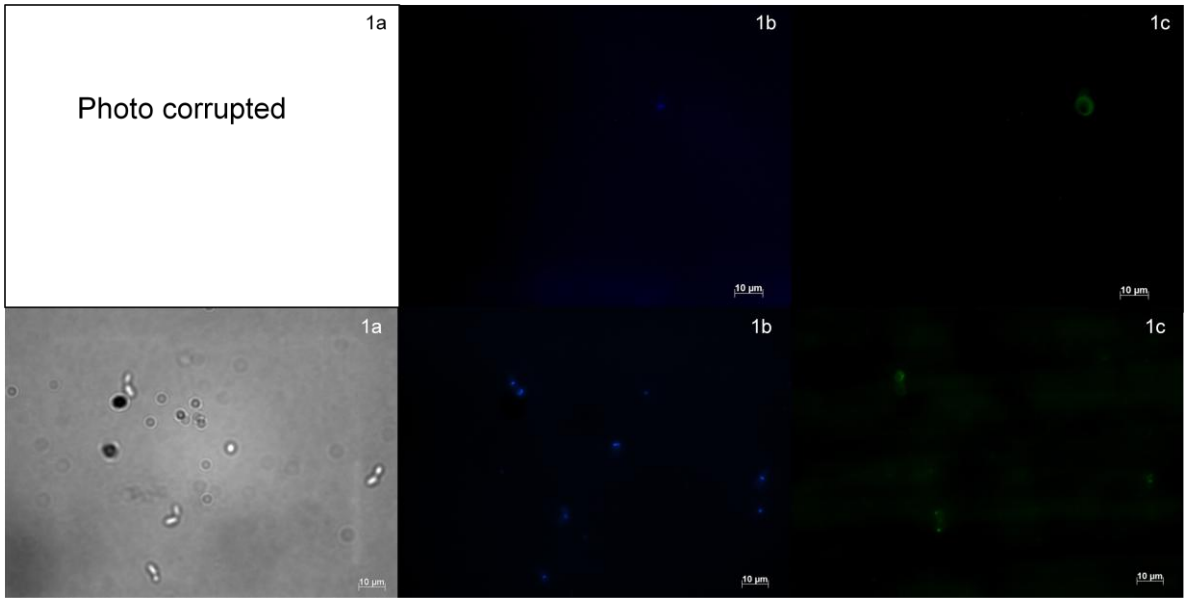

Figure 22. Immunofluorescence in *C. glabrata* ISA2163 incubated with PDβ for 24 h. PDβ functions as an antigen; first antibody anti-BLAD produced in rabbit; second antibody anti-rabbit produced in goat, conjugated with FITC. Bright field microscopy (a), DAPI filter (b) and FITC filter (c). Bar corresponding to 10 µm. The first photo corresponding to the bright field microscopy was corrupted being unable to show.

Figure 22 suggests that the activity of PDβ involves binding to the cell wall, since it is possible to observe a green fluorescence around the cells, corresponding to PDβ bounded to *the* cell wall, and not entering into the cell, after 24 h incubation. Regarding the staining with calcofluor white, the results corroborate those obtained in the growth curves experiments, which showed a destabilization of the cell wall resulting in an almost absence of cells stained with calcofluor white.

Like PDβ, there are other reports of antifungal proteins with the same mode of action, like the defensin RsAFP2 (Thevissen, *et al*., 2012). RsAFP2 bounds specifically to the cell wall of *C. albicans,* acting on a compound that does not exist in other yeast species, sphingolipid

glucosylceramide (GlcCer), and it does not enter into the cell. Furthermore, there are other reports of proteins with antifungal activity that act only on the surface of the cells without entering into the cytoplasm (Sass, *et al*., 2010; Schmitt, *et al*., 2010; Schneider, *et al*.,2010). As suggested previous, it is possible to conclude that PDβ somehow interferes with the integrity of the cell wall, being able to perform damages and destabilizing it. These damages in the cell wall are sufficiently harsh to block *C. glabrata* ISA 2163 cell multiplication.

IV. General conclusions

This work had three major goals: the biochemical characterization of PDβ oligomer and the understanding of the metabolic route involved in the β-conglutin degradation process; the characterization of PDβ stability and antifungal activity against an array of different species and finally, the attempt to understand PDβ mode of action by assessing their physiological and morphological effects on *C. glabrata.*

From the results obtained, it was possible to conclude that the degradation of β-conglutin 0 days into PDβ and its antifungal activity occurs gradually during the period of 7 days at 25°C and remains stable for at least 1 month. The degradation leads to a progressive accumulation of a 20 kDa polypeptide and a decrease of the polypeptides with higher molecular masses, which resembles the catabolism that naturally occurs in the *L. albus* cotyledons (Monteiro, *et al.*, 2010). However, whereas in the *L. albus* cotyledons the catabolism continues to occur, until day 14 of germination, originating almost exclusively a 20 kDa polypeptide, named Blad, that finally declines until complete disappearance, in this case, the process ends, suggesting that there is a missing piece (probably a missing enzyme) for further degradation. This process or the molecule necessary for it is both sensible to high temperatures and reducing environments. At high temperatures the degradation process is clogged which means that, probably, the trigger agent becomes unviable or inactive. A reducing environment, with the addition of 100 mM DTT, strongly interferes with the molecule or the process involved in the degradation and even has the ability to block this process after it had already started. Given these data, the hypothesis of a protease involved in the process that could be inhibited by a reducing agent emerged. With that in mind, various proteases inhibitors were added to the reaction medium (E-64, Pepstatin, EDTA and Pefabloc) to observe if one of them had the capability to prevent the degradation process. Only the addition of EDTA caused the inhibition of the protease, preventing the degradation pathway to proceed. This allowed us to assume that at least one of the molecules involved in the degradation process is a protease belonging to the class of metalloproteinases. The presence of a metalloproteinase was confirmed by a zymography gel, where was visualized that at early stages (until day 1) the metalloproteinase activity is absent or has a very low activity. The activity is only visible after the initiation of β-conglutin degradation, increasing when β-conglutin polypeptide pattern begins to change and remaining active when PDβ is already formed. This suggests that when β-conglutin is purified from dry seeds the metalloproteinase is inactive, being necessary an activator in order to the degradation process to be initiated. In a molecular exclusion chromatography analysis, is only visible one peak with 210 kDa that constitutes both the β-conglutin sample and later the PDβ. This result suggests that, probably, a subunit of β-conglutin oligomer with proteolytic activity, which needs to pass through an activation process, is the molecule responsible for the catabolic process. However, other sources of the protease could be envisaged, like a by-product from a contamination with a a bacteria. To eliminate the contamination hypothesis, several different types of sterile filters were tested along with ampicillin. The results obtained clearly confirmed that the source of the protease is not from a bacterial contamination, since β-conglutin was converted into PDβ even after filtered in 0.22 μm and with addition of ampicillin. However, from the filter testing other important observations were made. We discovered that the starting molecule or the activator becomes trapped

in a hydrophilic matrix since no degradation occurred when β-conglutin was filtered in hydrophilic filters. Despite the several attempts that were made to detach the molecule from the matrix filter no positive result or identification was achieved. In a final effort a new zymography electrophoresis, with the total vicilins from the dry seed as substrate, was made. This experiment had as a goal to isolate the bands with catalytic activity from the gel and send them for sequenciation aiming at the identification of the metalloproteinase. However, no identification of the metalloproteinase was achieved.

To pursuit the second major objective of this thesis, a general screening was made in three groups of organisms; yeasts, filamentous fungi and bacteria, in order to determine the general activity of PDβ. Two groups of yeasts species were tested in this screening: pathogenic and food spoilage yeasts. In several species of both groups the activity tests with PDβ showed that, when it has the ability to inhibit the cellular growth it also has the ability to kill 99.99% of the cells. The species that were found to be more susceptible are: *C. glabrata, C. krusei, C. neoformans, C. pomicola, C. stellimalicola, I. occidentalis, I. orientalis ,L. thermotolerans, P. fermentans, P. kluyveri and S. cerevisiae.* However, PDβ was unable to inhibit the growth of others yeasts species tested in the range of concentrations used. Positive results were also obtained with some of the phytopathogenic filamentous fungi tested, such as *Alternaria alternata, Botrytis cinerea, Colletotrichum acutatum and Colletotrichum gloeosporioide.* In the case of bacteria these preliminary studies indicate that probably PDβ does not seem to be bactericidal, since no growth inhibition was detected in the range of concentrations used.

From the screening results it was observed that *C. glabrata* ISA 2163 was one of the most susceptible species to PDβ. Given its growing importance as human pathogen this specie was selected to proceed the work. It was also confirmed that this susceptibility seems to be an intra-specific feature since a total of six different strains showed a similar behavior. To evaluate the physiological and morphological effects on *C. glabrata* ISA 2163 and using as golden standard two of the most common antifungal drugs used in the treatment of fungal infections -itraconazole and amphotericin B- PDβ MICs and MFCs were determined. The comparison of the MICs and MFCs values obtained for the three drugs showed that despite the doses of PDβ required to induce growth inhibition seemed to be higher than those from both itraconazole and amphotericin B, its molecular weight is also substantially higher (210 kDa), which means that the number of molecules necessary to induce the same inhibitory effect is quite similar between these drugs. To perform the growth curves due to an increase in the inoculum size, was necessary to readjust PDβ concentration. As so, new MICs and MFCs were determined with an inoculum size of 10^5 CFU/mL, for both PDβ and amphotericin B being respectively, 37.5 and 0.25 µg/mL for MIC and 150 and 2 µg/mL for MFC. Due to the inoculum size increase PDβ lost the capability to induce 99.99% cellular death, within the range of concentration tested. Therefore, the minimum fungicidal concentration of PDβ was considered, for further testing, as the concentration responsible for causing 90% cell death.

To elucidate the effect of PDβ in the growth of *C.glabrata* ISA 2163, studies in PDB medium on the viability and cellular integrity were performed . From the growth curves it was possible to see that the addition of both concentrations of PDβ (at the MIC and MFC) to the culture medium had an

intense effect on the growth of *C. glabrata* ISA 2163 showing a high decrease in the growth rate, when compared to the control fraction, resulting in lower optical density readings and in lower CFU/mL counts. This confirms that these concentrations have the capability to inhibit the cellular growth of the strain tested. Along with the growth curves it was evaluated the viability and cellular integrity of *C. glabrata* ISA 2163, by staining with FUN-1 and calcofluor white. During the first 4 h of incubation with PDβ there are no changes in the viability and integrity of the cells, in all conditions tested, since the presence of CIVS indicates metabolic activity and the fluorescence with calcofluor white is normal and equal to the control fraction, indicating cell wall integrity. However, after 8 hours of incubation with both concentrations of PDβ, occurred a turning point in the cell wall integrity since it was possible to see a decrease in the labeling with calcofluor white. After 24 h of incubation most of the cells incubated with PDβ are still metabolically active at this point. However, contrary to the control fraction, it appears that they are no longer able to multiplicate, giving the stabilization of the OD_{640nm} and CFU counts. In addition, the fractions that were exposed to PDβ presented gaps in the calcofluor white labeling, probably by a loss in the cell wall integrity. In the control fraction no changes could be observed. Given these results, an immunofluorescence assay was conducted in order to observe if the loss of wall integrity came as a result of PDβ binding to the cell wall. The results showed that the activity of PDβ probably involves binding to the cell wall since it was only possible to observe a green fluorescence around the cells, corresponding to PDβ bounded to the cell wall, but not inside of the cell, after 24 h incubation. Compiling all results obtained so far it is possible to conclude that PDβ interferes with the integrity of the cell wall damaging and destabilizing it, being these changes sufficiently severe to prevent *C. glabrata* ISA 2163 multiplication

. In the future, more studies have to be done in order to fully understand the mechanism of degradation from β-conglutin to PDβ, and to identify the specific metalloproteinase and its activator. In addition, it is necessary to better understand PDβ mode of action including the targets that are involved in the coupling of PDβ to cell wall of *C. glabrata* ISA 2163.

V. References

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VI. Annex

Annex I. Protein identification by mass spectra (MS) peptide mapping and sequencing analysis.

Sample name	Protein found in database	Entry name	Calculated MW	Score	Seq. cov.	Note
MET 1	No identification		0	0	0%	
MET 2	beta-conglutin precursor [Lupinus albus]	gi|46451223	62092	1050	63%	
BLAD	BLAD [Lupinus albus]	gi|77994351	20393	824	91%	

MET 2 - β-conglutin

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  1 MGKMRVRFPT LVLVLGIVFL MAVSIGIAYG EKDVLKSHER PEEREQEEWQ
 51 PRRQRPQSRR EEREQEQEQG SPSYPRRQSG YERRQYHERS EQREEREQEQ
101 QQGSPSYSRR QRNPYHFSSQ RFQTLYKNRN GKIRVLERFD QRTNRLENLQ
151 NYRIVEFQSK PNTLILPKHS DADYVLVVLN GRATITIVNP DRRQAYNLEY
201 GDALRIPAGS TSYILNPDDN QKLRVVFKLAI PINNPGYFYD FYPSSTKDQQ
251 SYFSGFSRNT LEATFNTRYE EIQRIILGNE DEQEYEEQRR GQEQSDQDEG
301 VIVIVSKKQI QKLTKHAQSS SGKDKPSDSG PFNLRSNEPI YSNKYGNFYE
351 ITPDRNPQVQ DLNISLTYIK INEGALLLPH YNSKAIYVVV VDEGEGNYEL
401 VGIRDQQRQQ DEQEEKEEEV IRYSARLSEG DIFVIPAGYP ISINASSNLR
451 LLGFGINADE NQRNFLAGSK DNVIRQLDRA VNELTFPGSA EDIERLIKNQ
501 QQSYFANGQP QQQQQQQSEK EGRRGRRGSS LPF
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Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
33 - 44	1494.78	1493.77	1493.76	10	1	K.DVLKSHERPEER.E
111 - 121	1419.70	1418.70	1418.68	11	1	R.QRNPYHFSSQR.F
113 - 121	**1135.54**	**1134.53**	**1134.52**	**7**	**0**	**R.NPYHFSSQR.F (Ions score 58)**
122 - 127	799.44	798.44	798.43	12	0	R.FQTLYK.N
133 - 138	785.51	784.50	784.49	8	1	K.IRVLER.F
135 - 142	1062.57	1061.57	1061.56	4	1	R.VLERFDQR.T
143 - 153	**1420.73**	**1419.72**	**1419.72**	**-1**	**1**	**R.TNRLENLQNYR.I (Ions score 24)**
146 - 153	1049.54	1048.53	1048.53	0	0	R.LENLQNYR.I
154 - 168	1727.00	1725.99	1726.00	-7	0	R.IVEFQSKPNTLILPK.H
169 - 182	**1557.81**	**1556.80**	**1556.79**	**6**	**0**	**K.HSDADYVLVVLNGR.A (Ions score 89)**
183 - 193	**1255.72**	**1254.71**	**1254.70**	**4**	**1**	**R.ATITIVNPDRR.Q (Ions score 28)**
194 - 205	1412.68	1411.67	1411.67	-1	0	R.QAYNLEYGDALR.I
194 - 222	3226.67	3225.66	3225.56	33	1	R.QAYNLEYGDALRIPAGSTSYILNPDDNQK.L
206 - 224	2102.09	2101.09	2101.08	2	1	R.IPAGSTSYILNPDDNQKLR.V
228 - 247	2307.11	2306.10	2306.13	-10	0	K.LAIPINNPGYFYDFYPSSTK.D
248 - 258	**1321.58**	**1320.58**	**1320.57**	**3**	**0**	**K.DQQSYFSGFSR.N (Ions score 82)**
259 - 268	**1166.59**	**1165.58**	**1165.57**	**7**	**0**	**R.NTLEATFNTR.Y (Ions score 44)**
259 - 274	**1984.98**	**1983.97**	**1983.97**	**4**	**1**	**R.NTLEATFNTRYEEIQR.I (Ions score 65)**
269 - 274	837.43	836.42	836.40	18	0	R.YEEIQR.I
275 - 289	**1864.88**	**1863.87**	**1863.85**	**10**	**0**	**R.IILGNEDEQEYEEQR.R (Ions score 83)**
275 - 290	2020.96	2019.96	2019.95	3	1	R.IILGNEDEQEYEEQRR.G
316 - 335	**2115.03**	**2114.02**	**2114.01**	**2**	**1**	**K.HAQSSSGKDKPSDSGPFNLR.S (Ions score 72)**
324 - 335	**1332.69**	**1331.68**	**1331.65**	**27**	**0**	**K.DKPSDSGPFNLR.S (Ions score 38)**
336 - 344	1051.51	1050.51	1050.50	7	0	R.SNEPIYSNK.Y
345 - 355	1374.65	1373.64	1373.63	11	0	K.YGNFYEITPDR.N
371 - 384	**1568.85**	**1567.84**	**1567.84**	**3**	**0**	**K.INEGALLLPHYNSK.A (Ions score 28)**
385 - 408	2721.38	2720.37	2720.38	-1	1	K.AIYVVVVDEGEGNYELVGIRDQQR.Q
409 - 422	1788.84	1787.83	1787.82	6	1	R.QQDEQEEKEEEVIR.Y
427 - 450	2533.32	2532.32	2532.32	-3	0	R.LSEGDIFVIPAGYPISINASSNLR.L
451 - 463	**1446.75**	**1445.74**	**1445.73**	**11**	**0**	**R.LLGFGINADENQR.N (Ions score 75)**
464 - 475	1333.70	1332.70	1332.72	-14	1	R.NFLAGSKDNVIR.Q
480 - 495	**1747.86**	**1746.85**	**1746.84**	**4**	**0**	**R.AVNELTFPGSAEDIER.L (Ions score 62)**

BLAD

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  1 RRQRNPYHFS SQRFQTLYKN RNGKIRVLER FDQRTNRLEN LQNYRIVEFQ
 51 SKPNTLILPK HSDADYVLVV LNGRATITIV NPDRRQAYNL EYGDALRIPA
101 GSTSYILNPD DNQKLRVVFK LAIPINNPGYF YDFYPSSTKD QQSYFSGFSR
151 NTLEATFNTR YEEIQRIILG NED
```

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
3 - 13	1419.66	1418.66	1418.68	-17	1	R.QRNPYHFSSQR.F
5 - 13	**1135.52**	**1134.52**	**1134.52**	**-4**	**0**	**R.NPYHFSSQR.F (Ions score 60)**
14 - 19	799.44	798.43	798.43	4	0	R.FQTLYK.N
14 - 21	1069.57	1068.56	1068.57	-10	1	R.FQTLYKNR.N
25 - 30	785.50	784.50	784.49	5	1	K.IRVLER.F
27 - 34	**1062.56**	**1061.56**	**1061.56**	**-5**	**1**	**R.VLERFDQR.T (Ions score 14)**
35 - 45	**1420.73**	**1419.72**	**1419.72**	**-3**	**1**	**R.TNRLENLQNYR.I (Ions score 26)**
38 - 45	**1049.53**	**1048.52**	**1048.53**	**-8**	**0**	**R.LENLQNYR.I (Ions score 5)**
46 - 60	**1726.99**	**1725.98**	**1726.00**	**-14**	**0**	**R.IVEFQSKPNTLILPK.H (Ions score 54)**
61 - 74	**1557.80**	**1556.79**	**1556.79**	**-2**	**0**	**K.HSDADYVLVVLNGR.A (Ions score 95)**
75 - 84	1099.60	1098.60	1098.60	-7	0	R.ATITIVNPDR.R
75 - 85	**1255.70**	**1254.69**	**1254.70**	**-8**	**1**	**R.ATITIVNPDRR.Q (Ions score 41)**
85 - 97	1568.77	1567.77	1567.77	-6	1	R.RQAYNLEYGDALR.I
86 - 97	1412.65	1411.64	1411.67	-23	0	R.QAYNLEYGDALR.I
86 - 114	3226.52	3225.51	3225.56	-14	1	R.QAYNLEYGDALRIPAGSTSYILNPDDNQK.L
98 - 114	1832.87	1831.87	1831.90	-16	0	R.IPAGSTSYILNPDDNQK.L
98 - 116	**2102.07**	**2101.06**	**2101.08**	**-8**	**1**	**R.IPAGSTSYILNPDDNQKLR.V (Ions score 66)**
120 - 139	2307.07	2306.07	2306.13	-26	0	K.LAIPINNPGYFYDFYPSSTK.D
120 - 150	3609.76	3608.75	3608.69	17	1	K.LAIPINNPGYFYDFYPSSTKDQQSYFSGFSR.N
140 - 150	**1321.57**	**1320.56**	**1320.57**	**-11**	**0**	**K.DQQSYFSGFSR.N (Ions score 85)**
151 - 160	**1166.57**	**1165.57**	**1165.57**	**-5**	**0**	**R.NTLEATFNTR.Y (Ions score 59)**
151 - 166	**1984.97**	**1983.97**	**1983.97**	**1**	**1**	**R.NTLEATFNTRYEEIQR.I (Ions score 56)**
161 - 166	837.42	836.41	836.40	10	0	R.YEEIQR.I