Host-induced changes in the cell surface *N*-linked glycoproteins, from *Aspergillus fumigatus.* Search for specific targets with potential for clinical therapy and/or diagnosis.

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This dissertation is presented to obtain a Master degree in Structural and Functional Biochemistry

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This dissertation was carried out at the Plant Physiology Laboratory at the Department of Natural Resources, Environment and Territory (DRAT), Instituto Superior de Agronomia of Universidade Técnica de Lisboa.

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Resumo

O fungo *Aspergillus fumigatus* é responsável por causar aspergilose invasiva pulmonar, uma doença fatal em pacientes imunodeprimidos. O desenvolvimento deste tipo de doenças está, geralmente, associado a uma resposta imunológica deficiente no hospedeiro, mas também, a alterações fenotípicas a nível celular do próprio fungo. A sequenciação do genoma completo do fungo, veio permitir o estudo do proteoma e dos seus constituintes, tornando possível explicar o porquê de tais alterações.

O conjunto das diferentes estruturas de natureza glicídica, os oligossacáridos, que constituem as glicoproteínas e glicolípidos presentes na membrana celular dos organismos, através de ligações *N*- e *O*-glicosídicas, são definidos como exoglicoma. Em estudos realizados observou-se que as alterações que ocorrem no exoglicoma celular de *A. fumigatus* são as principais causas do seu potencial infecioso. Como tal, a sua identificação e caracterização é essencial, de forma a aumentar o conhecimento do comportamento patogénico do fungo. Neste trabalho, foram utilizadas diversas técnicas experimentais, desenvolvidas em proteómica e glicómica, numa tentativa de identificar os principais componentes do proteoma da membrana celular de *A. fumigatus,* como também a estrutura dos *N*-oligossacáridos que constituem o exoglicoma do fungo. Foram utilizados dois métodos de deteção de glicoproteínas, que têm como base a ligação de lectinas a oligossacáridos específicos e a oxidação dos grupos glicosídicos, seguida da conjugação com um substrato cromogénico ou marcado. Numa tentativa de identificar as glicoproteínas que compõem o proteoma recorreu-se à espectrometria de massa, sendo, no entanto, os resultados inconclusivos. Certos factores, como a ausência de homologia entre os péptidos sequenciados das proteínas de membrana analisadas, com sequências proteicas já descritas, em *A. fumigatus*, foram avaliados e questionados, mas ainda sem resposta que suporte uma identificação.

Palavras-chave: *Aspergillus fumigatus*, exoglicoma, lectina, glicoproteínas, *N*-oligossacáridos

Abstract

The fungus *Aspergillus fumigatus* is responsible for causing invasive aspergillosis in human lungs, a fatal disease in immunocompromised patients. The development of such diseases is typically associated with a deficient immune response in the host as well as with phenotypic changes at cellular level of the fungus itself. The sequencing of the fungal genome has allowed the study of the proteome and its constituents, making it possible to explain the reason to such changes.

The collection of carbohydrate moieties present in *N*- and *O*-linked glycoproteins and glycolipids, which protrude outwards from the cell membrane, has been defined as the exoglycome. Furthermore, studies have demonstrated that changes suffered by the exoglycome of *A. fumigatus* are the main cause of the fungus infectious potential. Therefore, identification and characterization of the different carbohydrate structures that comprise the fungal exoglycome has become of great importance in order to increase the knowledge of the fungus pathogenicity.

In this study, several experimental techniques developed in proteomics and glycomics areas were used in an attempt to identify the main components of the cell membrane proteome of *A. fumigatus* as well as the *N*-linked oligosaccharides structure that comprise the fungal exoglycome. Two methods for glycoprotein detection were used that are based in the non-covalent binding of lectins to specific oligosaccharides and the oxidation of carbohydrate groups followed by conjugation with a chromogenic or tagged substrate. In an attempt to identify the glycoproteins that comprise the proteome was performed, mass spectrometry was used, however the results were inconclusive. Certain factors, such as lack of homology between the sequenced peptides from membrane proteins with protein sequences already described in the databases were evaluated and questioned, but with no conclusive answers.

Keywords: *Aspergillus fumigatus*, exoglycome, lectin, glycoproteins, *N*-linked oligosaccharides

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List of Abbreviations

1DE	One-dimensional electrophoresis
2DE	Two-dimensional electrophoresis
AMS	α-Mannosidase
Asn	Asparagine
ATP	Adenosine triphosphate
Con A	Concanavalin A
DCPIP	Dichlorophenolindophenol
DMF	*N,N*-Dimethylformamide
DTT	1,4-Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
Flu1P	Fluconazole resistance protein
G6PT	Glucose-6-phosphate transporter
GalNAc	*N*-Acetylgalactosamine
GlcNAc	*N*-Acetylglucosamine
Glut4	Glucose transporter
GPI	Glycosylphosphatidylinositol
H1N1	Influenza A virus
H5N1	Influenza A virus subtype
IA	Invasive Aspergillosis
IDPase	Inosine diphosphatase
IEF	Isoelectric focusing
kDa	Kilo Dalton
IPATIMUP	Institute of Molecular Pathology and Immunology of the University of Porto
MAL	*Maackia amurensis* lectin
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization – time-of-flight – mass spectrometer
MFS	Major facilitator superfamily
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

mV	Mili Volts
NADPH	Nicotinamide adenine dinucleotide phosphate
PAMPs	Pathogen-associated molecular patterns
pI	Isoelectric point
Pi	Inorganic phosphate
PMF	Peptide Mass Fingerprint
PMSF	Phenylmethylsulfonyl fluoride
PNA	Peanut agglutinin
PRRs	Pattern-recognition receptors
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Reducing polyacrylamide gel electrophoresis
Ser	Serine
TCA	Trichloroacetic acid
Thr	Threonine
TLRs	Toll-like receptors
UEA	*Ulex europaeus agglutinin*
UniProtKB	UniProt Knowledgbase
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight

1. Introduction

1.1 *Aspergillus fumigatus*

The genus *Aspergillus*, which includes almost 200 species, has a tremendous impact on public health, either beneficial as the workhorse of industrial applications and detrimental as plant and human pathogens (Dagenais and Keller, 2009).

The saprophyte *Aspergillus fumigatus* (Figure 1.1) has emerged as one of the most critical fungal pathogens in distinct clinical settings, being a major threat to the immunocompromised individual. *A. fumigatus* can be found worldwide and plays an important role in recycling carbon and nitrogen. Its natural ecological niche is the soil, wherein it survives and grows on organic debris (Latgé, 1999). This fungal species has a very simple biological cycle, one characteristic of which is its high sporulating capacity, which results in the ubiquitous presence of large numbers of conidia (1 to 100 conidia/m^3) in the air indoors and outdoors (Latgé, 2001).

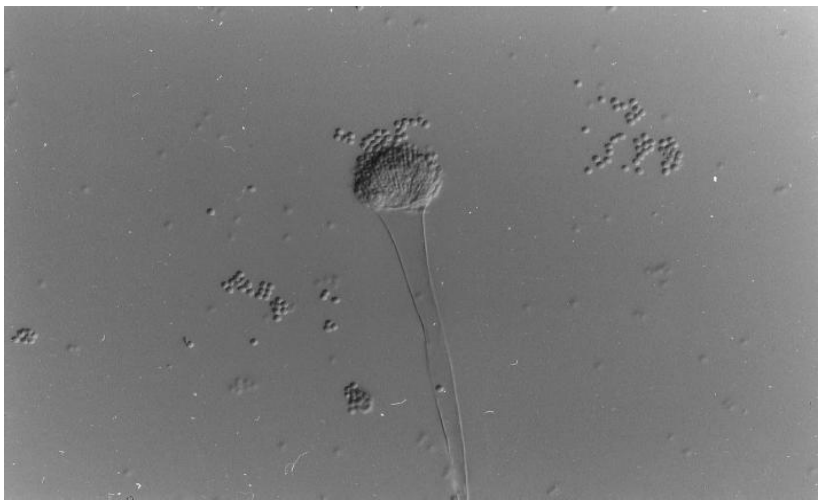


Fig. 1.1 – Light microscopy of typical *A. fumigatus* sporulating structures (Latgé, 1999).

The conidia released into the atmosphere have a diameter small enough (2 to 3 µm) to reach the lung alveoli (Latgé, 1999) and their dissemination simply relies on disturbances of the environment and strong air currents. In fact, *A. fumigatus* conidia are constantly inhaled by humans but rarely cause adverse effects, owing to a very successful response by the innate immunological system of healthy individuals. For most patients, disease occurs predominantly in the lungs, although dissemination to almost any organ may occur in the most severely predisposed. When a conidium overcomes the immune defense mechanisms, it germinates and produces a branched, septate vegetative mycelium that invades the lung tissues. The virulence of *A. fumigatus* can then be caused either by the production of fungal proteins that promote mycelia growth into the lung parenchyma and/or by structural features of the conidia which confer resistance to the host's antifungal mechanisms (Latgé, 2001). A compromised immune system due to immunosuppressive therapies and congenital defects may lead to the

development of invasive aspergillosis (IA) in experimental models, as well as in naturally occurring human infections. Under these conditions, the biological characteristics of the fungus (a fast-growing thermophillic species with small sized conidia and no specific nutritional requirements) may be sufficient for infection (Latgé, 2001).

In the past 10 to 20 years, the pathogenicity of this fungus has suffered several alterations due to the increasing number of immunosupressed patients and the severity of immunosupressing therapies. In spite of a pronounced increase in the incidence of IA, the pathogenicity of *A. fumigatus* is still poorly understood. Due to the unique nature of its infection process, understanding its molecular basis, in particular the host defense reactions might be the key to develop new therapies effective on immunocompromised hosts.

1.2 Pathogenesis of *Aspergillus fumigatus*

Of all human pathogenic species of *Aspergillus*, *A. fumigatus* is the major causative agent of human infections, followed by *A. flavus*, *A. terreus*, *A. niger* and the model organism, *A. nidulans* (Dagenais and Keller, 2009). *Aspergillus* species are exogenous fungi, which can colonize the respiratory mucosa of patients with underlying localized bronchopulmonary disorders, such as healed tuberculous cavities, bronchiectasis, and cystic fibrosis, as well as occasionally invading the respiratory mucosa regardless of systemic immunological conditions (Amitani and Kawanami, 2009). The noninvasive aspergilloma may arise from repeated exposure of the host to conidia as well as from pre-existent pulmonary cavities, as healed lesions on patients that have suffered from tuberculosis. IA is the most critical disease caused by this fungus on immunocompromised patients. The most susceptible individuals to this life-threatening disease are individuals with hematological malignancies such as leukemia; solid-organ and hematopoietic stem cell transplant patients; patients on prolonged corticosteroid therapy, which is commonly utilized for the prevention and/or treatment of graft-versus-host disease in transplant patients; individuals with genetic immunodeficiencies such as chronic granulomatous disease; and individuals infected with human immunodeficiency virus (Dagenais and Keller, 2009). Mortality rates range from 40% to 90% in high-risk populations and are dependent on factors such as host immune status, the site of infection and the treatment regimen applied (Lin and Teutsch, 2001).

Studying *A. fumigatus* molecules involved in virulence has been hampered by the lack of an identifiable sexual cycle, limiting experimental approaches to classical genetic analyses (Hohl and Feldmesser, 2007). Completion of the fungus genome sequencing accelerated gene structure and function studies, and allowed comparative genomic analyses with other sequenced *Aspergillus* species (*A. oryzae* and *A. nidulans*), as well as with other genera of pathogenic (e.g. *Candida albicans* and *Cryptococcus neoformans*) and nonpathogenic (e.g.

Saccharomyces cerevisiae) fungi. These studies showed that *A. fumigatus* does not share a common set of genes with other fungal pathogens (Hohl and Feldmesser, 2007).

1.2.1 Establishment of the disease

The primarily way to a human fungal infection is through conidia inhalation, followed by their deposition on bronchioles or alveolar spaces. On healthy individuals, *A. fumigatus* conidia that do not endure mucociliary removal encounter epithelial cells or alveolar macrophages, responsible for phagocytosis and elimination of conidia, as well as for primary inflammatory response initiation; this process recruits neutrophils to the site of infection. Fungal attributes, which allow the survival and growth of *A. fumigatus* within the host, together with a dysfunction on host defenses, increases the risk to develop IA. As conidia start developing, the factors which influence hyphal growth rate and resistance to infection mechanisms, invasion and dissemination, as well as secondary metabolite production, have a decisive effect upon disease establishment. However, these factors are secondary to the immune status of the host.

As mentioned before, there are two main pulmonary sites for infection: the lung epithelium and the alveola. Several observations (Latgé, 2003) suggest that the epithelium can serve as a focus of infection for IA establishment: (a) epithelial cells can engulf conidia which subsequently remain alive intracellularly; (b) the lung epithelium is damaged following immunosuppressive therapies and graft rejection, facilitating binding of conidia to altered or activated epithelial cells; and (c) corticosteroids, a risk factor for IA, reduce the release or efficacy of antimicrobial peptides and proteins constitutively synthesized at epithelial surface cells. With respect to epithelial cells, the soluble antimicrobial compounds that they secrete play a direct role in airway defense. Members of the defensin family of antimicrobial peptides have broad-spectrum activity against multiple microbes and are produced *in vitro* following the incubation of epithelial cells with *A. fumigatus* (Alekseeva *et al.,* 2009; Dagenais and Keller, 2009).

Several fungal derived factors may contribute to the ability of the fungus to bind and modulate the airway epithelium; it is the case, for example, of the presence of sialic acid residues on conidia, which contribute to *A. fumigatus* binding and uptake by epithelial cells (Dagenais and Keller, 2009). Surface-exposed sialic acid residues may be important for conidial dispersal and pulmonary deposition, as sialidase (an enzyme which catalyses the hydrolytic cleavage of glycosidic bonds between sialic acid and a hexose or hexosamine residue at the non-reduced terminal of oligosaccharidic side chains of glycoproteins, glycolipids and proteoglycans) treatment results in conidial agglutination. Adhesion of conidia to fibrinogen, to laminin (a basement membrane glycoprotein), and to fibronectin (an extracellular matrix component) is also partially mediated by specific proteins and sialic acid residues on the conidial surface (Dagenais and Keller, 2009). This interaction between sialic acid residues and fibronectin may be important for the establishment of aspergilloma or invasive disease. A higher density of sialic acid residues on *A. fumigatus* conidia may play a role in the predominance of human disease

associated with *this fungus.* This observation requires further analysis since limited density testing of other aspergilla has been performed (Hohl and Feldmesser, 2007). Removal of sialic acid residues diminishes conidial phagocytosis, but not surface binding, by cultured macrophages and type II pneumocytes (Hohl and Feldmesser, 2007). Therefore, immune function may be enhanced by sialylation, which may also provide a protected reservoir for conidia.

Regarding the alveola, conidia that have not been trapped intracellularly at the epithelial level end up in the alveola where they encounter the main lung phagocytic cell, the alveolar macrophage (Latgé, 2003). Alveolar macrophages are the primary resident phagocytic cells of the respiratory tract and a critical component of the host defense against *Aspergillus* conidia. These cells are able to phagocytize *Aspergillus* conidia in an actin-dependent manner, a process mediated by the recognition of pathogen-associated molecular patterns (PAMPs) by host cell pattern-recognition receptors (PRRs), including toll-like receptors (TLRs) and dectin-1 (a lectin; Dagenais and Keller, 2009). Conidial engulfment is very quick, lasting from 1 to 2 h, and is not affected by the immune status of the host (Latgé, 2003).

In addition to these pathogenic attributes, *A. fumigatus* produces a variety of secondary metabolites, including gliotoxin, which contributes to its pathogenicity, particularly during hyphal growth (Sugui *et al.,* 2007; Dagenais and Keller, 2009). The biological activity of gliotoxin is based on an internal disulfide bridge that can bind and inactivate proteins via a sulfide:thiol exchange, as well as via reactive oxygen species (ROS) produced by redox cycling between the oxidized and reduced forms of the toxin (Dagenais and Keller, 2009).

All the interactions mentioned above between fungal cells and host innate immune system, as well as interactions with the surrounding environment that may occur, rely upon a functional and permeable fungal cell wall. In fact, the initial sensing by the host innate immune system of fungal pathogens is most likely associated with components of the cell wall (Ferreira *et al.,* 2012). Moreover, localization of specific polysaccharides in the outermost part of the fungal cell wall is thought to be one of the first steps in the recognition of fungal pathogens by the host innate immune system (Ferreira *et al.,* 2012).

As previously noted, phagocytosis of *Aspergillus* conidia is believed to be a process mediated by the recognition of PAMPs by host cell PRRs (Dagenais and Keller, 2009) This interaction is crucial to defeat microorganisms as it boosts phagocytosis and cytokine release (Heesemann *et al.,* 2011), triggering a fierce chemical warfare between pathogen and host if pathogenesis is to be established. Therefore, understanding and determining the detailed structure of the fungal cell wall has become of great importance.

1.2.2 The fungal cell wall

All fungal cell walls are critical in maintaining cell shape and integrity in environments that range from the surface of grapes to human tissues, and are highly cross-linked structures, which adapt to highly variable growth conditions in a dynamic and flexible way (Cummings and Doering, 2009). The main structural components of the *A. fumigatus* cell wall are polysaccharides that can be divided in two groups, depending on their solubility in hot alkali (Bernard and Latgé, 2001). The alkali-insoluble fraction is primarily composed by galactomannans, chitin and β-1,3-glucans, and the alkali soluble fraction is composed mainly of α-(1,3)-glucans with some galactomannan (Bernard and Latgé, 2001). Galactomannans are covalently linked to the non-reducing ends of β-1,3/1,4-glucans, and are mainly coated with glycosylphosphatidylinositol (GPI) proteins, which contain *N*- and *O*-glycans derived primarily from the process of glycosylation (Cheng, 2011). Like all other eukaryotes, filamentous fungi possess three types of protein glycosylation, *N*-glycosylation of asparagines (Asn) residues, *O*-glycosylation of threonine (Thr) or serine (Ser) residues, and GPI-anchoring of the C-terminus of some proteins. Glycans are highly efficient vehicles for information storage and their biosynthesis requires a complicated non-template assembly line.

Carbohydrates from fungal cell walls have been used as prime targets to develop new drugs or by the pathogens themselves to their own benefit. For example, the antifungal echinocandins (e.g. caspofungin), target fungal cell wall β-1,3-glucan synthesis and are used clinically to treat yeasts (as *Candida albicans*) and IA (Ferreira *et al.,* 2012). Nevertheless, they do not completely inhibit *in vitro* growth of *A. fumigatus* since they are active only at the tips and branch points of the filaments, where β-1,3-glucan synthesis is most active (Ferreira *et al.,* 2012). Many of these carbohydrates, more specifically oligosaccharides, comprise both the cell wall and the plasma membrane, which protects the cytoplasm from the extracellular milieu. The plasma membrane of fungal pathogens contains many glycoproteins and glycolipids, whose oligosaccharide side-chains, collectively termed the exoglycome (discussed in section 1.3.2), hence excluding cell wall carbohydrates, are projected towards the cell exterior (Ferreira *et al.,* 2012). For this reason, the study of the plasma membrane glycoproteins and glycolipids, and therefore the exoglycome, is of great importance. Unlike pathogen cell wall carbohydrates, the study of the exoglycome has been somewhat 'neglected' as a target for developing effective ways to control fungal diseases. Indeed, only recently have appropriate powerful technological tools (based on mass spectrometry) been developed to a point capable of dealing with the study of this complex area of functional glycomics. Hosts often induce changes in the pathogen cell wall (and, most probably, in its exoglycome) during a host-pathogen interaction, which are used by the parasite to elude and/or circumvent host defenses. Therefore, identification of such host-induced alterations may prove extremely important for both treatment and diagnosis.

1.2.3 Fungal dissemination

A. fumigatus dissemination can occur through the lung and via the bloodstream to other organs. The growing hyphae that escape host defenses may invade the endothelial cell lining of blood vessels to gain access to the vasculature. Hyphal invasion occurs from the abluminal side to the luminal side of endothelial cells, inducing endothelial cell activation but little cell damage (Kamai *et al.,* 2006; Dagenais and Keller, 2009). Hyphal fragments can break off into the bloodstream and invade the endothelium at other sites, resulting in hematogenously disseminated disease (Dagenais and Keller, 2009).

1.3 The fungal plasma membrane

The fungal plasma membrane is the target for the largest group of antifungal and antimicrobial proteins (Theis and Stahl, 2004). Over 500 naturally occurring proteins have been reported that are believed to interact with lipid components of the plasma membrane, leading to pore formation, efflux of cellular components, and changes in the membrane potential (Tossi *et al.,* 2000).

The main function of the plasma membrane is to define the permeability barrier of cells and to serve as a matrix for proteins involved in a variety of cell functions such as energy targeting, signal transduction, solute transport, DNA replication, secretion, etc (Theis and Stahl, 2004). The cell membrane is formed by a lipid bilayer composed of sphingolipids, phospholipids, sterols and proteins. The plasma membrane proteins exhibit an enormous diversity of structures but share at least two common features (Ferreira *et al.,* 2012): a positive net charge under physiological conditions, which promotes interaction with negatively charged microbial surfaces; and an amphipathic structure that allows incorporation into pathogen membranes.

The fungal plasma membrane differs from those of higher eukaryotes with respect to embedded sterols (Theis and Stahl, 2004): cholesterol (zoosterols), campesterol, sitosterol, and stigmasterol (considered phytosterols) or ergosterol (present in the cell membrane of fungi). Ergosterol, and the ergosterol pathway, is reported to be the target to most antifungal agents currently used for clinical treatment of fungal infections.

Furthermore, membrane-acting antifungal and antimicrobial proteins have been shown to interact with phospho- and sphingolipids, which make up the major part of the plasma membrane.

1.3.1 Membrane proteins

In *A. fumigatus*, a large number of proteins that play important roles in cell survival and invasion are located in plasma membrane, endoplasmic reticulum, Golgi membrane, and other membrane systems (as mitochondria and vesicle-vacuole) (Ouyang *et al.,* 2010).

Regardless of being located in the same type of cellular structure, membrane proteins exhibit different functions and compositions. Each biological membrane has a phospholipid bilayer and

attached to it is a variety of membrane proteins which enable efficient cell performance. Membrane proteins vary according to the type of cell and their subcellular location. Moreover, they can be classified as integral or peripheral, and are organized in different domains. Extramembrane domains are usually involved in the signaling pathway of cells or in cell-cell interactions. On the other hand, intramembrane domains facilitate molecule movement through the membrane, by channel formation. Furthermore, there are domains close to the intracellular membrane layer which display a broad spectrum of functions, such as structural protein anchoring to the membrane to engage intracellular signaling pathways. In many cases, membrane protein functions and their polypeptide chain topology can be predicted based on homology between these proteins and other well characterized membrane proteins.

Integral membrane proteins are characterized by a hydrophobic domain which interacts directly with the hydrophobic core of the lipid bilayer. Non ionic detergents are widely used in solubilization and characterization of these proteins (Bordier, 1981). These non ionic detergents (as Triton X-100, Triton X-114, Brij 35, Brij 58, Tween 20, Tween 80, Nonidet P-40, octyl β-glucoside and Mega 8) have a hydrophilic head, free of electric charge, and are used to shatter lipid-lipid or lipid-protein interactions. They also possess a limited capacity to break interactions between proteins and are usually used to isolate active membrane proteins; also, they are often referred to as non-denaturing detergents. During protein solubilization, these detergents substitute a large amount of lipid molecules that are in direct contact with the hydrophobic domain of membrane proteins, leading to the formation of micelles – globular structures formed by an aggregate of surfactant molecules. In contrast to integral membrane proteins, which possess amphiphilic structures, hydrophilic proteins show little or no interaction with non-ionic detergents and their physico-chemical properties are not influenced by the presence of such detergents in solution (Bordier, 1981).

1.3.2 The Exoglycome

The word exoglycome was proposed by Ferreira *et al.* (2012) to represent the oligosaccharide side chains presented at the outer surface of the cell membrane, thus excluding the cell wall carbohydrates, whenever present, which tend to have a passive or “not-so-active” role in mediating cell communication. The exoglycome was then defined as the collection of carbohydrate moieties present in *N*- and *O*-linked glycoproteins and glycolipids which protrude outwards from the cell membrane (Ferreira *et al.,* 2012).

The biological roles played by these oligosaccharides may be grouped in (i) structural and unspecific, such as protection of the apoprotein from protease action, increase in water solubility and correct glycoprotein positioning, and (ii) functional and highly specific, such as regulation of cell–cell (discrimination between self and non-self), cell–matrix and cell–molecule interactions (oligosaccharide-lectin), protein quality control, receptors for viruses, bacteria and fungi, and in certain diseases such as cancer.

Specific recognition processes between fungal parasites and their host cell targets may be mediated by the interaction of carbohydrate-binding proteins on the surface of one type of cell with complementary sugars on the surface of another (Ferreira *et al.,* 2012).

1.3.3 Glycoproteins: N- *and* O-*glycans*

The glycome, which is the carbohydrate analog to the genome, proteome and metabolome, denote the total collection of carbohydrate species present in a biological unit (i.e. cell, tissue, organ, organism, species, and ecosystem) under any given set of environmental and physiological conditions. More recently, the concept of functional glycomics has emerged after discovering the array of biologically active roles played by certain carbohydrates, the set of which comprise the functional glycome. The exoglycome may therefore be considered as a subset of the functional glycome.

A. fumigatus cellular membrane glycoproteins contain different structures of oligosaccharide residues of great importance not only in cell biological processes but also in cell-cell and cell-molecule recognition, including those relating to pathogenic agents. The extensive stereochemistry, multiple hydroxyl groups and oxygen atoms, and accessible hydrophobic regions make oligosaccharides ideal ligands for precise interactions with recognition sites in proteins (Albersheim *et al.,* 1992). Regarding their role in biological processes, several theories have been reported (Varki, 1993), such as, for example: an aid in the conformation and stability of proteins; the provision of target structures for microorganisms, toxins and antibodies; the masking of such target structures; control of the half-life of proteins and cells; the modulation of protein functions; and the provision of ligands for specific binding events mediating protein targeting, cell-matrix interactions or cell-cell interactions. Also, the 'coating' by oligosaccharides of many apoprotein surfaces on glycoproteins may serve to protect the polypeptide chain from recognition by proteases or antibodies and the 'coating' of glycoconjugates covering a whole cell can present a 'glycocalyx' of substantial proportions (Varki, 1993).

The oligosaccharide side chains comprising the *A. fumigatus* exoglycome exhibit a large capacity for storing biological information, but also a high antigenic potential. The oligosaccharides are expressed in a cell-type specific and temporally manner to allow cell phenotypes to change dynamically in response to environmental stimuli (Zaia, 2008). They are synthesized in a non-template controlled manner and mature structures arise by the coordinated expression of numerous genes that code for glycosyltransferases, glycosidases, and other enzymes that synthesize and remodel oligosaccharide chains, as well as accessory enzymes involved in the synthesis and transport of nucleotide sugars (Ferreira *et al.,* 2012). Given these characteristics and the observations made regarding the early stages of a human fungal infection, which triggers a complex series of mechanisms of resistance/protection, the fungus will have to overcome these defense tools in order to succeed in establishing pathogenesis. It is therefore possible to assume that there are changes in the fungal exoglycome, so that the immune response is avoided, prevented or circumvented. Although the

importance of this fungus in clinical studies has become quite relevant, it remains unclear whether the *A. fumigatus* exoglycome changes during a human fungal infection.

It is certainly difficult to predict specific rules for oligosaccharide functions due to the effects of a changing glycosylation pattern, even within a given group of proteins (e.g. cell surface receptors or enzymes), which may be highly variable and quite unpredictable (Varki, 1993). Moreover, the same modification in glycosylation can have a dramatically opposite effect on *in vivo* function versus that observed under *in vitro* conditions (Varki, 1993).

Several reasons make carbohydrates complex to analyze and very difficult to synthesize (Ferreira *et al.,* 2012), including structural complexity of their linear or branched, oligo or polymer structures; the different configurations/conformations monosaccharides can adopt (ring opened or closed, different ring sizes and conformations); isomeric diversity, including the two possible anomeric stereochemical linkages between units (α and β); diversity of secondary modifications of monomers (e.g. methylation, sulphation, acetylation and phosphorylation); different modes of attachment for cell-surface oligosaccharides (including glycolipids and *N*- or *O*-linked glycoproteins); their indirect relationship to the genome; the range of molecular contexts in which the modifications are found and the fact that most carbohydrates lack chromophores or fluorophores, a property that makes their detection difficult.

From the technological point of view, a minimum amount of each oligosaccharide is required for the structural characterization of a fungal exoglycome. The extraction of a suitable amount of isolated fungal cells from the host turns out to be, most of the time, impossible, even from specific organs in which the fungus seems to be encountered in higher amounts (Ferreira *et al.,* 2012).

1.3.3.1 N- and *O*-glycans

Glycosylation is the most universal and structurally diverse form of posttranslational modification of proteins, and occurs by the attachment of a glycan to a protein either at an Asn residue, termed *N*-glycosylation, or at a Ser, Thr, hydroxylysine or hydroxyproline residue, called *O*-glycosylation (Deshpande *et al.,* 2008). Glycosylation can contribute to protein secretion, stability, and immunogenicity, and in the case of membrane glycoproteins and glycolipids, these oligosaccharides may mediate a cell's communication with the outside world (Deshpande *et al.,* 2008).

The oligosaccharides found on eukaryotic cell membranes are primarily defined according to the nature of the linkage regions (core) to the aglycone (protein or lipid) (Varki and Sharon, 2009). The large diversity of oligosaccharides makes a general discussion of all types of oligosaccharides and their glycoconjugates difficult. Here the focus will be on *N*- and *O*-linked oligosaccharides.

1.3.3.1.1 *N*-Linked oligosaccharides

All *N*-linked oligosaccharides are derived from the precursor $Glc_3Man_9GlcNAc_2$, which is attached to the protein during translation and subjected to various modifications in the Golgi and in the endoplasmic reticulum (An and Lebrilla, 2011). In addition, *N*-glycans share a common pentasaccharide core region (Varki and Sharon, 2009) and can be generally divided into three main classes (Stanley *et al.,* 2009): (i) high-mannose-type, in which only mannose residues are attached to the core; (ii) complex-type, in which "antennae" initiated by *N*-acetylglucosaminyltransferases (GlcNAcTs) are attached to the core; (iii) and hybrid-type, in which only mannose residues are attached to the Man-α-1,6 arm of the core and one or two antennae are on the Man-α-1,6 arm. These oligosaccharides are generally larger than *O*-linked oligosaccharides (typically 10 to 20 monosaccharide residues) with a single common core (An and Lebrilla, 2011).

Maturation of *N*-glycans comprises several biosynthetic processes that can be divided into three components: sugar additions to the core, mostly occurring in the *trans*-Golgi network (a highly dynamic series of interconnected tubules and vesicles at the trans face of the Golgi stack); elongation of branching *N*-acetylglucosamine (GlcNAc) residues by sugar additions; and "capping" or "decoration" of elongated branches (Stanley *et al.,* 2009). The most important "capping" or "decoration" reactions involve the addition of sialic acid, fucose, galactose, *N*-acetylgalactosamine (GalNAc), and sulfate (Stanley *et al.,* 2009).

Yeasts and other fungi typically produce high-mannose-type *N*-glycans by adding up to 100 mannose units, including β-linked mannoses, whereas formation of mammalian oligosaccharides generally involves the removal of mannose, followed by the addition of GlcNAc, galactose, fucose, and sialic acid (Kainz *et al.,* 2008). Filamentous fungi are known to carry small, high-mannose *N*-linked oligosaccharides (Deshpande *et al.,* 2008).

Understanding *N*-linked oligosaccharides pathways is important because they affect many properties of glycoproteins including their conformation, solubility, antigenicity, and recognition by oligosaccharide-binding proteins (Stanley *et al.,* 2009). For example, defects in *N*-linked oligosaccharide synthesis lead to a variety of human diseases (Stanley *et al.,* 2009).

Of all different types of glycosylation, the *N*-asparagine linked sugar chains are the easiest to manipulate in experimental systems (Varki, 1993), such as enzymatic or chemical removal of complete sugar chains, producing an aldehyde reducing end (An and Lebrilla, 2011); inhibition of initial glycosylation; alteration of oligosaccharide processing; elimination of specific glycosylation sites; and the study of natural variants and genetic mutants in glycosylation. *N*-Linked oligosaccharides may be purified by conventional ion-exchange and size-exclusion chromatography, high-pressure liquid chromatography (HPLC) methods, and affinity

chromatography on specific oligosaccharide-binding proteins called lectins (Stanley *et al.,* 2009).

1.3.3.1.2 *O*-Linked oligosaccharides

An *O*-linked oligosaccharide (or *O*-(Ser/Thr)-linked oligosaccharide) is typically attached to the apoprotein moiety of a *O*-glycoprotein by linking a GalNAc residue to a Ser or Thr residue, and can be extended into a variety of different structural core classes (Varki and Sharon, 2009). *O*-Glycosylation is diversified according to the origins of organisms with respect to sugar components and the linkage modes among sugars (Goto, 2007).

Regarding protein *O*-glycosylation in fungi, the structures of the O-linked oligosaccharides are highly dependent on the fungi. In filamentous fungi, *O*-linked oligosaccharides containing branched structures of carbohydrate chains and sugars, except for mannose, are more variable than those of yeasts (Goto, 2007). *O*-Mannosylation is commonly found in glycoproteins of many higher eukaryotes as well as in most fungi, including the filamentous fungi (Deshpande *et al.,* 2008).

A number of proteins localized in the cell wall, plasma membrane and organelles membranes are *O*-glycosylated and are related to proper cell morphology maintenance. However, very little is known about the glycosylated proteins and their function in filamentous fungi (Goto, 2007).

1.3.4 Lectins

1.3.4.1 General approach

Lectins are proteins of non-immune origin (thus excluding immunoglobulins) which bind in a stable manner (thus excluding enzymes and carbohydrate sensor/transport proteins) to carbohydrates (Ribeiro *et al.,* 2012). Lectins are a specific class of carbohydrate-binding proteins or receptors with a role in decoding the oligosaccharide-encoded messages.

Lectins have a ubiquitous distribution in nature, and plants are the richest source of lectins, namely legume seeds and the storage organs of plants in general (Ferreira *et al.,* 2012). Lectins are important tools used by viruses, cells and organisms to decipher the biological information encoded by glycocodes, which are capable for example of recognizing specific oligosaccharides projecting outwards from the cell membranes (Ribeiro *et al.,* 2012). As already mentioned above, such oligosaccharides are collectively termed the exoglycome (section 1.3.2). It has been reported that lectins are involved in diverse biological processes, such as clearance of glycoproteins from the circulatory system, adhesion of infectious agents to host cells, recruitment of leukocytes to inflammatory sites, cell interactions in the immune system, in malignancy and metastasis.

Lectins exhibit diversity of specificity towards simple sugars, although they regularly display a much higher binding affinity for oligosaccharides and glycoproteins, having the capacity to precipitate glycoproteins and branched oligosaccharides due to their usual multivalency (Ferreira *et al.,* 2012). This specific recognition between lectins and carbohydrates is of great importance as they have been used as reliable biochemical, cytochemical and histochemical probes in the study of subtle differences between the cell surface glycoconjugates of malignant and non-malignant cells that are otherwise undetectable with the available monoclonal antibodies. Recognition of specific oligosaccharides by lectins may explain, for example, human infection by flu viruses, how swine H1N1 and avian H5N1 viruses cross the species barrier and infect the human population, and many other exoglycome changes involved in dissemination of fungal diseases such as IA.

Screening large numbers of lectins for the capacity to target such oligosaccharides alterations could provide ways for an early detection/control of such ailments (Ferreira *et al.,* 2012). Nowadays, many different plant lectins have been detected, isolated and identified by conventional, laboratory biochemical techniques.

1.3.4.2 Physico-chemical properties

Although there are noticeable differences on carbohydrate specificity, lectins share common molecular properties as being usually comprised of two or four 25 to 30 kDa subunits (Lis and Sharon, 1986), each with a single, small carbohydrate combining site with the same specificity. Many are glycoproteins or metalloproteins containing a tightly bound Ca^{2+} and a transition metal ion, predominantly Mn^{2+}, per subunit, which are required for carbohydrate binding (Lis and Sharon, 1998). Lectins combine with carbohydrates by a network of hydrogen bonds and hydrophobic interactions, and coordination with metal ions (Lis and Sharon, 1998). The hydrogen bonds are formed between carbohydrate hydroxyl groups and amino groups, hydroxyl groups, and oxygen atoms of the protein.

1.3.4.3 Lectin classification according to carbohydrate specificity

According to the monosaccharide ligand towards which lectins exhibit the highest affinity, they are classified in seven main groups: mannose and glucose, galactose/*N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), fucose, *N*-acetylneuraminic acid (sialic acid), mannose-6-phosphate, and *N*-acetylgalactose-4-sulfate (Ambrosi *et al.,* 2005). All these sugars are in the D-configuration, except for fucose (Ambrosi *et al.,* 2005).

Although lectins are generally classified in seven groups, in this dissertation, affinoblotting and affinity chromatography (lectin column chromatography) assays were developed with lectins belonging to only four groups, namely concanavalin A (Con A), peanut agglutinin (PNA), *Maackia amurensis* lectin (MAL), and *Ulex europaeus* agglutinin (UEA). It has been reported that the cell wall surface glycoconjugates of medically important fungi, such as *A. fumigatus* and

others, exhibit *N*-acetyl-D-glucosamine and methyl-α-D-mannoside residues (as shown by lectin-binding assays; Leal *et al.,* 2011), which are recognized by MAL and Con A, respectively. Moreover, it has been described that lectins may be of value as important tools in mycological diagnostics, as well as in identification and typing fungal agents of isolates in culture, tissue samples and fixed histopathologic specimens (Leal *et al.,* 2011).

Con A (jack bean protein) belongs to the mannose/glucose group, which is the second major group. The ability of Con A to bind with high affinity to certain *N*-linked carbohydrates has made it a widely used tool to investigate the properties of normal and transformed cells, as well as to isolate carbohydrates, glyconjugates, and cells on Con A-affinity matrixes (Mandal *et al.,* 1994). As a tetramer, at neutral and alkaline pH, Con A consists of four identical subunits (Gunther *et al.,* 1973), and each subunit contains a binding site which binds ligands with unmodified hydroxyls at positions 3, 4, and 6 of an α-glucopyranose or α-mannopyranose unit or residue (Weatherman *et al.,* 1996).

PNA is also known as *Arachis hypogaea* lectin. PNA belongs to the galactose/ GalNAc group as well as the vast majority of lectins. It binds the carbohydrate sequence galactosyl-β(1,3)-*N*-acetylgalactosamine, and its activity is inhibited by lactose and galactose, which compete for the binding site of PNA (Lotan *et al.,*1975). A fairly stable and non-glycosylated lectin, PNA has proven as a potential structure-specific probe in glycobiology, especially as it sharply discriminates between sialylated and non-sialylated forms of its most powerful inhibitor carbohydrate group, the galactosyl-β(1,3)-*N*-acetylgalactosamine, unlike the jack fruit seed lectin, jacalin (Chacko and Appukuttan, 2001).

MAL belongs to the *N*-acetylneuraminic acid group, and is commonly used as a glycoanalytical tool to probe biological targets for α-2,3-linked sialic acids (Geisler and Jarvis, 2011). Immobilized MAL interacts with high affinity with complex-type Asn-linked oligosaccharides containing terminal sialic acid in α-2,3-linkage to galactose (Wang and Cummings, 1988). It has been referred that MAL binds most preferably to terminal α-2,3-Gal-β-1,4-Glc(NAc) in *N*-linked oligosaccharides (Geisler and Jarvis, 2011).

UEA is associated to the fucose residue owing to its capacity to bind to many glycoproteins and glycolipids containing L-Fuc α1-2-D-Galβ1-4-D-GlcNAc residues, which is the highest affinity ligand known to UEA (Loris *et al.,* 1998). Thus the inhibitory carbohydrate is α-L-fucose (fuc) (Blonski *et al.,* 2007).

1.3.4.4 Applications

In this dissertation only plant lectins were discussed (Con A, MAL, PNA and UEA) due to their wide application in basic and medical sciences. Hence, these proteins serve as important tools in scientific areas as biochemistry, cell biology and medicine, such as for example in the following applications (Rüdiger and Gabiu, 2001): purification of lectin-reactive glycoconjugates by affinity chromatography, oligosaccharide characterization by serial lectin affinity chromatography, glycome analysis (glycomics), characterization of cell surface presentation of

glycoconjugates and their preceding intracellular assembly and routing in normal and genetically engineered cells, analysis of mechanisms involved in correct glycosylation by lectin-resistant cell variants, detection of disease-related alterations of glycan synthesis, blood group typing and definition of secretor status, and cell marker for diagnostic purposes including infectious agents (viruses, bacteria, fungi, parasites).

1.4 Objectives

In order to achieve a better understanding of the host-induced changes upon the *Aspergillus fumigatus* cell membrane *N*-linked glycoproteins, this workplan aims, in a first stage, to identify the main components of *A. fumigatus* cell membrane proteome as well as to detect and identify the glycoproteins present in the cell membrane. The identification of the *N*-linked oligosaccharides (herein simply referred to as the *A. fumigatus* exoglycome), that are projected into the external milieu from the fungal cell membrane surface, will also be performed.
In due course, this workplan will also attempt to pinpoint the exact structural alterations which are induced by the host cells in both the cell membrane proteome and the *N*-linked exoglycome during attempted pathogenesis and disease. This may allow the identification of suitable disease markers which may be later used as targets to combat this serious human ailment.

2. Materials and Methods

2.1 Strain and cell culture

In this study the Asp1-56/03 strain (provided by the Instituto de Higiene e Medicina Tropical, New University of Lisbon) of *Aspergillus fumigatus* was inoculated in GYP medium (0.5% [w/v] yeast extract, 0.5% [w/v] peptone and 2% [w/v] glucose), for 2 to 3 days, at 37 °C, in a 3D culture system.

2.2 Membrane extraction and purification (Burghoom *et al.,* 2002)

A. fumigatus cells were collected by filtering through cheesecloth, maintained at 0 to 4 °C and resuspended in homogenization buffer (50 mM Tris-HCl pH 7.5, 0.3 M sucrose, 1% [w/v] glucose, 1 mM ethylenediamine tetraacetic acid [EDTA], 2 mM 1,4-dithiothreitol [DTT]) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were broken in a French pressure cell at 20.000 lb/in^2. After cell disruption, the cells were centrifuged in an Eppendorf F34-6-38 rotor for 20 min, at 3,024 *g*, recovering the supernatant. The crude cell membrane fraction was pelleted from the supernatant with a second centrifugation in a Beckman SW48 rotor, for 1 h, at 66,000 *g* and 4 °C. Crude membranes were washed in membrane wash buffer (10 mM Tris-HCl pH 7.5, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM DTT, 20% [v/v] glycerol) containing 0.5 mM PMSF and resuspended in membrane wash buffer. Purified plasma membranes were collected at the 42%-62% (w/w) sucrose interface of a step gradient containing 1 mM EDTA, 1 mM DTT and 10 mM Tris-HCl (pH 7.0) after centrifugation for 3 h at 66,000 *g* in a Beckman SW48 rotor, at 4 °C. The membranes were washed in membrane wash buffer for 1 h at 66,000 *g* in the SW48 rotor and resuspended in membrane wash buffer. For subsequent use, the membrane sample was stored at -80 °C.

2.3 Extraction of *A. fumigatus* plasma membrane proteome

For *A. fumigatus* plasma membrane total protein extraction enough solid urea, ammonium bicarbonate and sodium dodecyl sulfate (SDS) stock solution were added directly to the previously purified plasma membrane sample to prepare a denaturing buffer containing 8 M urea, 0.4 M NH_4HCO_3 and 0.1% (w/v) SDS. Afterwards, the sample was sonicated for 6 min at 4 °C with ultrasounds for homogenization.

2.4 Protein quantification

Protein concentration was determined following a modification of the Lowry method (Bensadoun and Weintein, 1976) using bovine serum albumin (0.5 mg/mL) as the standard. The readings were measured at 750 nm.

2.5 Marker enzymes of specific cell membrane systems

The catalytic activities of specific membrane marker enzymes were examined to confirm that the proteins extracted from *Aspergillus fumigatus* were indeed from the plasma membrane. The membrane markers used were from the vesicle-vacuole fraction (Chanda *et al.*, 2009), mitochondrial fraction (Chanda *et al.,* 2009), endoplasmic reticulum (Ugalde *et al.,* 1992), Golgi apparatus (Ugalde *et al.,* 1992) and plasma membrane (Perzov *et al.,* 2000).

2.5.1 Enzyme activity from the vesicle-vacuole membrane system

For the vesicle-vacuole membrane system, the catalytic activity of α-mannosidase (AMS) was assayed according to the method described by Chanda *et al.* (2009). Ten µL of the *A. fumigatus* purified plasma membrane sample (total protein range from 3 to 10 µg) was added to a reaction mix containing 0.5 mL of sodium succinate buffer (50 µM Na-succinate, pH 5.0); then, 3 µL of 0.1 M *p*-nitrophenyl substrate was added. The reaction was stopped by addition of 0.8 mL of 1 M Na_2CO_3 and the absorbance determined at 405 nm. The specific activity was expressed in nmol of *p*-nitrophenol produced per min per µg total protein.

2.5.2 Enzyme activity from the mitochondrial membrane system

For the mitochondrial membrane system, the catalytic activity of succinate dehydrogenase was measured according to Chanda *et al.* (2009). Ten µL of the *A. fumigatus* purified plasma membrane sample (total protein range from 3 to 10 µg) was added to a reaction mixture consisting of 0.3 M KH_2PO_4, 8.5 mM KCN and 50 µg of dichlorophenolindophenol (DCPIP, Na salt). Sodium succinate buffer (50 µM Na-succinate, pH 5.0) was then added to this reaction mixture and the absorbance was recorded at 600 nm. Enzyme specific activity was expressed in µmol DCPIP reduced per minute per µg total protein.

2.5.3 Enzyme activity from the endoplasmic reticulum membrane system

NADPH-cytochrome *c* oxido-reductase was used as an endoplasmic reticulum membrane marker according to Ugalde *et al. (1992)*. The reduction of cytochrome *c* was measured at 550 nm in the *A. fumigatus* purified plasma membrane sample. The 0.6 mL reaction mixture contained 0.45 mM cytochrome *c*, 50 mM KH_2PO_4 pH 7.2, and 3 mM NADPH, plus 50 mM NaCN and 0.4 µM antimycin A, which were included to inhibit cytochrome oxidase activity. The reaction was started with 20 to 50 µg protein.

2.5.4 Enzyme activity from the Golgi apparatus membrane system

Inosine diphosphatase (IDPase) activity, a commonly used marker of the Golgi body membrane system, was measured by the liberation of inorganic phosphate (Pi), according to Ugalde *et al.* (1992), associated with IDP hydrolysis after incubation for 10 min at 30 °C in a 0.5 mL reaction mixture containing 5 mM IDP, 5 mM $MgCl_2$, 10 mM piperazine-*N,N*-bis(2-ethane sulfonic acid (PIPES-Tris) pH 7.5, 0.1 mM Na_2MoO_4 and 40 to 100 µg *A. fumigatus* purified plasma

membrane protein. The reaction was stopped by addition of 0.1 mL 50 % (w/v) trichloroacetic acid (TCA). The liberation of inorganic phosphate (Pi) was measured at 700 nm.

2.5.5 Enzyme activity from the plasma membrane system

ATPase activity was used as a plasma membrane marker. According to the method described by Perzov *et al.* (2000), ATP hydrolysis associated to the *A. fumigatus* plasma membrane was assayed in 0.5 mL of a reaction medium consisting of 5 mM ATP (vanadate-free), 5 mM KCN, 1 mM NaN_3, and 10 mM 2-(N-morpholino)ethanesulfonic acid (MES)-KOH (pH 6.0) in the presence of 100 µM $NaVO_3$. The reaction was initiated by the addition of 0.1 mL from *A. fumigatus* plasma membrane gradient fractions, and allowed to proceed for 10 to 20 min at 30 °C. The ATPase activity was measured by the release of Pi associated to ATP hydrolysis according to the method described by Ames (1966), and the change in absorbance recorded at 700 nm.

2.6 Reducing, denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

For one-dimensional electrophoresis (1DE), the *A. fumigatus* plasma membrane proteome was washed with 10% (w/v) TCA to remove contaminants, and centrifuged at 15,800 *g*, for 10 min. The pelleted fraction was resuspended in 1 mL of acetone, and kept at -20 °C for 30 min. After protein precipitation, the sample was again centrifuged at 15,800 *g*, for 10 min. The pellet was finally resuspended in sample buffer (2% [w/v] SDS, 0.08 M Tris-HCl pH 6.8, 0.1 M β-mercaptoethanol (ME),15% glycerol and 0.006% *m*-cresol purple) for electrophoresis. To achieve full protein denaturation, the sample was heated at 100 °C for 3 min in a water bath.
Protein separation by SDS-PAGE was performed in a discontinuous buffer system according to the method proposed by Weber and Osborn (1969) and Laemmli (1970):

- Separation gel: 12.5% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide, 375 mM Tris-HCl pH 8.8, 0.03% (v/v) tetramethylethylenediamine (TEMED) and 0.03% (w/v) ammonium persulfate (PSA);
- Concentration gel: 5% (w/v) acrylamide, 0.13% (w/v) bis-acrylamide, 125 mM Tris-HCl pH 6.8, 0.05% (v/v) TEMED and 0,1% (w/v) PSA.

Electrophoresis took place under constant current intensity and voltage (70 mA and 220 V, respectively) conditions and 220 V.

2.7 Two-dimensional electrophoresis (2DE)

For complex mixtures of proteins which are not sufficiently resolved by 1DE, a multidimensional separation may be necessary in which components not satisfactorily resolved in the first dimension are separated in the second. The 2DE is the most common multidimensional separation technique used to separate complex mixtures of proteins.

Procedure: the 2DE method is typically initiated with an isoelectric focusing (IEF) step, which separates proteins according to their inherent isoelectric points (pI). First, a pH gradient is established along the gel, then an electric field is applied to the gel, making one end more positive than the other. At all pH values other than their individual pI, proteins will be charged. If they are positively charged, they will be pulled towards the more negative end of the gel, whereas if they are negatively charged they will be pulled to the more positive end of the gel. For this first step, the *A. fumigatus* plasma membrane proteome was previously resuspended in 1 mL of acetone, and kept at -20 °C for 30 min, which helps to remove the SDS fraction present that may interfere with the isoelectric focusing. This first separation step was carried on immobilized pH gel strips (Bio-Rad Laboratories, USA), 11 cm, with a pH interval of 3 to 10. The gel strips were hydrated in a rehydration buffer (7 M urea, 2 M thiourea, 4% [w/v] Nonidet P-40, 2% IPG buffer and 40 mM DTT). The application of mineral oil on the strip before IEF is necessary to avoid urea crystallization by water evaporation.

The IEF program was performed, in a Protean IEF Cell (Bio-Rad Laboratories, USA), according to the following parameters: rehydration – 50 V, for 12 h; step 1 – 250 V/h; step 2 – 500 V/h; step 3 – 8,000 V, 2.5 h; step 4 – 8,000 V, 30,000 V/h. After IEF, the strips are subjected to two incubations, 10 min each, with an equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% [v/v] glycerol and 2% [w/v] SDS) containing 1% (w/v) DTT in the first incubation and 2.5% (w/v) iodoacetamide in the second incubation.

The second part of 2DE is the SDS-PAGE step (section 2.6), performed in a polyacrylamide gel (40% [w/v] acrylamide, 1% [w/v] bisacrylamide, 1 M Tris-HCl pH 8.8, Milli-Q water, 10% [w/v] PSA and 0.03% [v/v] TEMED). The SDS-PAGE step was carried in the same way as described for 1DE, under a current intensity of 15 mA and changing it to 30 mA, after 15 min.

2.8 Polypeptide silver staining

To achieve a good visualization of the *A. fumigatus* plasma membrane proteome, the 1DE and 2DE gel polypeptides were stained following the silver stain method described by Blum *et al.* (1987).

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

The gels can be stored in stopping solution, at room temperature, with agitation, or washed with water before being dried.

2.9 Polypeptide staining with colloidal blue

For polypeptide sequencing by mass spectrometry (MS), the 2DE gels had to be stained with the colloidal blue (Commassie G) staining method described previously by Neuhoff *et al.* (1988). This method requires higher amount of protein per band/spot than the silver stain method, about 400 µg per gel (in case of a mini-gel, ca. 100 µg is enough).

Procedure: *A. fumigatus* plasma membrane polypeptides were fixed in a 2% (v/v) phosphoric acid and 50% (v/v) methanol solution, for 2 h or overnight. Subsequently, the gel was washed three times with Milli-Q water, 30 min each wash. In the incubation process the gel was maintained in a 34% (v/v) methanol, 17% (w/v) ammonium sulfate and 2% (v/v) phosphoric acid solution, for 1 h. Lastly, the staining solution (1.1% [w/v] Commassie G in 34% [v/v] methanol) was added to the incubation solution, and the gel was stained for several days (no more than 5 days).

2.10 Glycopolypeptide gel stain with Pro-Q Emerald 300

The Pro-Q Emerald 300 glycoprotein stain (Life Technologies, Invitrogen) reacts with periodate-oxidized carbohydrate groups, creating a bright green fluorescent signal on glycoproteins. The Pro-Q Emerald 300 reagent method depends greatly upon adequate fixation and washing to remove SDS from the proteins and washing after the oxidation reaction to remove residual periodate, which can interfere with staining.

Procedure: proteins were separated by standard one-dimensional SDS-polyacrylamide gel electrophoresis, before the staining procedure. After the SDS-PAGE, the gel was immersed in ~ 100 mL of fix solution (50% [v/v] methanol and 5% [v/v] acetic acid) and incubated, at room temperature with agitation, for 45 min. This fixation step was repeated to ensure that the SDS is fully washed out of the gel. The second step of this procedure was to incubate the gel in ~ 100 mL of wash solution (3% [v/v] glacial acetic acid) with gentle agitation for 10-20 min (this step is repeated once). The oxidation step of the carbohydrates follows, where the gel was incubated in 25 mL of oxidizing solution (addition of 250 mL of 3% [v/v] acetic acid to the kit bottle containing 2.5 g of periodic acid) with gentle agitation for 30 min. The gel was then washed again with the wash solution for 10-20 min (this step is repeated twice more). To prepare the Pro-Q Emerald 300 staining solution the Pro-Q Emerald 300 stock solution (addition of 6 mL of *N,N*-dimethylformamide [DMF] to the vial containing the Pro-Q Emerald 300 reagent) was diluted 50-fold into Pro-Q Emerald 300 staining buffer. For the staining step the gel must incubate in the dark in 25 mL of Pro-Q Emerald 300 staining solution while gently agitating for 90-120 min. Finally, the gel was washed in ~ 100 mL of wash solution at room temperature for 15-20 min (this step was repeated once) and images were taken. All images were obtained and photographed on a ChemiDoc™ XRS+ (Bio-Rad laboratories, USA).

2.11 Western blotting and affinoblotting

2.11.1 Western blotting

To perform the western analysis, the separated *A. fumigatus* plasma membrane polypeptides were subsequently transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham Hybond^{TM}-P, GE Healthcare, UK) in a semi-dry blotting system (Bio-Rad Laboratories, USA).

Procedure: proteins separated by SDS-PAGE were blotted onto a PVDF membrane (previously soaked for 15 min in transfer buffer: 39 mM Trizma base (Tris), 48 mM glycine, 0.1% [w/v] SDS, 20% [v/v] methanol, pH 8.3) at 15 V for 45-50 min using a semidry transfer unit. Before membrane soaking in transfer buffer, the membrane must be activated in methanol for a few minutes. After protein transfer, the membrane was heated to 100 °C in water, for 10 min, and kept in methanol for polypeptide fixation. Total polypeptides in the membrane were reversibly visualized with Ponceau S. In brief, the membrane was washed for 1 min with 100% (v/v) methanol, incubated with 0.026 M Ponceau S, 1.8 M TCA, and 1.2 M sulfosalicylic acid, and washed again with methanol.

2.11.2 Affinoblotting

Polypeptides separated by SDS-PAGE and blotted onto a PVDF membrane were also utilized in the detection of glycopolypeptides by the method developed by Faye and Chrispeels (1985). For glycopolypeptide detection, four lectins were used as probes, namely concanavalin A, peanut agglutinin (PNA), *Maackia amurensis* lectin (MAL) and *Ulex europaeus* agglutinin I (UEA). All lectins used in this procedure were acquired in biotinylated form (Galab Technologies GmbH, Germany).

Procedure: the membrane, containing the fixed polypeptides, was washed for 5 min with water, for 1 min with 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl (TBS), immersed for 1 h in TBS containing 0.1% (v/v) Tween-20 (TBST), and incubated in TBST containing the specific lectin (25 µg/mL concanavalin A, 15 µg/mL PNA, 15 µg/mL MAL or 5 µg/mL UEA), 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (TBSTS). After 1 h, the membrane was washed (4 x 10 min) with TBSTS, incubated for 1 h in TBSTS containing 0.5 µg/mL avidin, and washed four times (10 min each) with TBSTS and once (5 min) with TBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (TBSS). Finally, an enhanced chemiluminescence (SuperSignal West Femto/Pico Chemiluminescent Substrate) method was used as the detection system (Thermo Scientific Pierce), according with the manufacturer's instructions. All images were obtained and photographed on a ChemiDoc^{TM} XRS+ (Bio-Rad laboratories, USA).

2.12 *A. fumigatus* plasma membrane protein isolation by lectin affinity chromatography

2.12.1 Affinity chromatography on concanavalin-A-Sepharose gel

The Concanavalin-A-Sepharose (GE Healthcare, UK) chromatography gel has a particular lectin (Con-A) immobilized on a Sepharose 4B matrix and is specific for molecules which contain α-**D**-mannopyranosyl,α-**D**-glucopyranosyl or sterically related residues. Con-A is a metalloprotein and contains two metal binding sites. At neutral and alkaline pH, Con-A exists as a tetramer of four identical subunits of approximately 26 kDa each (Gunther *et al.,* 1973). Below pH 5.6, the lectin dissociates into active dimmers of 52 kDa (Gunther *et al.,* 1973). Acetylation, succinylation or other derivatizations can also produce stable forms with dimeric structures (Gunther *et al.,* 1973). Con-A has a pI of about 5.0 and requires calcium or magnesium ions at each of its four saccharide binding sites. As such, to preserve the binding activity of the Con-A molecule below pH 5.0, excess Mg^{2+} and Ca^{2+} must be present. This will ensure maintenance of an active Con-A metal complex.

The *A. fumigatus* plasma membrane protein sample was dialysed to the column binding buffer before being applied on the column (Ø = 1.1 cm; h = 1.2 cm) containing Con-A-Sepharose gel.

Procedure: the column was prepared with 500 µL affinity gel, and equilibrated with binding buffer (20 mM Tris-HCl, pH 7.4) containing 0.5 M NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. To start the separation process, 2 mg of *A. fumigatus* membrane proteins was applied to the column; the first eluted fraction was collected and its absorbance read at 280 nm. The column was washed several times with binding buffer (1 mL fractions were collected) and each fraction was read at 280 nm. This process ended when the absorbance values of the collected fractions reached zero or stabilized, indicating that all *A. fumigatus* membrane proteins that did not bind to the gel matrix had been eluted. To elute all *A. fumigatus* membrane proteins bound to the Con-A gel, the column was washed with elution buffer (20 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 M methyl-α-**D**-mannopyranoside, as the lectin binding-inhibiting sugar). All spectrophotometric readings were made at 280 nm.

After each assay the column was regenerated by washing it with two different buffers (3 times each buffer): i) 0.1 M Tris-HCl, pH 8.5, containing 0.5 M NaCl; ii) 0.1 M sodium acetate, pH 4.5, containing 0.5 M NaCl. The Con-A-Sepharose gel was preserved in 0.1 M sodium acetate buffer, pH 6.0, containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.01% [w/v] sodium azide.

All non-binding protein fractions were pooled, as well as all binding protein fractions, and were both dialysed against Milli-Q water, pH adjusted to 7.5. Both samples were lyophilized after dialysis, for protein concentration.

2.12.2 Affinity chromatography on Affisep-PNA-adsorbent gel

Arachis hypogaea lectin or peanut agglutinin (PNA) was purchased in purified form after isolation from peanut seeds and purification by affinity chromatography. The lectin has a

molecular mass of 110 kDa and consists of four identical subunits of approximately 27 kDa each (Lotan *et al.*, 1975). This lectin is specific for terminal β-galactosyl residues.

The *A. fumigatus* plasma membrane protein sample was dialysed against the affinity gel binding buffer before being applied onto the Affisep-PNA-Adsorbent (Galab Technologies GmbH, Germany) gel containing column (Ø = 1.1 cm; h = 1.2 cm)

Procedure: like the Con-A-Sepharose column, the PNA column was prepared with 500 µL affinity gel and equilibrated with binding buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 8.0) containing 0.5 M NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Again, 2 mg of *A. fumigatus* membrane protein was applied to the column. Collection of non-bound and bound protein fractions was performed as described above for the Con-A-Sepharose affinity chromatography. The main difference resides on the buffers used along the process: i) the elution buffer (20 mM HEPES, pH 8.0, containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 M **D-**galactose (the lectin binding-inhibiting sugar); ii) the washing buffer (binding buffer containing 2 M NaCl); iii) the preservation buffer (20 mM HEPES, pH 8.0, containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.02% [w/v] sodium azide). All spectrophotometric readings were made at 280 nm.

All non-binding protein fractions were pooled, as well as all binding protein fractions, and were both dialysed against Milli-Q water, pH adjusted to 7.5. Both samples were lyophilized after dialysis, for protein concentration.

2.12.3 Affinity chromatography on Affisep-MAL-adsorbent gel and Affisep-UEA-adsorbent gel

Maackia amurensis lectin (MAL) is the leucoagglutinin or mitogenic isolectin form from *Maackia* seeds. This multimeric lectin dissociates into 38 and 40 kDa molecular mass bands in SDS gels in the presence of reducing agents but migrates as a single 75 kDa band under nonreducing conditions (Geisler *et al.,* 2011). It has a pI of about 4.6 and has been reported to have a native molecular mass of about 130 kDa when estimated by gel filtration (Geisler *et al.,* 2011). This lectin binds glycoconjugates having galactosyl (β-1,4) *N*-acetyl-**D**-glucosamine structures. The inhibiting sugar for this lectin is lactose.

Ulex europaeus agglutinin I (UEA) is a glycoprotein with a molecular mass of 63 kDa, although multimeric aggregates have been reported. UEA I has two subunits, one of about 31 kDa and another of 32 kDa. UEA I binds to many glycoproteins and glycolipids containing α-linked **L**-fucose residues, such as ABO blood group glyconjugates. The inhibiting sugar for this lectin is **L**-fucose.

Two samples of *A. fumigatus* plasma membrane protein were dialysed to the affinity gel binding buffer, which is identical for Affisep-MAL (Galab Technologies GmbH, Germany) and Affisep-UEA (Galab Technologies GmbH) adsorbents.

Procedure: once again, both columns (Ø = 1.1 cm; h = 1.2 cm) were prepared with 500 µL lectin-affinity gel, equilibrated with binding buffer (20 mM Bis-Tris, pH 6.0) containing 0.5 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, and 2 mg of *A. fumigatus* membrane protein was applied

to each column. Collection of non-bound and bound protein fractions was performed as described above (sections 2.12.1 and 2.12.2). The main difference resided, once again, on the buffers used along the process: i) the elution buffer for MAL (20 mM Bis-Tris, pH 6.0, containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 M α-lactose); ii) the elution buffer for UEA (20 mM Bis-Tris, pH 6.0, containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 M **L**-fucose); iii) the washing buffer (binding buffer containing 2 M NaCl); iv) the preservation buffer (20 mM Bis-Tris, pH 6.0, containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.02% [w/v] sodium azide). All spectrophotometric readings were made at 280 nm.

All non-binding protein fractions were pooled, as well as all binding protein fractions, and were both dialysed against Milli-Q water, pH adjusted to 7.5. Both samples were lyophilized after dialysis, for protein concentration.

2.13 MALDI-TOF-MS analysis and protein identification by peptide mass fingerprinting

Subfractions of the *A. fumigatus* plasma membrane proteome obtained after affinity chromatography with four different lectins (bound and nonbound fractions; section 2.11), were separately submitted to 1DE (section 2.6) and stained with the colloidal blue method (section 2.9). After analysis of each resulting proteome map, the fractioned lectin-bound polypeptides were excised from each gel. Each polypeptide was individually sequenced by mass spectrometry (MS).

Each selected *A. fumigatus* polypeptide was incubated with porcine modified trypsin, which cleaves at specific sites of its aminoacid sequence, generating a typical 'fingerprint' composed of several peptides with a distinct pattern of molecular masses which is, most often, exclusive to a given protein. These peptides constitute the peptide mass fingerprint (PMF) of the protein. After tryptic digestion, the peptide samples were analyzed by *matrix-assisted laser desorption/ionization – time-of-light – mass spectrometry* (MALDI-TOF-MS), which allows their identification.

All analyses were performed at the Proteomics Department of the Institute of Molecular Pathology and Immunology of the University Of Porto (IPATIMUP) and executed with a 4700 MALDI TOF/TOF Proteomics Analyzer.

Protein identification was determined by comparing the PMF of the unknown *A. fumigatus* protein with the theoretical molecular masses of the peptides which are generated by virtual digestion of each of the proteins listed in databases like UniProt Knowledgbase (UniProtKB) or Swiss-Prot. PMF is the method-of-choice for protein identification in proteome research because it is a simple and sensitive technique and generally originates high confidence levels for protein identification (information given by IPATIMUP). Protein identity is checked out for plausibility taking trypsin autolysis and cleavage faults, as well possible contaminations into account.

2.14 Oligosaccharide-chain purification and quantification from *A. fumigatus* plasma membrane proteome

A. fumigatus plasma membrane proteome preparation was executed according to the method described by Kim *et al.* (2006), with the purpose to purify the oligosaccharide structures coupled to glycoproteins of the fungal membrane. Protein samples were treated with proteinase K to release *N*-glycans linked to an Asn residue.

2.14.1 Oligosaccharide release and purification

Procedure: proteinase K (0.5 mg/mL) was added to a fraction of *A. fumigatus* total membrane protein and incubated at 37 °C, for 16 h. After proteolytic digestion, the sample was centrifuged at 18,000 *g* and 4 °C, for 30 min. The enzymatic reaction was interrupted by addition of formic acid (5% [v/v]) before centrifugation. The supernatant was tapped, transferred into a new Eppendorf tube, and then dried in a speed vacuum. To extract the *N*-linked oligosaccharide side chains, the enzyme peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (PNGase-F, EC 3.5.1.52) was added to the membrane protein sample. After a 17 h incubation period, at 37 °C, proteins were precipitated by adding 4 volumes of cold acetone and the mixture was kept on ice for 30 min. The mixture was then centrifuged (at 18,000 *g* and 4 °C, for 10 min) and the protein fraction was retained in the pellet. The supernatant, containing the *N*-linked released oligosaccharides, was transferred into a new Eppendorf tube and centrifuged (at 18,000 *g* and 4 °C, for 10 min). Finally, the acetone was evaporated and the glycans subjected to desalting and delipidation processes.

2.14.2 Oligosaccharide quantification

The process of oligosaccharide quantification was performed according to the phenol-sulfuric acid hexose assay described by Hounsell (1998).

Procedure: twenty five µL of *A. fumigatus* oligosaccharide sample was transferred to a microplate well and 25 µL of 4% (v/v) aqueous phenol was added. The mixture rested for 5 min before adding 200 µL of H_2SO_4. After glycosidic cleavage, monosaccharide concentration was obtained by absorbance readings at 492 nm.

3. Results and Discussion

3.1 Determination of enzyme activity on different membrane systems

To confirm the origin of the protein sample used during this work was indeed from *A. fumigatus* plasma membrane, several membrane specific enzymatic assays (section 2.5) were performed on different membrane systems, namely: the vesicle-vacuole, the mitochondrial, the endoplasmic reticulum, the Golgi apparatus and the plasma membrane. These studies examined the catalytic activities of specific membrane markers, such as α-mannosidase (AMS; Figure 3.1A), succinate dehydrogenase (SDH; Figure 3.1B),inosine diphosphatase (IDPase; Figure 3.1C), NADPH-cytochrome *c* oxido-reductase (Figure 3.1D) and ATPase (Figure 3.1E). If the protein sample is indeed from the plasma membrane, the catalytic activity value for the membrane marker (ATPase) should be higher for the plasma membrane fraction.

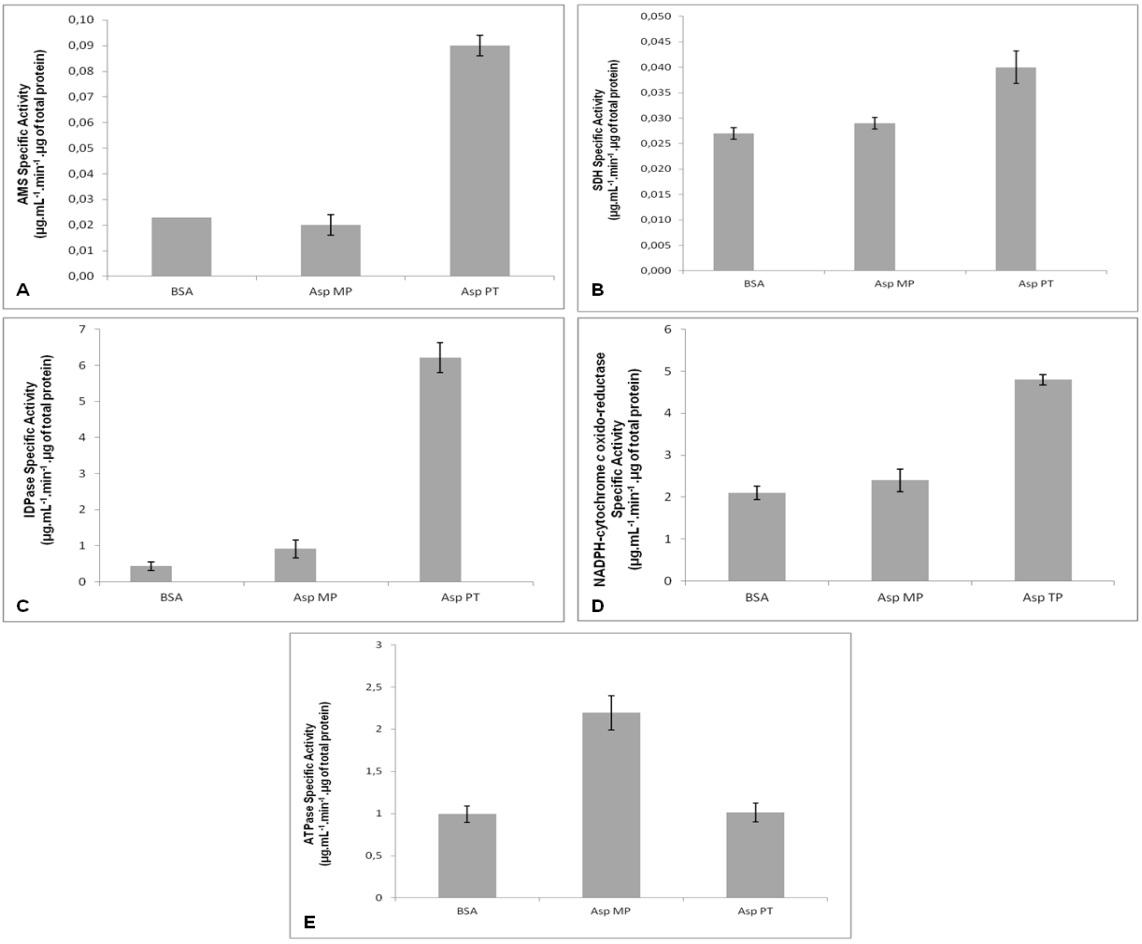


Fig. 3.1 – Measurement of marker enzyme specific activities from whole cells, total purified cell membrane sand bovine serum albumin (BSA, negative control). Protein extracts were prepared from whole cells (Asp PT) and total cell membrane fraction (Asp MP). The specific activities of AMS (**A**) (vacuole marker; specific activity was expressed in µg of *p*-nitrophenol reduced/ mL .min. µg total protein), SDH (**B**) (mitochondrial marker; specific activity was expressed in µg of DCPIP reduced/ mL .min. µg total protein), IDPase (**C**) (Golgi apparatus marker; specific activity was expressed in µg of Pi/ mL .min. µg total protein), NADPH-cytochrome *c* oxido-reductase (**D**) (endoplasmic reticulum marker; specific activity was expressed in µg of cytochrome *c* reduced/ mL .min. µg total protein), and ATPase (**E**) (plasma membrane marker; specific activity was expressed in µg of Pi produced per/ mL .min. µg total protein) were measured.

In Figure 3.1A-E it is shown the specific activity measurements performed for each enzyme marker. As a negative control the BSA protein was used that should present no enzyme activity. However, from the spectra analysis the contrary was verified; an enzymatic activity (BSA, Figure 3.1A-E) has been detected that may be due to some lack of experimental parameters adjustment during the assay. To compare with the other measurements made for the whole cell (Asp PT, Figure 3.1A-E) and total cell membrane (Asp MP, Figure 3.1A-E) fractions, the BSA values were chosen as base line.

Naturally, all of the enzyme markers (AMS, SDH, IDPase and NADPH-cytochrome *c* oxido-reductase) should show some catalytic activity in the whole cell fraction. In fact, this was observed as shown in the specific activity data on Figure 3.1A-D. For the specific activity spectrum of the plasma membrane marker the opposite was verified as the catalytic activity of ATPase was higher in the total cell membrane fraction (Asp MP, Figure 3.1AE). As mentioned in the material and methods section, there are some adjustments that could be made to the protocols for enzyme activity determination, however the data allowed to conclude that the methods used for membrane and plasma membrane proteome extraction, of *A. fumigatus*, was efficient.

3.2 One dimensional electrophoretic (1DE) analysis of the plasma membrane proteome profile of *A. fumigatus*

After *A. fumigatus* membrane protein quantification using a modification of Lowry method, polypeptide separation was achieved by 1DE (100 µg of total cell membrane protein was loaded in each lane; Figure 3.2).

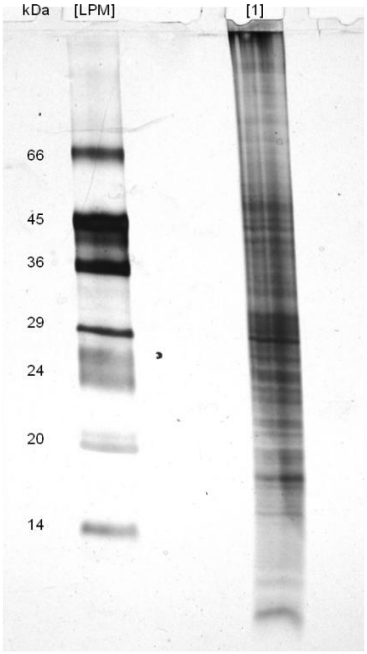


Fig. 3.2 – Electrophoretic analysis of *A. fumigatus* plasma membrane proteins, through a 17.5% (w/v) polyacrylamide gel. The lanes contain [LPM] low protein molecular mass markers (14-66 kDa). [1] Plasma membrane protein from *A. fumigatus* (100 µg). The gel was silver stained.

The 1DE gel (presented in Figure 3.2) was silver stained (Blum *et al.*, 1987; section 2.8) to obtain a greater sensitivity, showing the presence of different plasma membrane polypeptides, with a good band distribution ranging between 14 and 66 kDa. Several polypeptide bands detected show a high polypeptide concentration, whereas others are present in lower amounts. The latter ones are not usually visualized in gels stained with colloidal blue. So, in a first attempt to have a deeper look of the plasma membrane proteome it was necessary to use an appropriate staining method, enabling the detection of as many polypeptide bands as possible.

3.3 Two dimensional electrophoretic (2DE) analysis and evaluation of the plasma membrane proteome profile of *A. fumigatus*

The 2DE method is often used to analyze complex protein mixtures due to its capacity to separate the individual polypeptide according to their pI and to its molecular weight.

In Figure 3.3 the spot distribution is shown, regarding cell membrane polypeptides, resulting from 2D electrophoresis. The polypeptides are distributed along the pH interval 3 to 10, and are widely spread, though there is a highest polypeptide concentration in the upper side of the gel, between 24 and 66 kDa. It is possible to say that this polypeptide distribution in the 2DE gel confirms the band size distribution visualized in the 1DE gel (Figure 3.2).

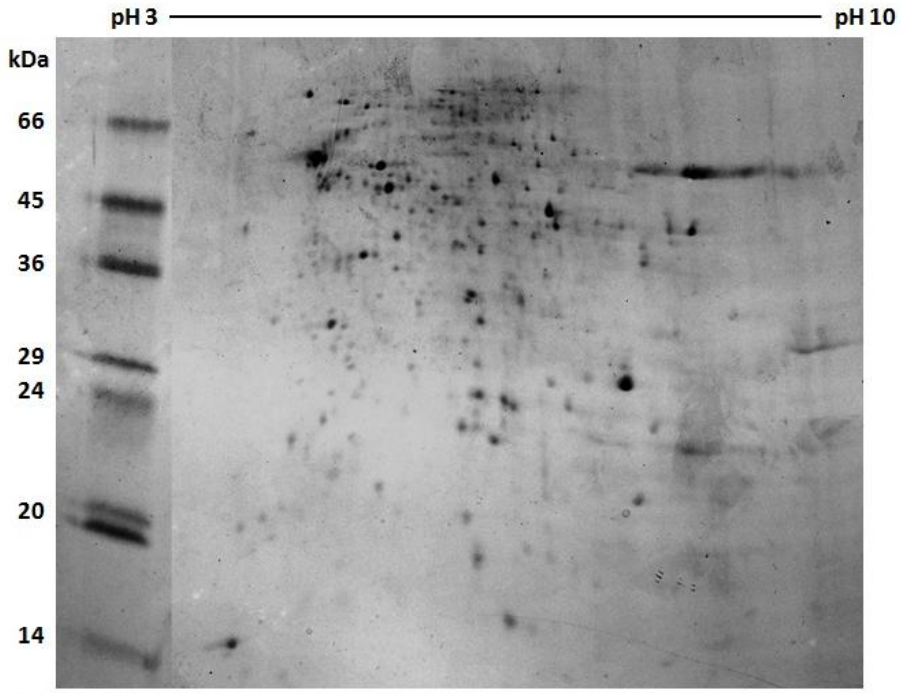


Fig. 3.3 – 2DE electrophoresis of the cell membrane proteome, of *A. fumigatus,* through a 17.5% (w/v) polyacrylamide gel. Four hundred µg of total cell membrane protein was loaded. The gel was stained for total polypeptides with colloidal blue. In the left side, the molecular mass markers (14-66 kDa) are indicated.

After 1DE and 2DE proteome analysis, this work aimed to identify the largest possible number of glycosylated cell membrane proteins that may be detected by SDS-PAGE or on Western blotting membranes (section 2.11.1). The *A. fumigatus* plasma membrane is heavily

glycosylated and the oligosaccharide side-chains present in its glycoproteins, already collectively designated here as the exoglycome, are believed to be involved in fungal recognition/protection processes and may be subjected to host-induced changes during disease attempted pathogenesis. The characterization of these glycoproteins will lead to a better understanding of the oligosaccharide moieties (in this work, the *N*-linked oligosaccharides) and will allow the identification of the ones which might suffer alterations during fungal infection.

3.4 ***A. fumigatus*** **plasma membrane glycoproteome profile analysis through fluorescence methods**

There is an increase consciousness of the posttranslational modifications significance, such as glycosylation, in the production of recombinant proteins and in the proteomic studies of development and disease. This means that the development of, techniques for the identification and characterization of the oligosaccharides attached to proteins need to be established (Packer *et al.*, 2002). In this work, after separation of the proteins by either 1DE or 2DE, the initial step was the identification of the glycoproteins and further characterization.

Two methods have been widely used for detecting glycoproteins in gels or on membrane blots: the non-covalent binding of lectins to specific oligosaccharides and the oxidation of carbohydrate groups followed by conjugation with a chromogenic or tagged substrate (Mehta-D'souza, 2012). In this work, to detect the *A. fumigatus* plasma membrane glycoproteins two methods using fluorescence were used: the Pro-Q Emerald 300 gel stain method (Figure 3.4; section 2.10) and lectin affinoblotting (Figures 3.5 and 3.6; section 2.11.2.

3.4.1 One dimensional electrophoretic (1DE) analysis using the Pro-Q Emerald 300 gel stain method

Initial detection of glycoproteins *in vitro* is routinely accomplished on SDS-Page gels and Western blots. Pro-Q Emerald 300 dye is a fluorescent hydrazide that can be used to detect glycoproteins directly in gels or on blots, relying upon the oxidation of carbohydrate groups (Mehta-D'souza, 2012). Depending on the glycoprotein and the degree of glycosylation, as little as 0.5 ng of glycoprotein can be detected per band in a polyacrylamide gel (Mehta-D'souza, 2012).

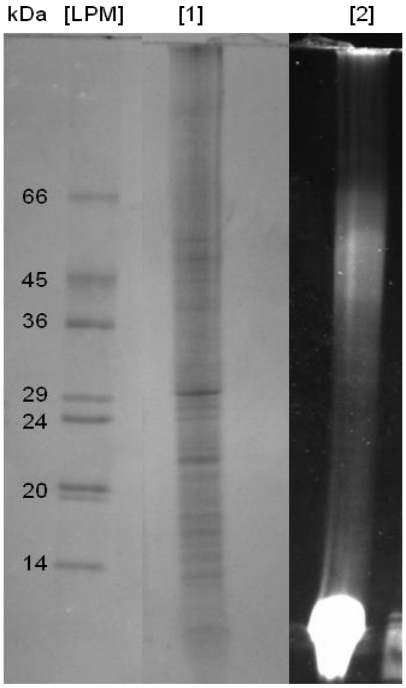


Fig. 3.4 – Detection of glycoproteins in polyacrylamide gel using Pro-Q Emerald 300 dye. [LPM] Low protein molecular mass markers (14-66 kDa). [1] Electrophoretic analysis through a 17.5% (w/v) polyacrylamide gel of *A. fumigatus* plasma membrane proteins. The gel was stained with colloidal blue and 100 µg of total cell membrane protein was loaded. [2] Glycoprotein detection using Pro-Q Emerald 300 gel stain method after electrophoresis through a 17.5% (w/v) polyacrylamide gel (250 µg of total cell membrane protein was loaded).

Through the Pro-Q Emerald 300 stain method it was possible to detect a large range of glycopolypeptides with molecular weights between 14 and 66 kDa (lane [2], Figure 3.4). The image clear shows the inexistence of a specific band/or glycopolypeptide. Also, the image on Figure 3.4, lane (2), shows that the higher amount of oligosaccharides linked to glycopolypeptides has molecular weights between 36 and 66 kDa. This data also shows a strong positive signal (that can be seen at the bottom of lane [2]) that is believed to be unlinked saccharides that are present in the sample due to the plasma membrane protein extraction method (section 2.3) where sucrose gradients were used.

Although it was possible to detect glycopolypeptides in 1DE, no results were obtained for 2DE using the same detection method and the reason for this is unclear. Glycoprotein detection in 2DE would give a more specific idea of which proteins are glycosylated.

The glycoprotein detection method in a 1DE showed that, definitely, there is a considerable amount of glycoproteins in the plasma membrane sample used through this work and, this was just the first approach in the identification of glycosylated proteins.

3.4.2 One dimensional electrophoretic (1DE) and two dimensional electrophoretic (2DE) analysis by lectin affinoblotting

Lectins are most appropriate for the detection of structural subclasses of glycoproteins using methods that are quite similar to standard immunoblotting approaches. Typically, direct lectin conjugates of biotin in conjunction with reporter enzyme conjugates of streptavidin or conjugates of a lectin and reporter enzyme are used in combination with chromogenic, fluorogenic or chemiluminescent substrates (Hart *et al.,* 2003).

They are usually classified on the basis of the monosaccharides with which they interact best, but it is important to note that complex glycoconjugates are generally found to be much better

ligands. In addition, the position of a particular monosaccharide in a glycan chain (i.e., to what it is attached) will affect lectin binding (Packer *et al.*, 2002).

Given the fact that no results were obtained for glycoprotein detection in 2DE with the Pro-Q Emerald 300 stain method, which is a general oligosaccharide detection method, it was necessary to change to a more specific method that would specifically detect carbohydrates and glycoconjugates. This was achieved through lectin affinoblotting using different types of lectins.
In this work 1DE (Figure 3.5) and 2DE (Figure 3.6) analyses by lectin affinoblotting were performed using the following lectins: Con A, PNA, MAL and UEA. These lectins belong to different carbohydrate groups, namely, the mannose/glucose group (Con A), the galactose/*N*-acetylgalactosamine (GalNAc) group (PNA), the *N*-acetylneuraminic acid/sialic acid group (MAL), and the fucose group (UEA).

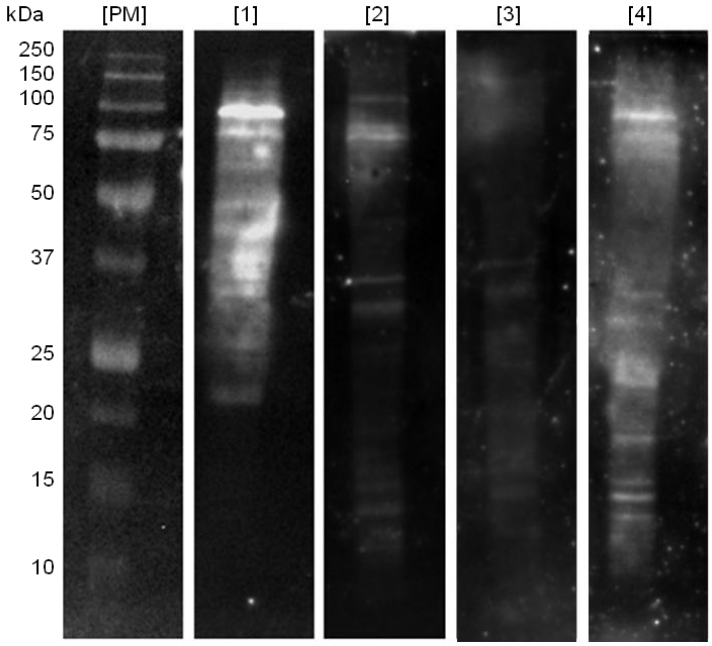


Fig. 3.5 – Lectin affinoblot detection of cell membrane oligosaccharides of *A. fumigatus*, Polypeptides were separated by 1DE (through a 17.5% [w/v] polyacrylamide gel) and blotted onto a PVDF membrane. 150 µg of total cell membrane protein was loaded. [PM] Protein molecular mass markers (10-250 kDa). The affinoblots for oligosaccharide detection were performed with 4 different lectins, namely: [1] Con A; [2] PNA; [3] MAL; and [4] UEA.

The four 1DE lectin affinoblots (lanes [1] to [4], Figure 3.5) showed different band pattern distribution between them, with good resolution in comparison to the results achieved with the Pro-Q Emerald 300 stain method, which indicates the presence of different glycopolypeptides. Furthermore, from this results and aligning the protein bands of the same molecular weight, it is possible to assume that, probably, the same polypeptide have more than one oligosaccharide linkage type.
Comparing signal intensity, the blots showed that there is higher intensity in the Con A affinoblot (lane [1], Figure 3.5) that could indicate a stronger linkage between the lectin used and the corresponding carbohydrate. The MAL affinoblot appears to show lower signal intensity that

might be due to a weaker linkage between lectin and carbohydrate. However when the analysis was made through a 2DE analysis, as shown in Figure 3.6C, the 2DE MAL affinoblot does not follow the same pattern. The different signal intensity could be justified by the fact that in a SDS-PAGE the same electrophoretic band can represent different proteins or polypeptides from different proteins with the same molecular weight, where the oligosaccharide residues might overlap the *N*-acetylneuraminic acid/sialic acid residues that are the target for MAL. The same assumptions can be taken for the blots of PNA and UEA.

Regarding the protein molecular weight, band distribution in the 1DE affinoblots and spot distribution in the 2DE affinoblots, all are evenly distributed.

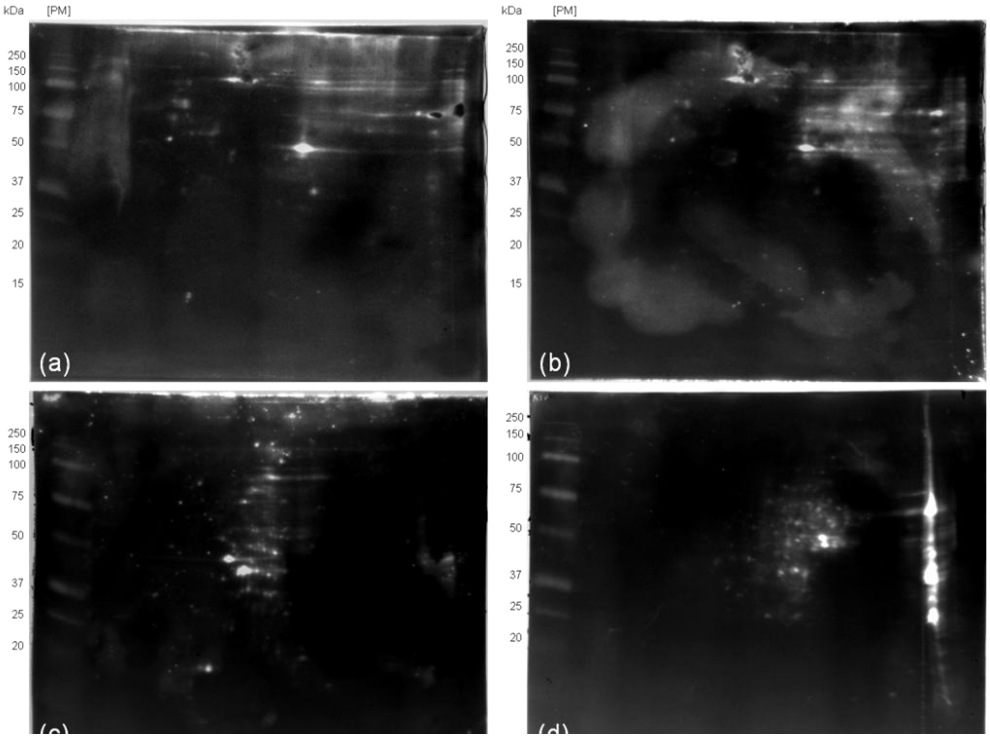


Fig. 3.6 – Lectin affinoblot detection of cell membrane oligosaccharides of *A. fumigatus*, Polypeptides were separated by 2DE (through a 17.5% [w/v] polyacrylamide gel) and blotted onto a PVDF membrane. 500 µg of total cell membrane protein was loaded in each gel. [PM] Protein molecular mass markers (10-250 kDa). The affinoblots for oligosaccharide detection were performed with 4 different lectins, namely: (a) concanavalin A; (b) peanut agglutinin; (c) *Maackia amurensis* lectin; and (d) *Ulex europaeus* agglutinin.

As expected, the 2DE lectin affinoblots showed different patterns for spot distribution. In each blot is possible to detect individual spots, each one representing a different glycopolypeptide, being the MAL and UEA blots (Figure 3.6C and D, respectively) with the highest amount of spots.

The lectin affinoblot results for 1DE and 2DE showed that the lectin approach for plasma membrane glycoprotein detection was the correct choice, for now, although there are certain restrictions to it uses due to the fact that does not represents the entire glycoproteome. The

next step was the isolation of the *A. fumigatus* cell membrane glycoproteins by lectin affinity chromatography.

3.5 Isolation of *A. fumigatus* plasma membrane proteins by lectin affinity chromatography and one dimensional electrophoresis (1DE)

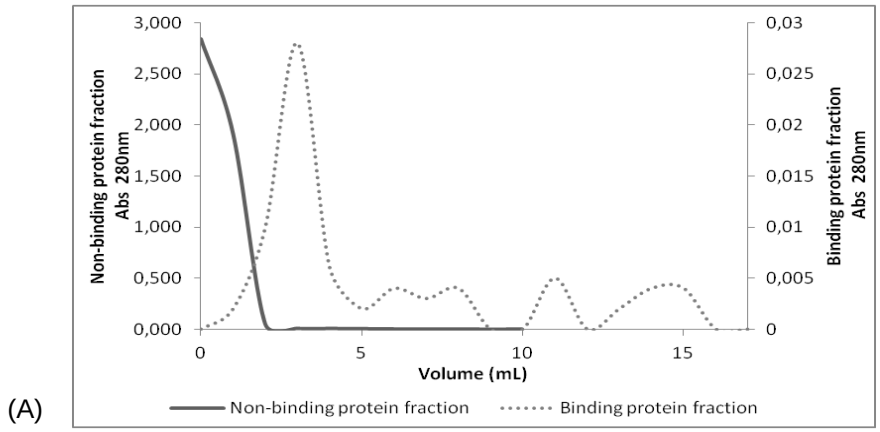
Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix. There are several biological molecular interactions often used in this technique, but lectin affinity chromatography is the most frequently employed specific purification procedure for glycoproteins (GE Healthcare Handbook). In this work this method aimed to separate glycoproteins from non-glycosylated proteins by column chromatography, each one prepared with different lectin matrices (section 2.12). The lectins chosen were Con A, PNA, MAL and UEA, as expected.

3.5.1 Affinity chromatography on concanavalin-A-Sepharose gel

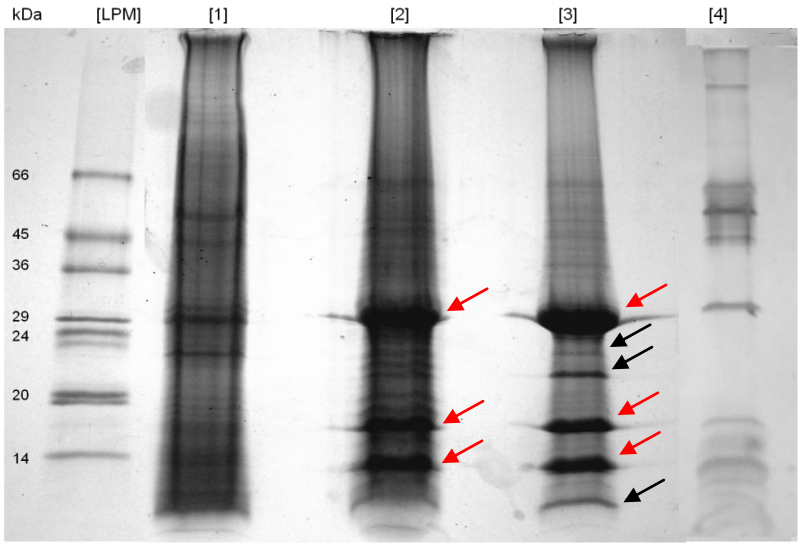
As mentioned before in section 2.12.1, Con A is specific for molecules which contain α-D-mannopyranosyl,α-D-glucopyranosyl or sterically related residues. This lectin has high affinity to *N*-linked carbohydrates and, nowadays, is a widely used tool in a variety of assays such as carbohydrates and glycoconjugates isolation.

When the fungal membrane glycoproteins bind to the gel, a lectin binding-inhibiting sugar is required to elute the bound membrane glycoprotein from the affinity gel. Fractions that represent all glycoproteins that were able to bind to the Con A gel. For Con A the inhibiting sugar used was methyl-α-D-mannopyranoside.

For each fraction of non-binding and binding protein eluted from the Con A chromatography column the absorbance values, at 280 nm, were registered and presented in the graph on Figure 3.7A. Furthermore, a 1DE gel (Figure 3.7B) was performed to verify the purification level of each collected fractions.



(A)



(B)

Fig. 3.7 – Electrophoretic analysis of the cell membrane proteome of *A. fumigatus*. (A) Affinity chromatography on Con A-Sepharose gel. Elution was performed with 0.5 M methyl-α-**D**-mannopyranoside, as the inhibiting sugar. One mL fractions of non-bound and bound protein were collected for each absorbance reading at 280 nm. (B) 1DE through a 17.5% (w/v) polyacrylamide gel was performed with the collected fractions of non-bound protein [2] and bound protein [3]. One hundred µg of total cell membrane protein sample [1] and a negative control [4] were also loaded. [LPM] Low protein molecular mass markers (14-66 kDa). The black arrows (←) indicate the electrophoretic bands that were extracted from the gel for posterior protein identification by PMF. The red arrows (←) indicate a possible contamination with Con A.

By analyzing the data shown in Figure 3.7, it is possible to visualize a major absorbance band eluted from the column in the first 5 mL, for the non-binding protein fraction, which makes it plausible to say that the total polypeptide was eluted in those fractions. As for the binding proteins, the major absorbance band was between the 2nd and 6th mL, indicating that most of the binding proteins present were eluted in that interval. Other absorbance bands, with lower peak values, can be identified for the binding protein fraction, which might also indicate that a small amount of proteins was collected before stabilization.

To better understand the results given by the chromatogram, a 1DE gel was performed. Apart from the fractions collected from the chromatography column, the 1DE gel was also loaded with a sample of total cell membrane protein and a negative control (lanes [1] and [4], respectively, Figure 3.7B), which should only show electrophoretic bands eluted from the Con A column. Although the negative control bands help to distinguish between Con A and plasma membrane

proteins, it can also show if the chromatography column is releasing the lectin as well. In this affinity chromatography assay, the release of Con A from the column is clearly observed (lane [4], Figure 3.7B). This may bring some difficulty in glycoprotein identification due to the fact that the bands chosen as the ones that supposedly correspond to plasma membrane glycoproteins, which must not be contaminated with the lectin Con A.

Regardless of Con A contamination on the binding protein lane (red arrows, lane [3], Figure 3.7B), it was possible to detect several electrophoretic bands that were extracted for protein identification by peptide mass fingerprint (PMF) (indicated with a black arrow, Figure 3.7B).

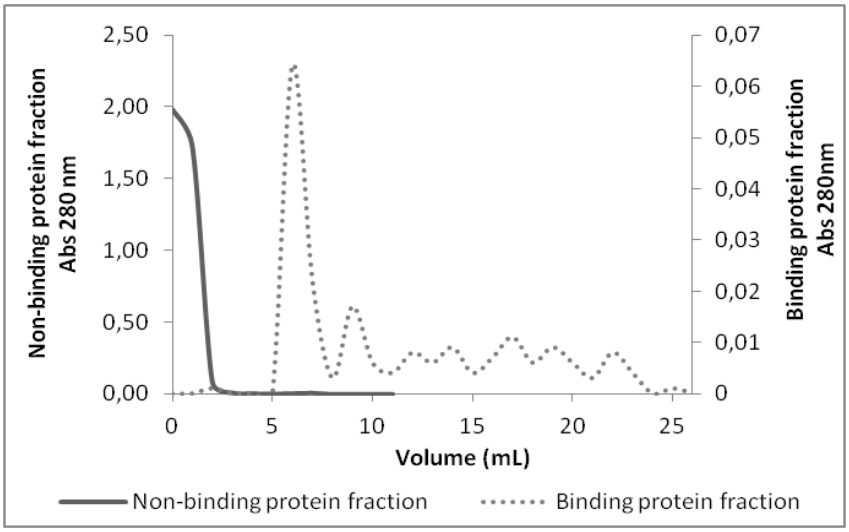
3.5.2 Affinity chromatography on Affisep-PNA-adsorbent gel

As previously mentioned (section 2.12.2), PNA is specific for terminal β-galactosyl residues; more specifically, it binds the carbohydrate sequence galactosyl-β(1,3) *N*-acetylgalactosamine. Because of its specificity towards **D**-galactose residues at non-reducing terminal positions of glycoconjugates, the peanut lectin is a useful tool for histochemical and cell-surface studies.

Regarding the lectin binding-inhibiting sugar used to elute the membrane bound protein fractions, which correspond to the glycoproteins that were able to bind to the PNA gel, the inhibiting sugar was **D**-galactose.

For each fraction of non-binding and binding protein eluted from the PNA chromatography column the absorbance values, at 280 nm, were registered and presented in the graph depicted in Figure 3.8A. A 1DE gel (Figure 3.8B) was also performed to confirm the purification level of each collected fractions.

(A)



(B)

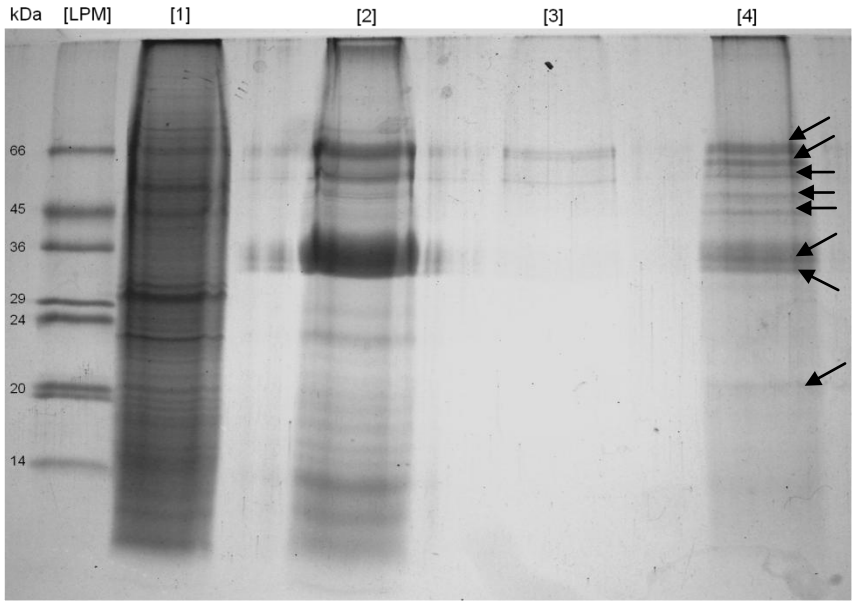


Fig. 3.8 – (A) Affinity chromatography of the cell membrane proteome of *A.* fumigatus on Affisep-PNA-adsorbent gel. Elution was performed with 0.5 M **D-**galactose, as the inhibiting sugar. One mL fractions of non-bound and bound protein were collected for each absorbance reading at 280 nm. (B) 1DE through a 17.5% (w/v) polyacrylamide gel was performed with the collected fractions of non-bound protein [2] and bound protein [4]. One hundred µg of total cell membrane protein sample [1] and a negative control [3] were also loaded. [LPM] Low protein molecular mass markers (14-66 kDa). The black arrows (←) indicate the electrophoretic bands that were extracted from the gel for posterior protein identification by PMF.

By analyzing the chromatogram it is possible to visualize a major absorbance band in the first 5 mL of elution, for the non-bound protein fraction, which makes it plausible to say that the majority of the proteins were eluted in those fractions. As for the bound proteins, the major absorbance band was between the 6th and 9th mL, indicating that most of the bound proteins present were eluted in that interval. Other absorbance bands, with lower level peak values, can be identified for the binding protein fraction, which might also indicate that a small amount of proteins was collected before stabilization.

To better understand the results given by the chromatogram, a 1DE gel was performed. Apart from the fractions collected from the chromatography column, the 1DE gel was also loaded with

a sample of total cell membrane protein and a negative control (lanes [1] and [3], respectively, Figure 3.8B), which should only show electrophoretic bands eluted from the PNA column. Once again, the negative control electrophoretic bands helps to distinguish between PNA and plasma membrane proteins, and to verify if the chromatography column is releasing the lectin as well. Unlike the Con A column, for PNA the negative control did not show any sign of released lectin from the column (lane [3], Figure 3.8B), although it is possible to detect electrophoretic bands between 45 and 66 kDa, which it is believed to be residual plasma membrane proteins as the PNA electrophoretic bands should appear between 25 and 37 kDa.

Although there are no visible electrophoretic bands for PNA in the negative control lane that does not indicate that during elution of the bound protein fractions there was no PNA release as well. Therefore, there is still the possibility of PNA contamination on the bound protein lane (lane [4], Figure 3.8B), which may interfere with future protein identification.

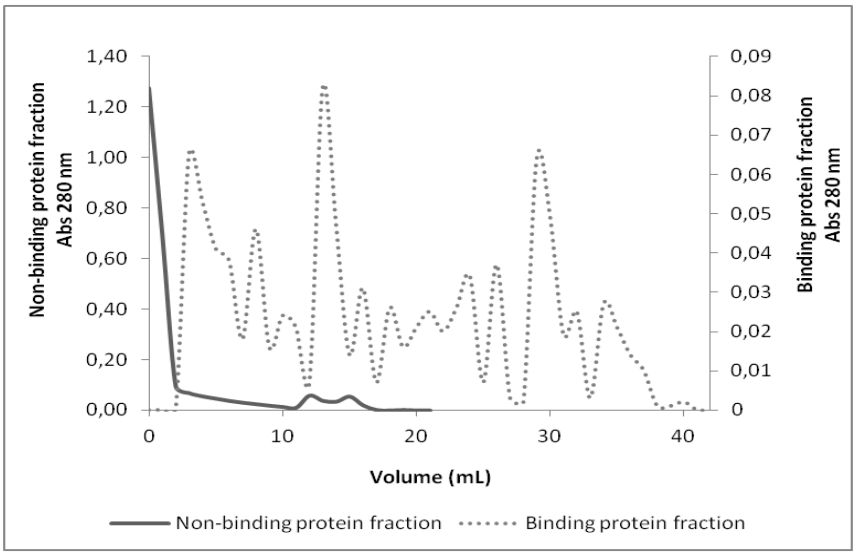
All the detectable protein bands of the eluted fraction (lane [4], Figure 3.8B) were extracted for protein identification by PMF.

3.5.3 Affinity chromatography on Affisep-MAL-adsorbent gel

As referred previously in this dissertation (section 1.3.4.3), MAL is commonly used as glycoanalytical tool to probe biological targets for α-(2-3)-linked sialic acids and interacts with high affinity with complex-type Asn-linked oligosaccharides containing terminal sialic acid in α-(2-3)-linkage to galactose. Lactose was used as the lectin binding-inhibiting sugar.

For each fraction of non-bound and bound protein eluted from the MAL chromatography column the absorbance values, at 280 nm, were registered and presented in the chromatogram on Figure 3.9A. Furthermore, a 1DE gel (Figure 3.9B) was performed to verify the purification level of each collected fractions.

(A)



(B)

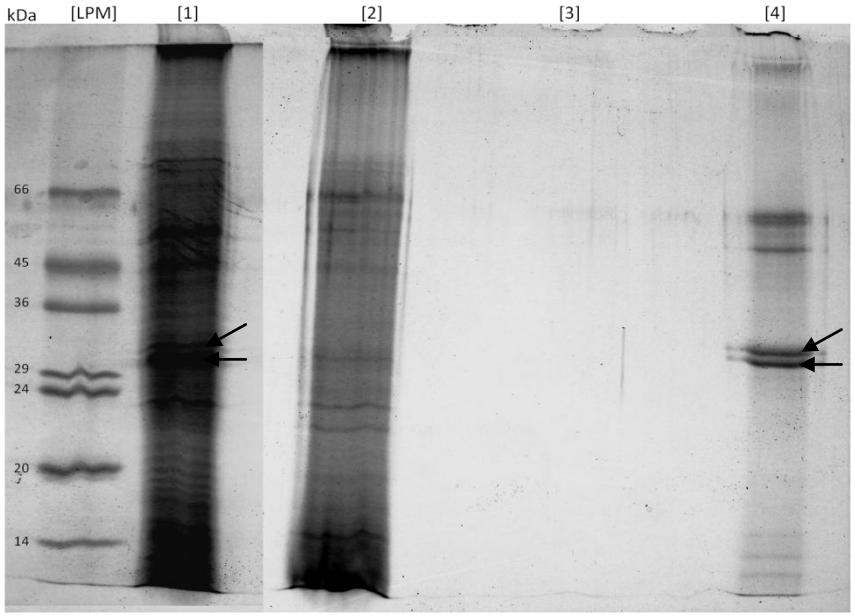


Fig. 3.9 – (A) Affinity chromatography of the cell membrane proteome of *A. fumigatus* on Affisep-MAL-adsorbent gel. Elution was performed with 0.5 M α-lactose, as the inhibiting sugar. One mL fractions of non-bound and bound protein were collected for each absorbance reading at 280 nm. (B) 1DE through a 17.5% (w/v) polyacrylamide gel was performed with the collected fractions of non-bound protein [2] and bound protein [4]. One hundred µg of total cell membrane protein sample [1] and a negative control [3] were also loaded. [LPM] Low protein molecular mass markers (14-66 kDa). The black arrows (←) indicate the electrophoretic bands that can be detected on lane [1] and [4], having the same molecular weight.

By analyzing the data shown in Figure 3.9A it is possible to observe a major absorbance band in the first 8 mL of elution, for the non-bound protein fraction, which makes it plausible to say that the total polypeptide was eluted in those fractions. As for the bound proteins, there was a marked variation in the absorbance values as shown in the graph. However, it is possible to detect three major absorbance peaks at 3, 13 and 29 mL. Between these three main absorbance bands, other bands with lower peak values can be identified.

To better understand the results given by the chromatogram, a 1DE gel (Figure 3.9B) was performed. Apart from the fractions collected from the chromatography column, the 1DE gel was

also loaded with a sample of total cell membrane protein and a negative control (lanes [1] and [3], respectively, Figure 3.9B), which should only show electrophoretic bands eluted from the MAL column. Again, the negative control bands help to distinguish between MAL and plasma membrane proteins, and to verify if the chromatography column is releasing the lectin as well. Like the PNA column, MAL negative control did not show any sign of released lectin from the column (lane [3], Figure 3.9B), that should appear at 38 and 40 kDa. Although there are no visible electrophoretic bands for MAL in the negative control lane or in the bound protein lane (lane [4], Figure 3.9B), there is still the possibility of MAL contamination, which may interfere with future protein identification.

The bound protein lane shows that there are, at least, seven electrophoretic bands that may belong to plasma membrane glycoproteins. It is important to verify if the detected bands on the bound protein lane are detectable in the total cell membrane protein lane (lane [1], Figure 3.9B) as well, to make sure that those bands are from plasma membrane glycoproteins and not from MAL. In this case, it is possible to align two of the seven detected bands with lane [1], with a molecular weight of approximately 30 and 31 kDa (indicated with a black arrow, Figure 3.9B).

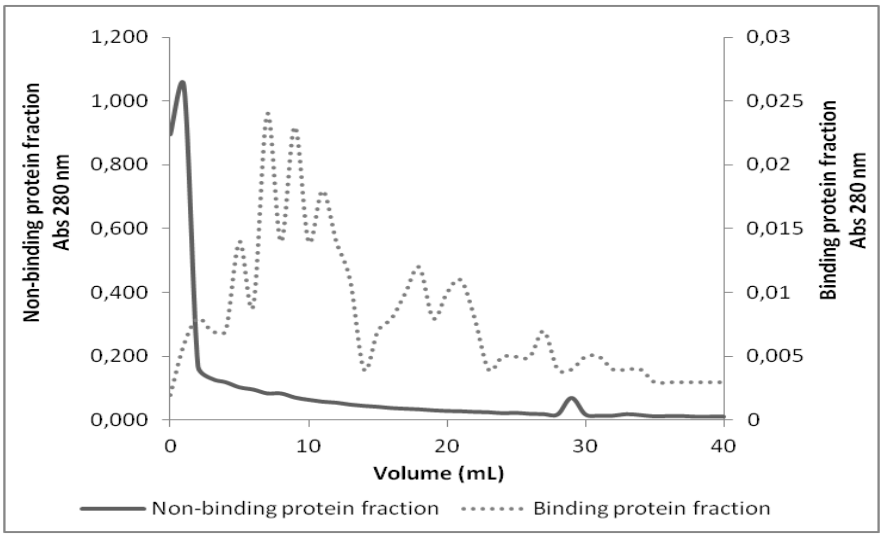
All detectable electrophoretic bands on lane [4] (Figure 3.9B) were extracted for protein identification by PMF.

3.5.4 Affinity chromatography on Affisep-UEA-adsorbent gel

The UEA is a fucose specific legume lectin and has the capacity to bind to glycoproteins and glycolipids that contain L-Fuc α1-2-D-Galβ1-4-D-GlcNAc residues, which is its highest affinity ligand. The sugar used to elute the bound protein fractions to UEA was α-L-fucose.

For each fraction of non-bound and bound protein eluted from the UEA chromatography column the absorbance values, at 280 nm, were registered and presented in the graph on Figure 3.10A. Furthermore, a 1DE gel (Figure 3.10B) was performed to verify the purification level of each collected fractions.

(A)



(B)

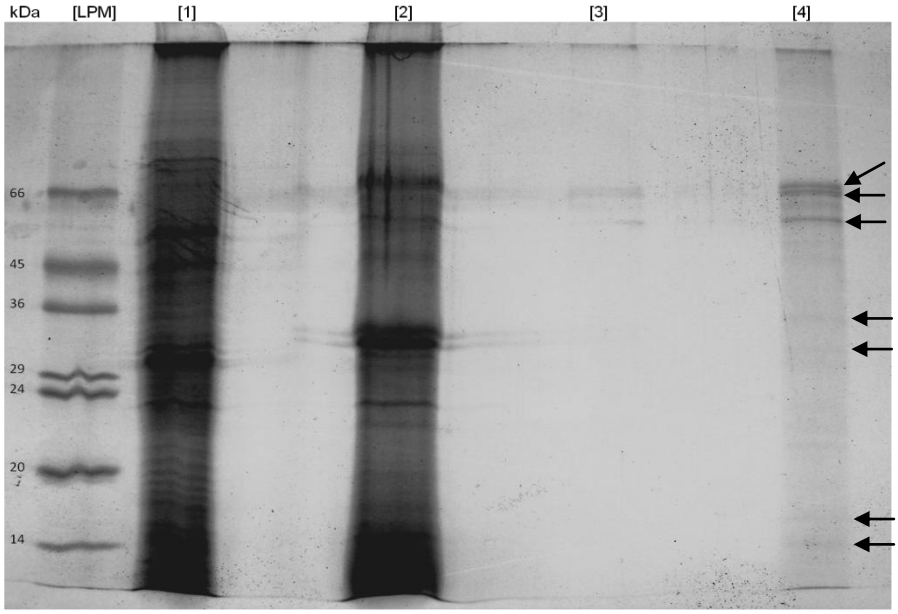


Fig. 3.10 – (A) Affinity chromatography of the cell membrane proteome of *A.* fumigatus on Affisep-UEA-adsorbent gel. Elution was performed with 0.5 M **L**-fucose, as the inhibiting sugar. One mL fractions of non-bound and bound protein were collected for each absorbance reading at 280 nm. (B) 1DE through a 17.5% (w/v) polyacrylamide gel was performed with the collected fractions of non-bound protein [2] and bound protein [4]. One hundred µg of total cell membrane protein sample [1] and a negative control [3] were also loaded. [LPM] Low protein molecular mass marker (14-66 kDa). The black arrows (←) indicate the electrophoretic bands that were extracted from the gel for posterior protein identification by PMF.

By analyzing the data shown in Figure 3.10A it is possible to observe a major absorbance band eluted from the column in the first 11 mL, for the non-bound protein fraction, which makes it plausible to say that the total polypeptide was eluted in those fractions. As for the bound proteins, in comparison to the results obtained for the MAL affinity chromatography, once again there was a marked variation in the absorbance values as shown by the data. However, it is possible to detect three major absorbance peaks at 7, 9 and 11 mL. Besides those three absorbance bands, other bands with lower level peak values were identified before absorbance stabilization.

To better understand the results given by the chromatogram, a 1DE gel (Figure 3.10B) was performed. Apart from the fractions collected from the chromatography column, the 1DE gel was also loaded with a sample of total cell membrane protein and a negative control (lanes [1] and [3], respectively, Figure 3.10B), which should only show electrophoretic bands eluted from the UEA column.

Like the PNA and MAL column, UEA negative control did not show any sign of released lectin from the column (lane [3], Figure 3.10B), although it is possible to detect electrophoretic bands between 45 and 66 kDa that is believed to be residual plasma membrane proteins as the UEA bands should appear between 31 and 32 kDa.

Though there are no visible electrophoretic bands for UEA in the negative control lane or in the bound protein lane (lane [4], Figure 3.10B), there is the possibility of UEA contamination on the bound protein lane that may interfere with future protein identification.

It is possible to detect at least seven electrophoretic bands on lane [4] (indicated with a black arrow, Figure 3.10B) that may belong to plasma membrane glycoproteins. Again, it is important to verify if the detected bands on the bound protein lane are detectable in the total cell membrane protein lane (lane [1], Figure 3.10B) as well, to make sure that those bands are from plasma membrane glycoproteins and not from UEA. Unlike the 1DE gel obtained for the MAL column (Figure 3.9B), on the 1DE gel obtained for the UEA column is quite difficult to compare both lanes (total cell membrane protein and bound protein fraction).

All detectable electrophoretic bands on lane [4] (Figure 3.10B) were extracted for protein identification by PMF.

3.5.5 1DE comparison of the lectin-bound protein fractions collected from all four affinity chromatography columns

After conclusion of the lectin affinity chromatography assays and the 1DE for all four lectins, namely, Con A, PNA, MAL and UEA, a comparison between the electrophoretic lanes of the bound protein fractions (Figure 3.11) was made.

This was useful to visualize the achieved patterns of electrophoretic bands between the four studies, which are unevenly distributed through the 1DE gel (Figure 3.11). It is also interesting to see if there are protein bands that have matching molecular weights that may indicate the possibility that the same polypeptide have more than one oligosaccharide linkage types (this attempt of alignment was also possible with the lectin affinoblots on section 3.4.2; Figure 3.5). But only through mass spectrometry for protein identification it will be possible to have an idea of which type of glycoproteins were detected with the experimental techniques performed throughout this work.

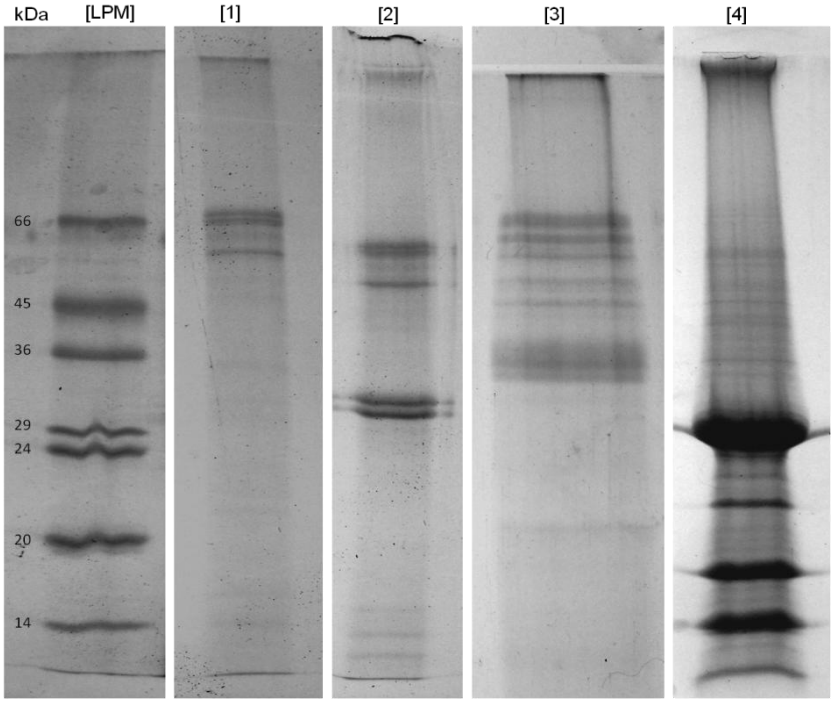


Fig. 3.11 - 1DE comparison of the lectin-bound protein fractions collected from all four affinity chromatography columns. [LPM] Low protein molecular mass markers (14-66 kDa). The lanes correspond to the bound protein fractions of UEA [1], MAL [2], PNA [3] and Con A [4].

3.5.6 *Sequential purification of lectin-binding proteins*

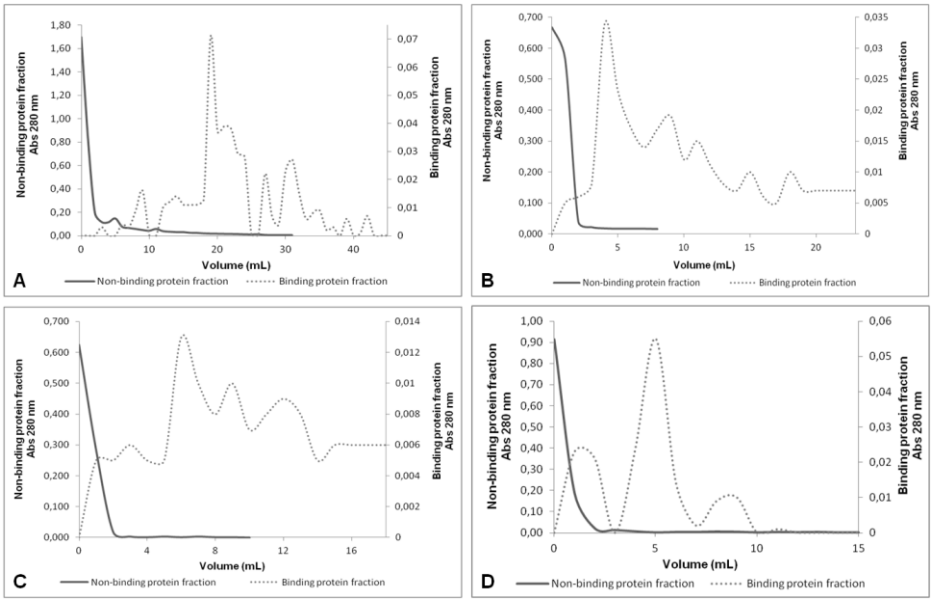
It was referred previously that it may be possible that the same glycoprotein presents more than one oligosaccharide linkage types. Thus, it is possible that certain oligosaccharide linkages may mask other linkages during affinity chromatography. As an example, it is known that PNA discriminates between sialylated and non-sialylated forms of its most powerful inhibitor carbohydrate group, the Galβ1→3GalNAc-, and inferences regarding the carbohydrate structure responsible for PNA binding in histochemical and Western blot analyses have been blurred by the suggestion that PNA also recognizes Galβ1→4GalNAc (LacNAc) groups (Chacko and Appukuttan, 2001), which is the most preferably group; more specifically the terminal Siaα2-3Galβ1-4Glc(NAc) residue, for MAL in *N*-linked oligosaccharides (Geisler and Jarvis, 2011).

To better understand if that interference does happen, a sequential purification of lectin-binding proteins through affinity chromatography was performed in order to observe if the electrophoretic patterns of the bound protein fractions were different from the ones obtained with the isolated lectin affinity chromatography assays. This assay also helped to “clean” the non-bound protein fractions (lane [2], Figure 3.12II), as the same fraction was loaded to every chromatography column in a sequential way as is shown in Figure 3.12II.

Comparing the electrophoretic band patterns of the bound protein fractions obtained with the sequential purification with the ones obtained in each 1DE gel of the isolated lectin affinity chromatography assays, there is clearly a difference between them. In this assay, apart from the MAL binding protein lane (lane [3], Figure 3.12II), which has a good similarity in the distribution of electrophoretic bands, the same did not occur with the other lectins. In the other

binding protein lanes, of PNA, UEA and Con A (lanes [4], [5] and [6], respectively; Figure 3.12II) the release of bound lectin from the column is evident and a major part of the electrophoretic bands obtained with the isolated assays are not present. Though there should be some sign of plasma membrane binding proteins on the last three lanes (lanes [4], [5] and [6]; Figure 3.12II), this may suggest that there was some interference between lectin-protein binding after the original cell membrane sample passed through the MAL chromatography column.

(I)



(II)

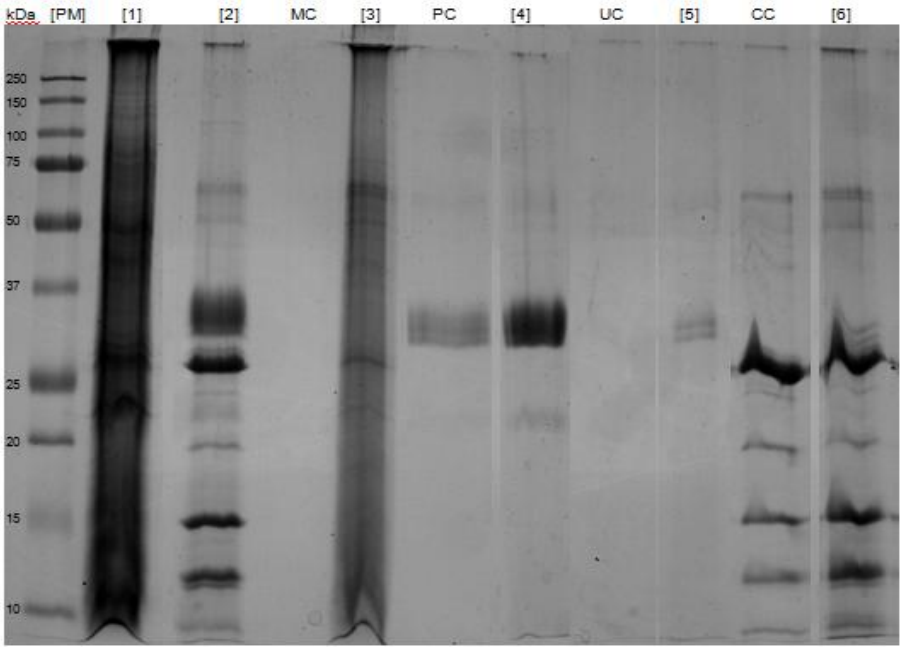


Fig. 3.12 – Sequential purification of lectin-binding proteins. (I) Affinity chromatography chromatogram for: (**A**) MAL, (**B**) PNA, (**C**) UEA and (**D**) Con A. (II) 1DE through a 17.5% (w/v) polyacrylamide gel was performed with the collected fractions of non-bound protein [2] and bound protein ([3] MAL; [4] PNA; [5] UEA; [6] Con A). One hundred µg of total cell membrane protein sample [1] and a negative control of each column ([MC] MAL; [PC] PNA; [UC] UEA; [CC] Con A) were also loaded. [PM] protein molecular mass markers (10-250 kDa).

3.6 Protein identification by peptide mass fingerprint (PMF)

Peptide Mass Fingerprinting (PMF) is an analytical technique used to identify proteins. The unknown protein of interest is first cleaved into smaller peptides, whose absolute masses can be accurately measured with a mass spectrometer (in this work, a MALDI-TOF). These masses are then compared to either a database containing known protein sequences or even the genome. This is achieved by using computer programs that translate the known genome of the organism into proteins, then, theoretically, cut the proteins into peptides, and calculate the absolute masses of the peptides from each protein. After that the masses of the peptides of the unknown protein are compared with the theoretical peptide masses of each protein encoded in the genome. The results are statistically analyzed to find the best match.

In this work, a first attempt of glycoprotein identification was performed with the resulting isolated proteins from two of the lectin affinity chromatography assays (with Con A and PNA). In Figure 3.7 and 3.8 the protein bands that were extracted from the 1DE gel, for protein identification by PMF, are indicated with a black arrow (←). From the 1DE gel of the Con A affinity column were extracted a total of three bands of the bound protein fraction (lane [3], Figure 3.7) and from the 1DE gel of the PNA affinity column were extracted a total of eight bands of the bound protein fraction (lane [4], Figure 3.8).

Protein identification needs to take into account several statistical parameters, such as protein and ion scores. Protein and ion scores above the significance level are considered to be statistically non-random at the confidence interval (C.I.%) of 95%. The closer the C.I.% is to 100%, the more likely the protein is correctly identified.

Tables 3.1 and 3.2 present the results for protein identification of the resulting isolated proteins from the Con A and PNA affinity column, respectively. The mass spectra obtained for the presented samples (Con A1, Con A2, Con A3, PNA 2, PNA 3 and PN5; Tables 3.1 and 3.2) are shown in Appendix A.

As mentioned before in this section, three electrophoretic bands, referring to different proteins, were extracted from the 1DE gel performed for the Con A affinity column and were identified as Con A1, Con A2 and Con A3 (Table 3.1). The peptides of each sample, resulting from enzymatic digestion with trypsin, showed homology with the peptides generated by virtual digestion of each of the proteins listed in the databases used for PMF.

Table 3.1 – Protein identification determined by PMF. The protein samples were extracted from the 1DE gel performed for the Con A affinity chromatography (Figure 3.7B, section 3.5.1).

Sample	Sequenced Peptides (Da)	Databases (taxonomic entries)	Identified Proteins	Protein Score C.I.%
Con A1 (≈ 23 kDa)	1318, 1572, 2103, 2256 and 2474	UniProtKB (*Aspergillus*)	Glycosylphosphatidylinositol anchor related protein (*Trichoderma atroviride*)	98.731
Con A2 (≈ 21 kDa)	893, 1572, 1800, 2103 and 2117	UniProtKB (*Aspergillus*)	40S ribosomal protein S9 (*Aspergillus kawachii*)	100
Con A3 (≈ 12 kDa)	931, 956, 974, 1247 and 1261	UniProtKB (*Eukaryotes*)	Predicted protein (*Chlamydomonas reinhardtii*)	97.895

Peptides from the Con A1 sample showed homology with a membrane protein, glycosylphosphatidylinositol (GPI) anchor related protein (found in *Trichoderma atroviride*, a filamentous fungus commonly found in soil) and presented a C.I.% of 98.731%, which is statistically significant. The amino acid sequence of the GPI anchor related protein shows one conserved domain, the PIG-F domain. This domain belongs to the GPI biosynthesis protein family and is involved in GPI anchor biosynthesis (Hong *et al.*, 2000). Also, through bioinformatic tools, using the YinOYang 1.2 Server and NetNGlyc 1.0 Server (Center for Biological Sequence Analysis, Denmark), a prediction of *O*-glycosylated sites and *N*-glycosylated sites, respectively, was made. The GPI anchor related protein showed one possible *N*-glycosylated site (Figure 3.13) and ten possible *O*-glycosylated sites (Figure 3.14), being the two most viable at position 2 (Ser) and 218 (Thr) of the amino-acid sequence.

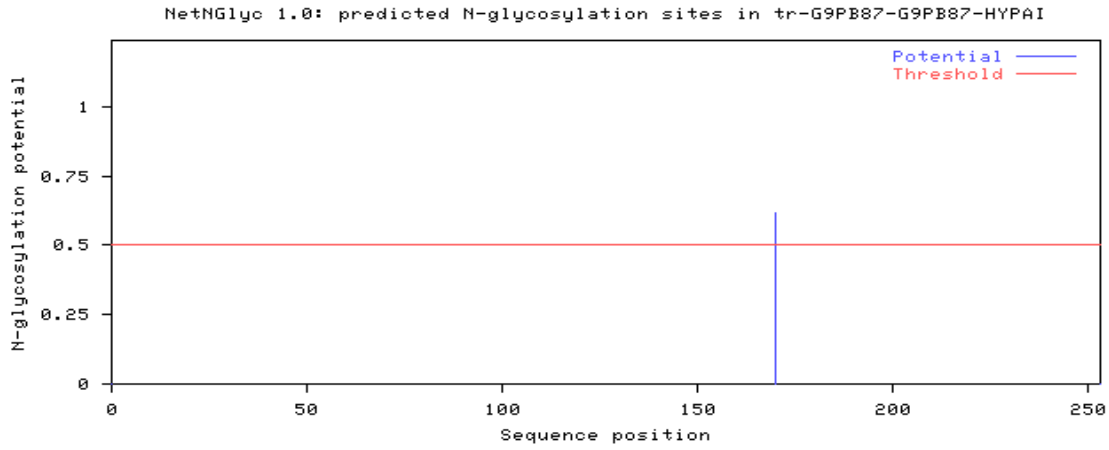


Fig. 3.13 – Predicted positions of *N*-glycosylated sites for the GPI anchor related protein.

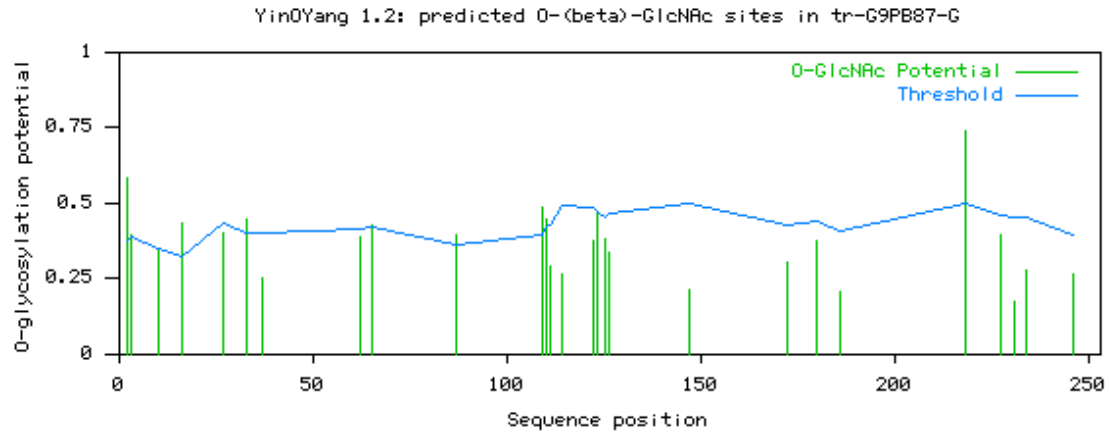


Fig. 3.14 – Predicted positions of *O*-glycosylated sites for the GPI anchor related protein.

The identified peptides from the Con A2 sample showed homology with the 40S ribosomal protein S9, from *Aspergillus kawachii*, and presented a C.I.% of 100%, which is statistically significant. This protein is a structural constituent of the ribosome and the conserved domains that can be found for its amino-acid sequence are related with subunits (like 40S) of ribosomal proteins. As a ribosomal protein it cannot be a membrane protein and, in fact, this result can be due to a contamination of the sample with other constituents of the cell during the extraction and purification protocols. However, it is indeed very complicated to explain concerning its origin and the specificity that is attributed to the lectins affinoblots.

The same bioinformatic tools were used to predict the *N*- and *O*-glycosylated sites of the identified protein. No sites were predicted for *N*-glycosylated site in the 40S ribosomal protein S9 sequence. Nevertheless, there were two site predictions for *O*-glycosylated sites (Figure 3.15), at positions 9 (Ser) and 161 (Thr) of the amino-acid sequence. Again, it is not easy to explain how this protein was purified with the used methodology.

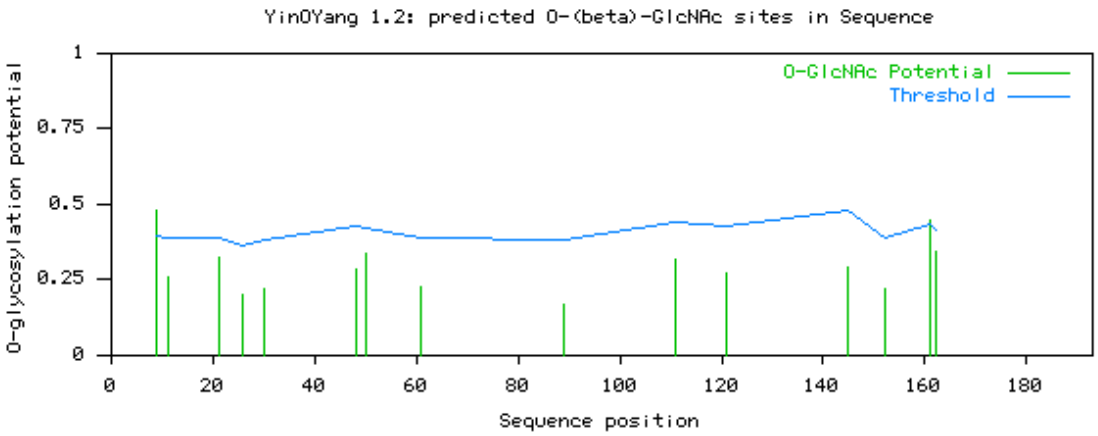


Fig. 3.15 – Predicted positions of *O*-glycosylated sites for the 40S ribosomal protein S9.

The Con A3 sample peptides had homology with a predicted protein, from *Chlamydomonas reinhardtii*, with a C.I.% of 97.895%, whose functional specifications have not been described yet. The same bioinformatic tools were used to predict the *N*- and *O*-glycosylated sites of the identified protein. The identified protein showed two possible *N*-glycosylated sites (Figure 3.16), at positions 28 (Asn) and 1021 (Asn), and thirty one possible *O*-glycosylated sites (Figure 3.17), being the two most viable at position 738 (Thr) and 739 (Thr) in the amino-acid sequence.

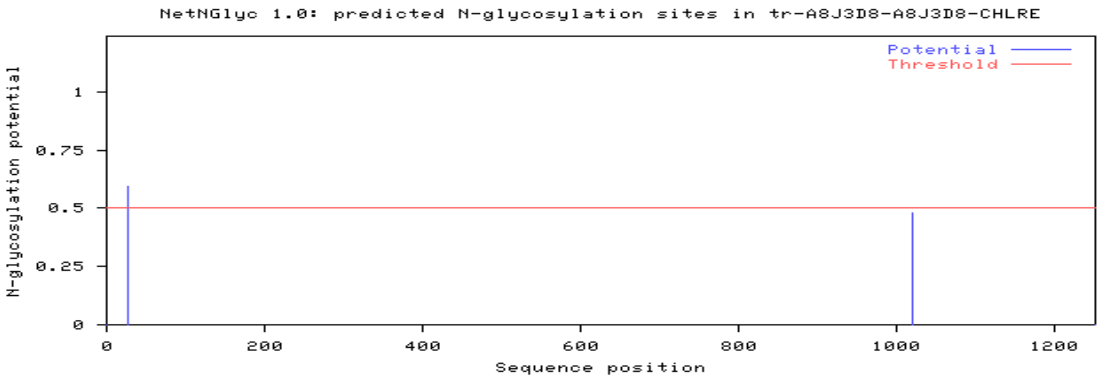


Fig. 3.16 – Predicted positions of *N*-glycosylated sites for the predicted protein.

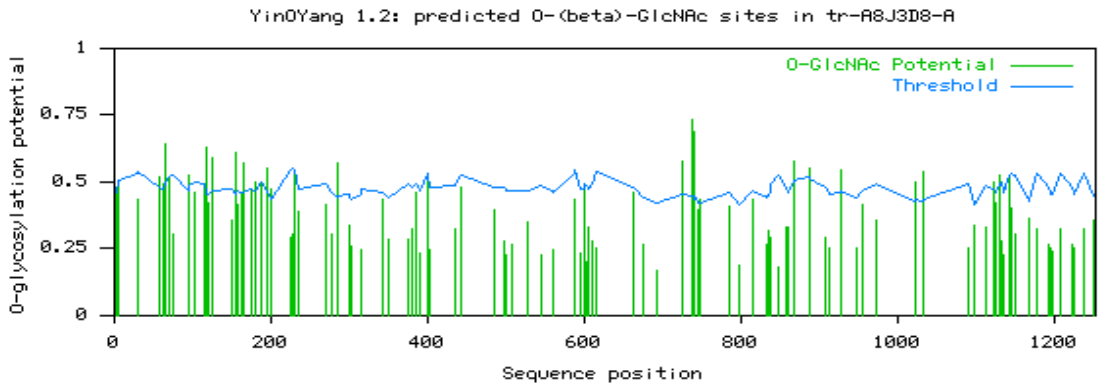


Fig. 3.17 – Predicted positions of *O*-glycosylated sites for the predicted protein.

As previously referred, eight electrophoretic bands, were extracted from the 1DE gel performed for the PNA affinity column, but only three showed significant PMF results (PNA 2, PNA 3 and PNA 5; Table 3.2).

Table 3.2 – Protein identification determined by PMF. The protein samples were extracted from the 1DE gel performed for the PNA affinity chromatography (Figure 3.8B, section 3.5.2).

Sample	Sequenced Peptides (Da)	Databases (taxonomic entries)	Identified Proteins	Protein Score C.I.%
PNA 2 (≈ 45 kDa)	837, 1135, 1321, 1412 and 1878	UniProtKB (*Aspergillus*)	Fluconazole resistance protein (*Candida albicans*)	80.794
PNA 3 (≈ 35 kDa)	779.948, 964, 1142 and 1321	UniProtKB (*Aspergillus*)	WD repeat protein (*Aspergillus kawachii*)	57.983
PNA 5 (≈ 20 kDa)	1149, 1334, 1463, 1494 and 2196	UniProtKB (*Aspergillus*)	C6 transcription factor (*Neosartorya fumigata*)	37.852

Peptides from the PNA 2 sample showed homology with a fluconazole resistance protein (Flu1P), from *Candida albicans*, with a C.I.% of 80.794%. The amino acid sequence of Flu1P showed several conserved domains, such as the major facilitator superfamily (MFS) domain, which is part of a large and diverse group of secondary transporters that includes uniporters,

symporters and antiporters (NCBI, 19.09.12). MFS proteins facilitate the transport across cytoplasmic or internal membranes of a variety of substrates including ions, sugar phosphates, drugs, neurotransmitters, nucleosides, amino acids, and peptides. They do so using the electrochemical potential of the transported substrates. Some MFS proteins have medical significance in humans such as the glucose transporter Glut4, which is impaired in type II diabetes, and glucose-6-phosphate transporter (G6PT), which causes glycogen storage disease when mutated (NCBI, 19.09.12). Other conserved domains, like PRK11102, PRK15402, PRK11043, PRK11551, and PRK10473, are part of a multidrug efflux system.

The same bioinformatic tools were used to predict the *N*- and *O*-glycosylated sites of the identified protein. The identified protein showed three possible *N*-glycosylated sites (Figure 3.18), at positions 3 (Asn), 21 (Asn) and 568 (Asn), and six possible *O*-glycosylated sites (Figure 3.19), being the most viable at position 24 (Thr) in the amino-acid sequence.

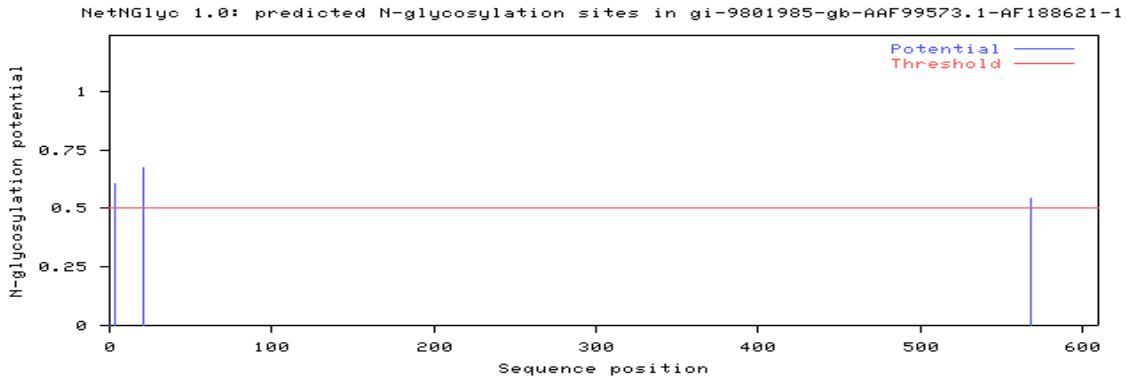


Fig. 3.18 – Predicted positions of *N*-glycosylated sites for the fluconazole resistance protein.

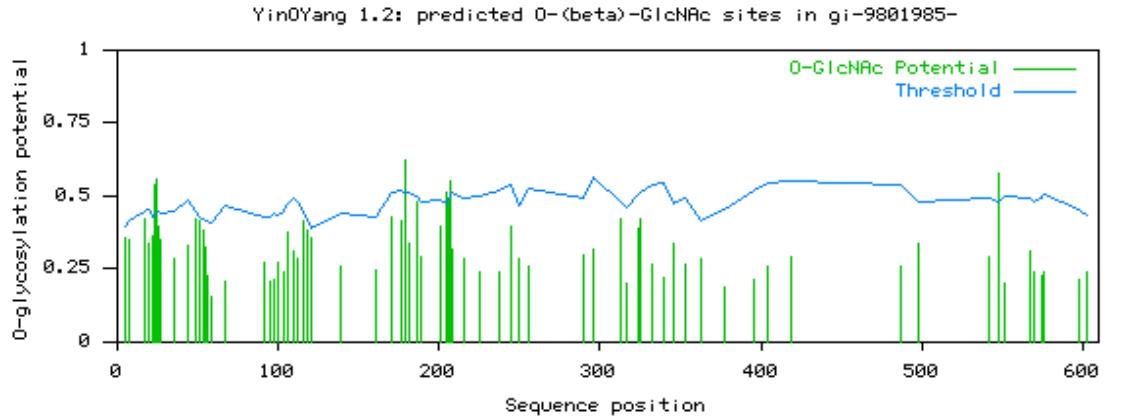


Fig. 3.19 – Predicted positions of *O*-glycosylated sites for the fluconazole resistance protein.

The peptides acquired for PNA 3 sample showed homology with a WD repeat protein, again from *A. kawachii*, with a C.I.% of 57.983%. The WD repeat protein contains the WD40 domain which repeats itself several times in the amino acid sequence. The WD40 domain is found in a number of eukaryotic proteins that cover a wide variety of functions including adaptor/regulatory

modules in signal transduction, pre-mRNA processing and cytoskeleton assembly (NCBI, 18.09.12).

The same bioinformatic tools were used to predict the *N*- and *O*-glycosylated sites of the identified protein. The identified protein showed three possible *N*-glycosylated sites (Figure 3.20), at positions 24 (Asn), 142 (Asn) and 269 (Asn), and sixteen possible *O*-glycosylated sites (Figure 3.21), being the most viable at position 144 (Thr) and 153 (Thr) in the amino-acid sequence.

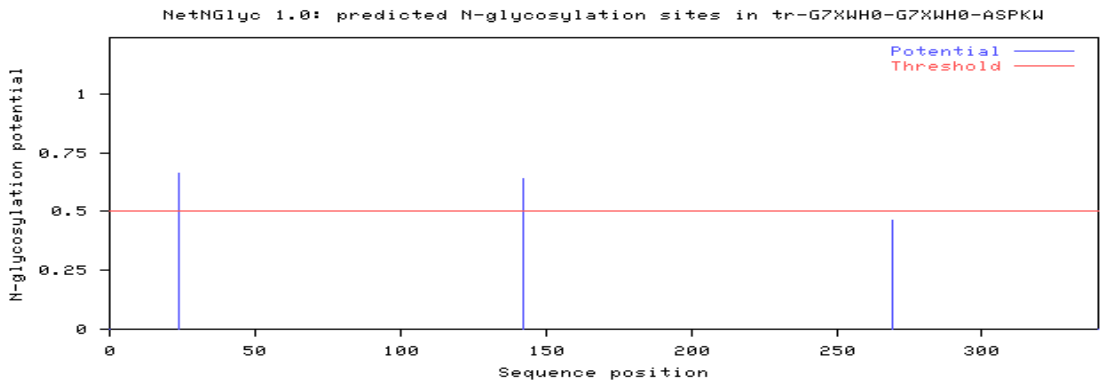


Fig. 3.20 – Predicted positions of *N*-glycosylated sites for the WD repeat protein.

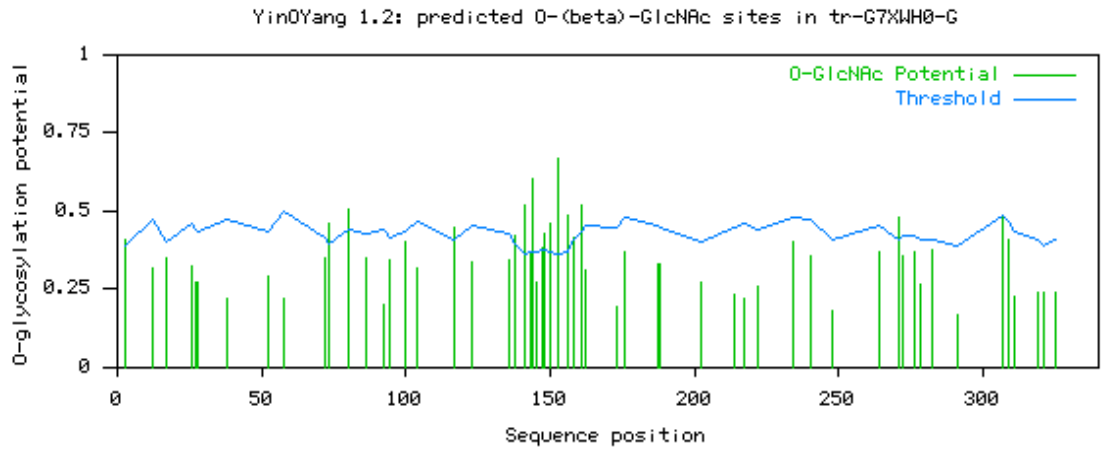


Fig. 3.21– Predicted positions of *O*-glycosylated sites for the WD repeat protein.

The PNA 5 sample resulting peptides showed homology with a C6 transcription factor, from *Neosartorya fumigate* (also known as *Aspergillus fumigatus*), with a C.I.% of 37.852%. The amino acid sequence for this transcription factor possess three different conserved domains, namely, the fungal_trans, GAL4 and Zn_clus domains. The fungal_trans domain is present in the large family of fungal zinc cluster transcription factors that contain an N-terminal GAL4-like C6 zinc binuclear cluster DNA-binding domain, and it has been suggested that this region plays a regulatory role.

The same bioinformatic tools were used to predict the *N*- and *O*-glycosylated sites of the identified protein. The identified protein showed four possible *N*-glycosylated sites (Figure 3.22), being the most viable at positions 79 (Asn) and 501 (Asn). Twenty seven possible *O*-glycosylated sites (Figure 3.23) were found, being the most viable at positions 36 (Thr) and 632 (Ser) in the amino-acid sequence.

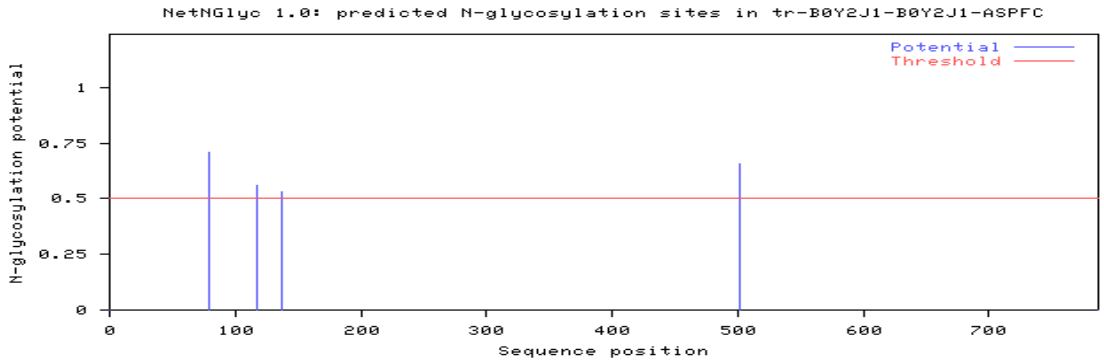


Fig. 3.22– Predicted positions of *N*-glycosylated sites for the C6 transcription factor.

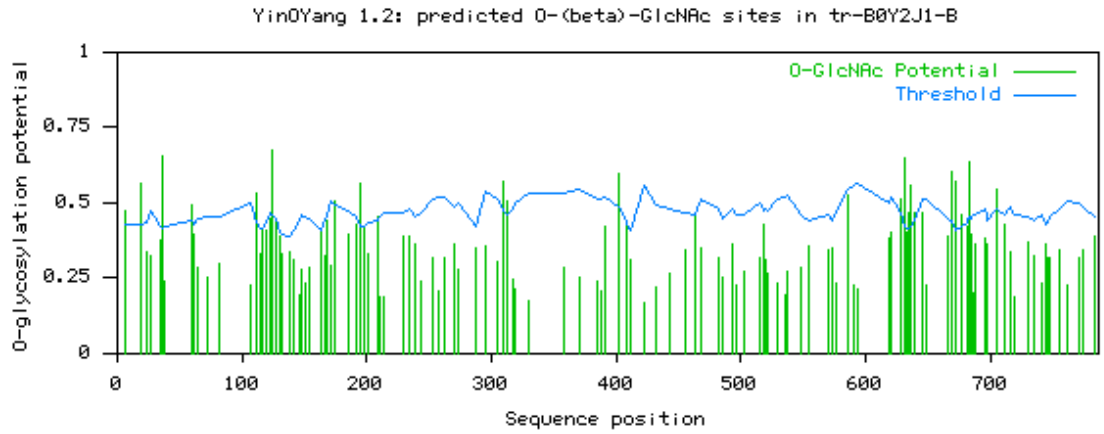


Fig. 3.23– Predicted positions of *O*-glycosylated sites for the C6 transcription factor.

The previously described proteins showed homology to the analyzed peptides (from PNA 2, PNA 3 and PNA 5 samples), however their C.I.% were in the cases of PNA 3 and PNA 5 far below from 100% indicating that these proteins may have been incorrectly identified.

Besides the bound protein fractions obtained from the Con A and PNA affinity chromatography assays, electrophorectic bands of the bound protein fraction were also extracted from the 1DE gel (Figure 3.9 and 3.10) performed for MAL and UEA affinity chromatography assays. These polypeptide bands were sent to IPATIMUP for protein identification by PMF, but we are still waiting for the sequencing results.

3.7 Quantification of glycoproteins linked oligosaccharide levels present in an *A. fumigatus* cell membrane protein sample

A first attempt of oligosaccharide quantification and purification (section 2.14) was performed in order to have a better understanding of the amount of linked oligosaccharides isolated that are possible to be used in further testing.

To release the *N*-linked oligosaccharide side chains samples were treated with PNGase-F, which is an amidase that cleaves between the innermost *N*-acetylglucosamine (GlcNAc) and Asn residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins (Maley *et al.,* 1989).

The value obtained for oligosaccharide concentration was 3.1 μg/μL ± 0.3. This low value was expected and is correlated with the low amount of glycoproteins purified during the former steps of the work.

4. Conclusion

Fungal pathogens have a deleterious impact on human health as they claim hundreds of thousands of human lives each year. Invasive infections caused by these fungal pathogens may reach mortality rates of up to 80%. The lack of suitable fungicides and the fungal capacity to grow in organs such as the brain or lungs explain their devastating effects. For example, in immunocompromised individuals, invasive aspergillosis displays mortality rates of 20 to 90%, often exceeding 50% in high-risk groups, even with antifungal treatment. This patient population is expanding due to the increasing use of transplantation for end organ disease, the development of immunosuppressive therapies for autoimmune and neoplastic disease, and the human immunodeficiency virus/AIDS pandemic (Ferreira *et al.,* 2012).

The molecular interactions which take place during attempted pathogenesis between host and fungal pathogen often display an exquisite complexity. Such interactions may be compared to an open warfare, whose major weapons are proteins and their metabolites. The outcome of the war results in the establishment of resistance or pathogenesis.

Carbohydrates from fungal cell walls have been used as prime targets to develop new drugs or by the pathogens themselves to their own benefit. Many of these carbohydrates, more specifically oligosaccharides, comprise both the cell wall and the plasma membrane, which protects the cytoplasm from the extracellular milieu. The plasma membrane of fungal pathogens contains many glycoproteins and glycolipids, whose oligosaccharide side-chains, collectively termed the exoglycome, are projected towards the cell exterior. For this reason, the study of the plasma membrane glycoproteins and glycolipids, and therefore the exoglycome, is of great importance.

Throughout this work, a first attempt to identify the main components of *A. fumigatus* cell membrane glycoproteins and its exoglycome was made. Membrane proteins are generally very difficult to work with due to their conformational characteristics and their hydrophobicity. For example, one greatest single obstacle is the ability to maintain solubilized proteins in fully dispersed monomeric micelles without inactivating the protein.

In this work, it was very important to choose an effective extraction and purification method to acquire the largest amount possible of protein sample to proceed to more specific studies. For glycoprotein detection different methods were used achieving some successful results. Isolation of cell membrane glycoproteins was also possible through lectin affinity chromatography that aimed to separate glycoproteins from non-glycosylated proteins by column chromatography.

Moreover, a first attempt of glycoprotein identification was performed with the resulting isolated proteins from two of the lectin affinity chromatography assays (with Con A and PNA). From the peptide mass fingerprint (PMF) analyses, it was possible to find homology between the isolated glycoproteins and other already described proteins. Two of the identified proteins, namely, glycosylphosphatidylinositol (GPI) anchor related protein and fluconazole resistance protein are membrane proteins found in microorganisms that are closely related to *A. fumigatus*.

Futhermore, the glycosylation predictions made by bioinformatic tools showed that these proteins have potential glycosylated sites.

Although these PMF results might indicate that the sample used throughout this work was indeed isolated and purified, and that it was possible with the used methodology to work with cell membrane glycoproteins, they are not enough to make acceptable protein identification. These insufficient results raise the question whether there has been a sequencing error or a major problem with the extraction and purification protocols performed for plasma membrane protein isolation. This work will proceed in order to answer these questions and to accomplish the desired objectives.

5. References

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Appendix

Appendix A – Obtained spectra for the selected isolated proteins, of *A. fumigatus*, from the Con A and PNA affinity column, by MALDI-TOF mass spectrometry (section 3.6).

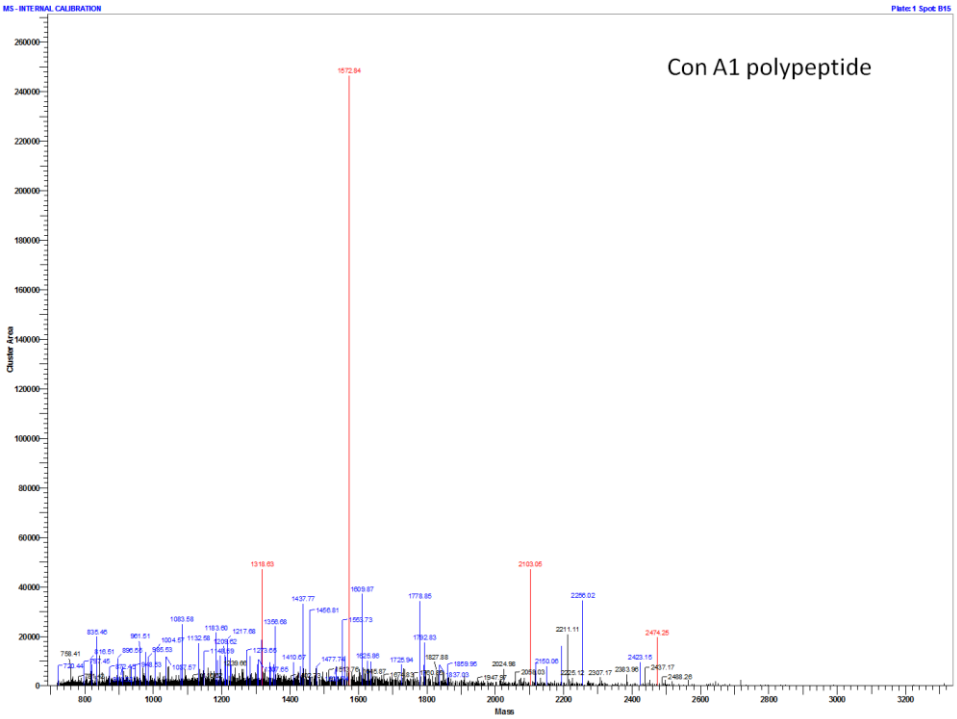

Fig. 6.1 – MALDI-TOF mass spectrum of Con A1 polypeptide.

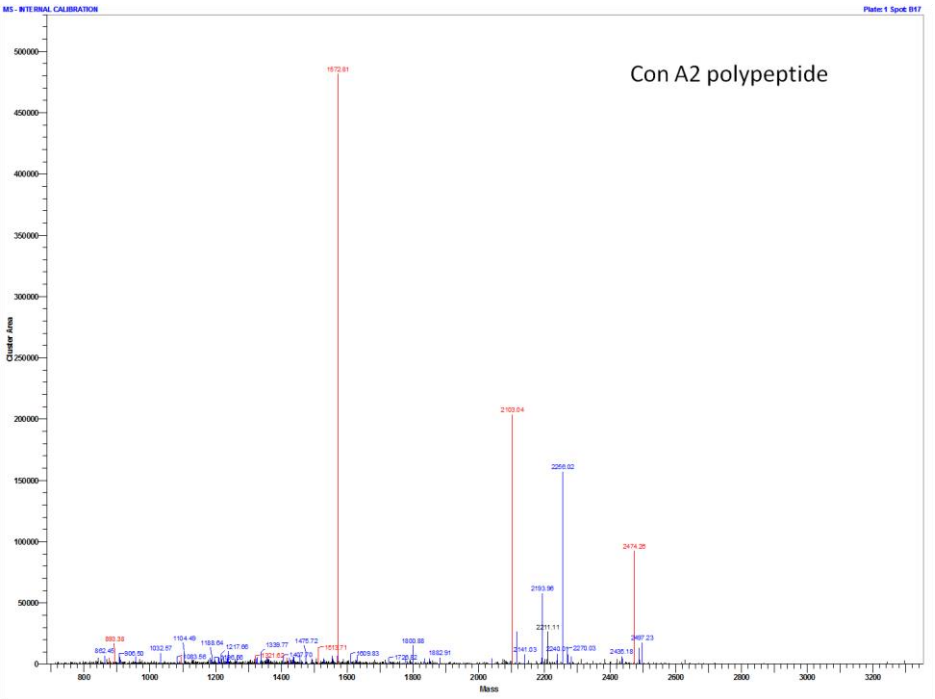

Fig. 6.2 – MALDI-TOF mass spectrum of Con A2 polypeptide.

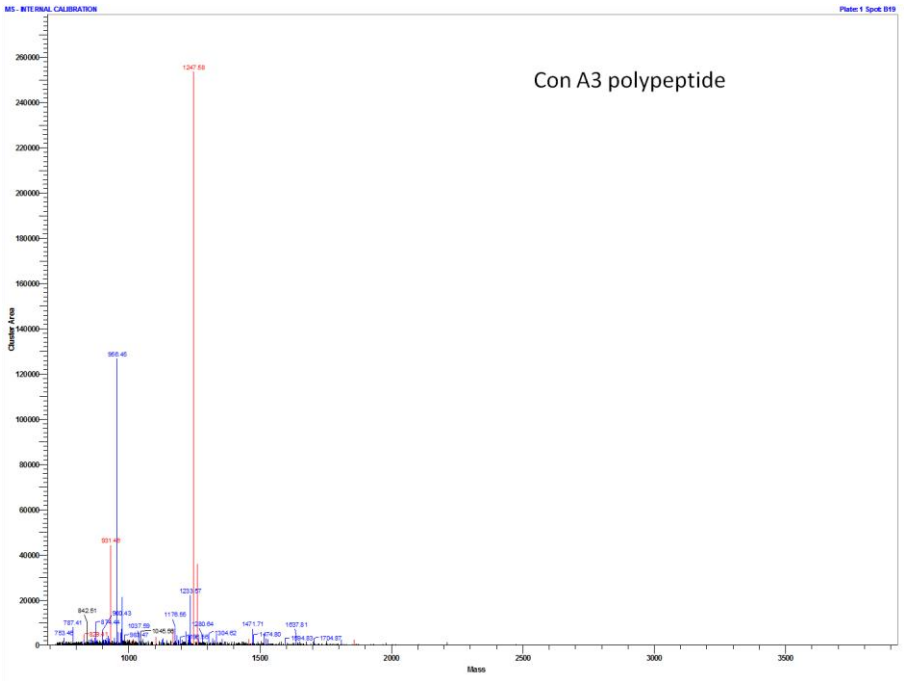

Fig. 6.3 – MALDI-TOF mass spectrum of Con A3 polypeptide.

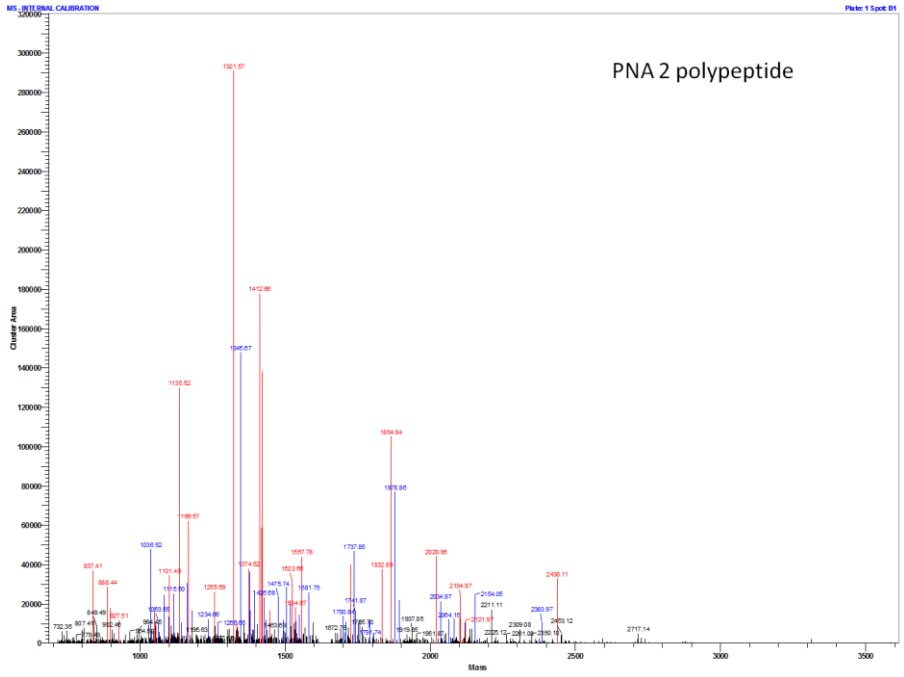

Fig. 6.4 – MALDI-TOF mass spectrum of PNA 2 polypeptide.

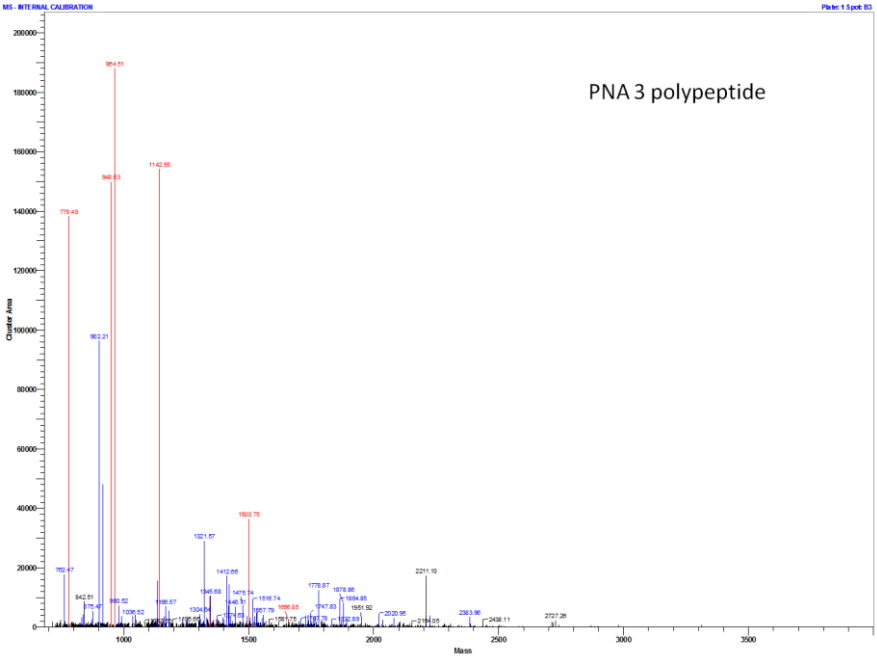

Fig. 6.5 – MALDI-TOF mass spectrum of PNA 3 polypeptide.

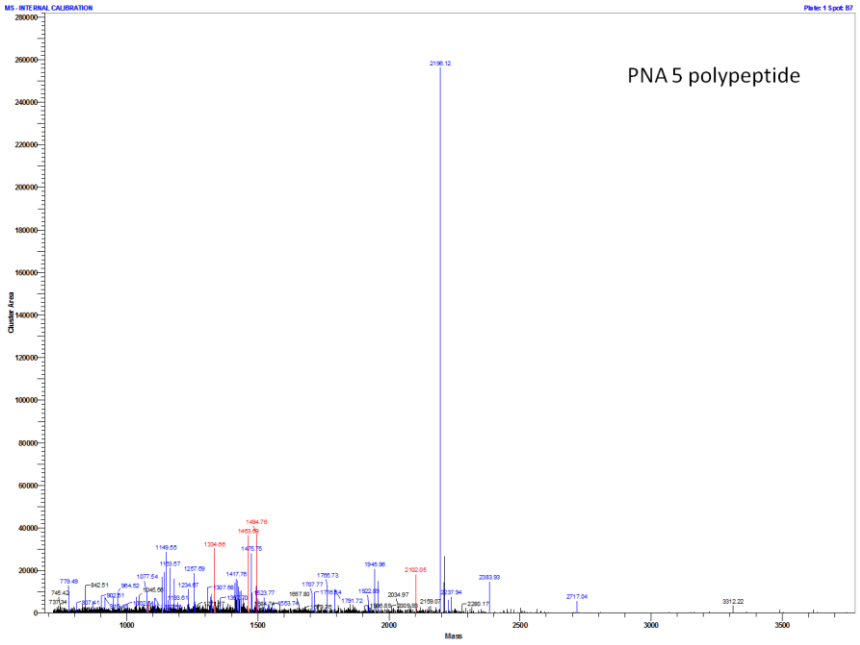

Fig. 6.6 – MALDI-TOF mass spectrum of PNA 5 polypeptide.