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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Purification and Characterization of Metalloproteinases and Chitinase from *Laetiporus sulphureus*

Diana Cristina Simões Maurício

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professora Doutora Paula Veríssimo (Universidade de Coimbra).

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2014

“Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.”

Thomas Edison

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SUMMARY

Higher fungi proteins are known for their singular properties and specially, they are considered safe for human consumption due to the long-term exposure, namely in nourishment. Hence, they are becoming recognized as a potential source of new and improved proteins.

Several hydrolytic proteins have already been purified and characterized in basidiomycetes. In this are included proteases with superior characteristic such as thermostability and high pH range resistance. Other proteins such as lectins, laccases, xylanases, among others, were also found and purified in basidiomycetes, with potentially interesting applications in industry.

Laetiporus sulphureus is a wood-rotting basidiomycete creating large fruiting bodies. Several interesting activities of secondary metabolites were already identified. Although very little is known about its hydrolytic potential.

Thereby, this study focused on the identification of the biotechnological potential of *Laetiporus sulphureus*. Thus enzymatic and protein characterization of the fruiting body was carried out, with special attention to hydrolytic activities.

Furthermore, in this work hydrolases purifications were carried out by a serie of processes such as: 85% Ammonium Sulphate precipitation, gel filtration chromatography and anion exchange chromatography. Two metalloproteinases, one with 86.7 kDa and other with 232.2 kDa, were isolated and characterized. A chitinase was also partially purified, although its visualisation in SDS-Page was not achieved.

In conclusion, this study reveals the great biotechnological potential of *Laetiporus sulphureus* since this is just the beginning of the analysis to this macrofungi. So, “Chicken of the woods” shows itself as an uncharted pool of hydrolytic activities.

Key Words: *Laetiporus sulphureus; Metalloproteinase; Chitinase*

RESUMO

Proteínas de fungos superiores são conhecidas pelas suas propriedades únicas e, especialmente, são consideradas seguras para consumo humano devido ao longo tempo de exposição, nomeadamente na alimentação. Desta forma, estes estão a começar a ser reconhecidos como uma possível fonte de novas e melhoradas proteínas.

Várias hidrolases já foram purificadas e caracterizadas em basidiomicetes. Nestas estão incluídas proteases com características superiores como a termoestabilidade e resistência a uma vasta gama de pHs. Outras enzimas como lectinas, lacases, xilanases, entre outras, foram também encontradas e purificadas em basidiomicetes, com possíveis aplicações interessantes na indústria.

Laetiporus sulphureus é um basidiomicete que causa o apodrecimento da madeira e que forma grandes corpos frutados. Já foram identificadas algumas atividades de metabolitos secundários interessantes. Contudo, muito pouco é conhecido no que diz respeito ao seu potencial hidrolítico.

Assim sendo, este estudo incide na identificação do potencial biotecnológico do *Laetiporus sulphureus*. Desta forma, foi levada a cabo a caracterização enzimática e proteica do corpo frutado, tendo em especial atenção as atividades hidrolíticas.

Além disso, neste trabalho realizaram-se purificações de hidrólases através de uma série de processos, tais como: Precipitação com 85% de Sulfato de Amónia, cromatografia de exclusão molecular e cromatografia de troca aniónica. Duas metaloproteases, uma com 86.7 kDa e outra com 202.2 kDa, foram isoladas e caracterizadas. Uma quitinase também foi parcialmente purificada, contudo a sua visualização em SDS-Page não foi conseguida.

Concluindo, este estudo revela o grande potencial biotecnológico *do Laetiporus sulphureus*, uma vez que isto é apenas o início da análise a este macrofungo. Assim, a "Galinha do bosque" revela-se como uma fonte de atividades hidrolíticas inexplorada.

Palavras-chave: *Laetiporus sulphureus; Metaloprotease; Quitinase*

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Ls – *Laetiporus sulphureus*
Ala-AMC – Alanine-4-Methyl-Coumaryl-7-Amide
Arg-AMC – Arginine-4-Methyl-Coumaryl-7-Amide
Phe-AMC – Phenylalanine-4-Methyl-Coumaryl-7-Amide
Met-AMC – Methionine-4-Methyl-Coumaryl-7-Amide
Leu-AMC – Leucine-4-Methyl-Coumaryl-7-Amide
BzArg-AMC – Benzoyl-Arginine-4-Methyl-Coumaryl-7-Amide
Lys-AMC – Lysine-4-Methyl-Coumaryl-7-Amide
Ala-Ala-Phe-AMC – Alanine-Alanine-Phenylalanine-4-Methyl-Coumaryl-7-Amide
Phe-Arg-AMC – Phenylalanine-Arginine-4-Methyl-Coumaryl-7-Amide
Gly-Pro-AMC – Glycine-Proline-4-Methyl-Coumaryl-7-Amide
SucLLVT-AMC–N-Succinyl-Leucine-Leucine-Valine-Tyrosine-4-Methyl-Coumaryl-7-Amide
Gly-Pro-Arg-AMC – Glycine-Proline-Arginine-4-Methyl-Coumaryl-7-Amide
SucAAPP-AMC - N-Succinyl-Alanine-Alanine-Proline-Phenylalanine-4-Methyl-Coumaryl-7-Amide
BSA – Bovine Serum Albumin
PFaBloc - 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride
DTT – Dithiothreitol
EDTA – Ethylenediaminetetraacetic acid
E-64 - *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane
TPCK - Tosyl phenylalanyl chloromethyl ketone
TLCK – Tosyl lysine Chloromethyl Ketone
kDa – kilodalton
AMC – Methyl-Coumaril-7-Amide
MUC– 4-Methylumbelliferone-β-Cellobiose
MUGlc – 4-Methylumbelliferone-β-D-Glucopyranose
4-MUP – 4-Methylumbelliferone-Phosphate
MU-NAG - 4-Methylumbelliferone-N-Acetyl-Glucosaminide
AS – Ammonium Sulphate
ON – Overnight
RPM – Rotations Per Minute
RT – Room Temperature
SDS – Sodium Dodecyl Sulphate
SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TEMED – N, N, N', N'-tetramethylethylenediamine

INTRODUCTION

1.1 - Fungi

In the recent decades, interest in biologically active fungal compounds has increased. Fungi are the third largest group of eukaryotes after animals and plants. A total of 140 000 fungal species, also called higher fungi, that form what is commonly called mushrooms and belong to the phyla Ascomycetes and Basidiomycetes are registered. Of these, around 2000 species are considered edible, and only about 200 have traditionally been harvested for food or for medicinal and other preparations (Erjavec, Kos et al. 2012).

Higher fungi have diverse morphological, physiological and metabolic characteristics that support their diverse lifestyles that include parasites, saprotrophs that degrade dead organic matter mainly of plant origin, and symbionts that form lichens and mycorrhyzae.

It is known that fungal species, specially the so called mushrooms, have been used for medicinal purposes since the early stages of development of the Oriental culture. For thousands of years, higher fungi have been known as an important source for treatment of human diseases (Karaman, Jovin et al. 2010).

Biotechnologically valuable bioactive proteins of higher fungi have already been found in fruiting bodies, cultivated mycelium or its supernatant. Therefore, Ascomycetes and Basidiomycetes are starting to be taken in consideration as a new powerful, natural and consumer friendly form of production of bioactive proteins with an essential role in biotechnology and medicine fields (Hilden, Hakala et al. 2009, Elvan, Ertunga et al. 2010, Wong, Ng et al. 2010).

Only 10% of higher fungi species are known, making them an enormous uncharted pool of potentially useful new molecules and substances (Lindequist, Niedermeyer et al. 2005, Hibbett, Binder et al. 2007, Wasser 2011).

1.1.2 - Basidiomycetes properties

Basidiomycota phyla, which covers Basidiomycetes represents about 34% of all species of fungi known to Man. Basidiomycetes are considered higher fungi, since they form fruiting bodies and include most of the so called mushrooms.

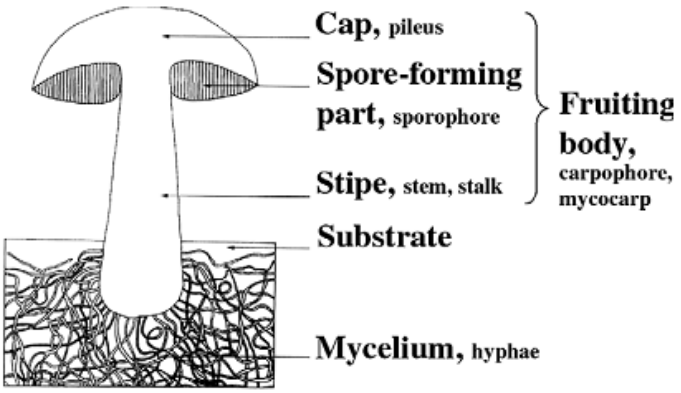

Figure 1 - Sketch of Basidiomycetes morphology(Kalač 2009).

Higher fungi are known as a source of proteins and secondary metabolites with impressive bioactivity. Although medicinal mushrooms (MMs) already have an established history of use in ancient and oriental therapies, essentially Basidiomycetes (Wong, Ng et al. 2010).

In recent years MMs have been the target of special attention due to the fact that they show strong pharmacological and biological activities based mainly on the uncharacterized substances or extracts, including antiviral (Lindequist, Niedermeyer et al. 2005), antibacterial (Alves, Ferreira et al. 2012), antifungal (Wong, Ng et al. 2010), antiparasitic (Tateno and Goldstein 2003), detoxifying, immunomodulatory (Wasser 2002), antitumor(Zhang, Cui et al. 2007, Novaes, Valadares et al. 2011, Lin, Wong et al. 2013), antioxidant(Karaman, Jovin et al. 2010, Liu, Du et al. 2014), radical scavenging, anti-inflamatory (Hwang 2011), antihiperlipidemic or antihypercholesteromic, hepatoprotective and antidiabetic effects (Hwang and Yun 2010).

Basidiomycetes are currently evaluated for their nutritional value and acceptability, since they have been consumed as food, as well as for their pharmaceutical ingredients for centuries.(Adrio and Demain 2003, Wasser 2011).

Therefore, edible higher fungi are excellent sources of protein, have low-fat content and are free of cholesterol. The fungi as food and feed are very nutritive since they contain essential and nonessential amino acids (Ghorai, Banik et al. 2009). Fruiting bodies have been consumed directly fresh or processed and used as delicacies (Kalač 2009, Klaus, Kozarski et al. 2013) being a prospect of successful business model (Karaman, Jovin et al. 2010).

Basidiomycetes polysaccharides have already been proven to prevent oncogenesis, show direct antitumor activity against various metastatic tumours preventing its metastasis (Zhang, Cui et al. 2007), as stated above. Their activity is especially beneficial when in conjunction with chemotherapy (Novaes, Valadares et al. 2011, Lin, Wong et al. 2013).

Likewise, Basidiomycetes' polysaccharides are able to induce gene expression of various immunomodulatory cytokines and cytokine receptors (Wasser 2011). Thereby they can be considered as multicytokine inducers. The secondary metabolites (alkaloids, lactones, terpenoids, metal chelating agents and antibiotics) have been considered to also have therapeutic interest, since they show importance for the immune function of the organism.

Higher fungi accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Phenolics are one of the major groups of nonessential dietary components (Erjavec, Kos et al. 2012) that have been associated with the inhibition of atherosclerosis and cancer.

The beneficial effects produced by the MMs are not summarized to drugs but also as a novel class of products known as: dietary supplements (DSs), functional foods, nutriceuticals, mycopharmaceuticals, and designer foods that produce healthy benefits through everyday use as a part of a healthy diet. The expression "mushroom nutriceuticals" was invented do to the growing interest in traditional ways for treatment or appeasing of various physiological disorders and the recognition of numerous biological activities of higher fungi products (Wasser 2011).

1.1.2.1 – Proteins from Basidiomycetes

The great advantage of proteins from higher fungi is their uniqueness, since they often differ from microbial, animal and plant proteins due their already proven thermostability and broad pH range (Berends, Scholtmeijer et al. 2009, Alves, Ferreira et al. 2012). Another advantage of bioactive ingredients from mushrooms is their perceived and, with a proper choice of source material, actual safety for human consumption and application because of long-term experience with their effects on human health (GRAS status) (Wong, Ng et al. 2010).

In this line of thought, higher fungi are rapidly becoming recognized as a very promising source of novel proteins. Several proteins showing special and unique features have been parcially isolated, including lectins, lignocellulolytic enzymes, protease inhibitors and hydrophobins. They can offer solutions to several medical and biotechnological problems such as microbial drug resistance, low crop yields, environmentally friendlier plant protection, and demands for renewable energy (Cohen, Persky et al. 2002, Adrio and Demain 2003, Sabotic, Trcek et al. 2007).

1.1.2.1.1 – Proteases

Proteases catalyse hydrolytic reactions (Sumantha 2006) cleaving peptide bonds. So, proteases can be endopeptidases if cleave internal bonds, aminopeptidases if cleave N-terminal peptide bonds or carboxipeptidases, if cleave C-terminal peptide bonds (van der Hoorn 2008). These enzymes are distributed in five groups based on the characteristics of their active sites: serine proteases, cysteine proteases, aspartic proteases and metalloproteases (van der Hoorn 2008).

As has already been thoroughly studied, proteases from microorganisms display many unique characteristics regarding substrate specificity, catalytic mechanism, activation mechanism, thermostability and pH range (Sabotic, Trcek et al. 2007). Thereby, higher fungi could represent another source of proteases with novel characteristics, as proteases play a major role in the physiology, morphogenesis and metabolism of fungi (Nakamura, Iketani et al. 2011).

However, the knowledge of proteolytic activity in basidiomycetes, popularly called mushrooms, is limited to proteases involved in nutrient utilization in saprophytic and mycorrhizal basidiomycetes and to virulence factors in pathogenic species (Wong, Ng et al. 2010, Wasser 2011).

In the pharmaceutical industry proteases are important targets for drug design against a diverse set of diseases (Yike 2011). Because of their broad potential many investigations are focused on the discovery and characterization of novel naturally occurring proteases from sources that have so far been overlooked (Yike 2011).

Saprophytes are able to digest dead organic matter by means of enzymes and turn it to compounds that can be reused as nutrients. In addition to general degradation, a regulatory role of extracellular proteases in promoting ligninolytic activity in white-rot basidiomycetes under nutrient limitation was shown in some higher fungi (Karaman, Jovin et al. 2010, Sabotic and Kos 2012).

Several proteases have already been purified from basidiomycetes. Fibrinolytic metalloproteases were purified from *Armillariella mellea* (Kim 1999) and *Flammulina velutipes* (Shin 1998). A serine protease with fribrinolytic activity was also purified from fruiting body of *Paecilomyces tenuipes* (Kim, Choi et al. 2011) *Cordyceps militaris* (Choi, Cha et al. 2011). Being Fibrin the main protein component of blood clot (Danesh, Whincup et al. 2001), these proteases purified from basidiomycetes can potentially become a new source of fibrinolytic enzymes, showing basidiomycetes as a new source for helping treating cardiovascular diseases. Some examples are presented in Table I and II and Figures 2 and 3.

Table I -Effects of various protease inhibitors on activity of the fibrinolytic enzyme from *C.militaris* fruiting bodies (Choi, Cha et al. 2011).

Metal ions and inhibitors	Concentration (mM)	Relative activity (%)
None	–	100.0
EDTA[a]	5	72.8
EGTA[b]	1	66.0
PMSF[c]	1	0.0
TPCK[d]	1	0.0
TLCK[e]	1	75.9
Pepstatin A	0.5	92.4
Aprotinin	0.1	46.9
1,10-Phenanthroline	1	0.0

The enzyme (2 μg) was incubated in 50 mM Tris–HCl buffer (pH 7.0) with various protease inhibitors for 1 h at 37 °C. Following incubation, enzyme activity was assayed using 1% azocasein.

[a] Ethylenediaminetetraacetic acid.
[b] Ethyleneglycolbis(beta-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.
[c] Phenylmethylsulfonyl fluoride.
[d] *N*-α-tosyl-L-phenylalanine chloromethyl ketone.
[e] *N*-α-tosyl-L-lysine chloromethyl ketone.

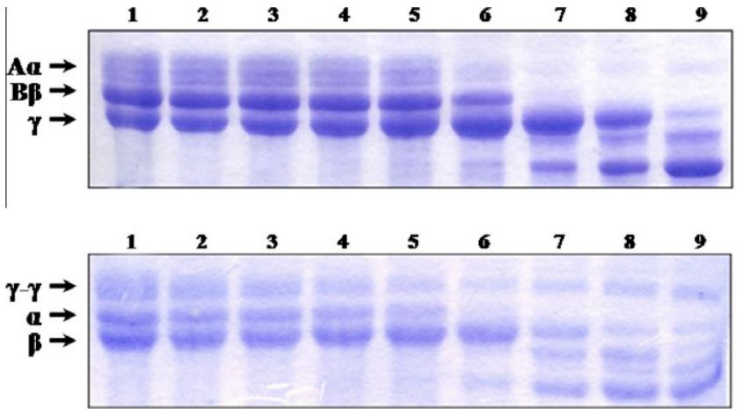

Figure 2 - Degradation patterns of fibrinogen (A) and fibrin (B) by the fibrinolytic enzyme from *C. militaris* fruiting bodies. Lanes 1 – 9 are degradation products after 0, 5, 10, 20, 30, 60, 120 and 240 min incubation, respectively (Choi, Cha et al. 2011).

As shown in Table I a chymotrypsin-like serine protease was purified from fruiting bodies of *C. militaris,* inhibited by PMSF and TPCK. It is clear that the fibrinolytic enzyme purified has similar degradation pattern of fibrinogenolysis (Choi, Cha et al. 2011). The purified enzyme hydrolized the Aα-chain within 60 min of incubation and the Bβ-chain after only 2h of incubation (Figure 2).

This study strongly suggests that this fibrinolytic enzyme can become a potential source for developing therapeutic agents for thrombosis (Choi, Cha et al. 2011)

Other fibrinolytic enzymes were already found in basidiomycetes, as was already stated. A metalloproteinase was already purified from the Fruiting bodies of *A. mellea* (Kim 1999).

Table II - Inhibitors effect in purified protease (Kim 1999).

Reagents	Concentration	Residual activity (%)
None		100
Ca^{2+}	1 mM	104
Co^{2+}	1 mM	119
Zn^{2+}	1 mM	111
Cu^{2+}	1 mM	92
Mg^{2+}	1 mM	110
Fe^{2+}	1 mM	105
Hg^{2+}	1 mM	0
PMSF	1 mM	100
Pepstain A	0.2 mM	106
E-64	1 mM	100
1,10-phenanthroline	1 mM	55
EDTA	1 mM	11
2-mercaptoethanol	2 mM	100

E-64: trans-epoxysuccinyl-L-leucylamido-(4-quanidino)-butane. PMSF: phenylmethylsulfonyl fluoride.

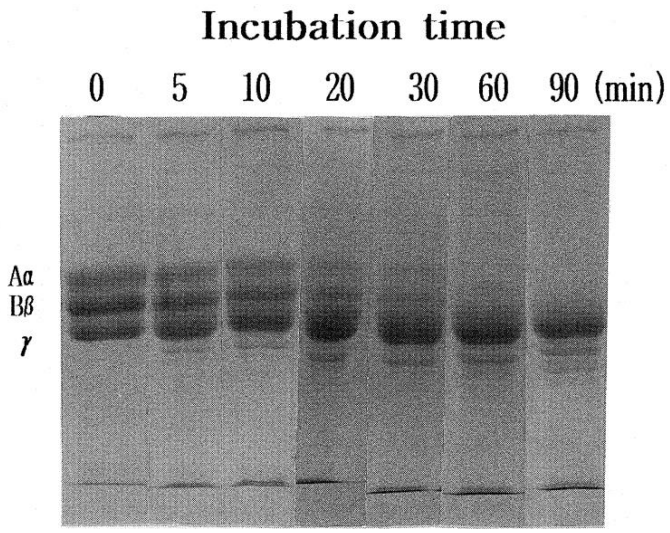

Figure 3 - Degradation of Human fibrinogen by the purified fibrinolytic enzyme (Kim 1999).

This purified metalloprotease was inhibited by EDTA, as shown in Table II. It is clear in Figure 3 that the fibrinolytic enzyme was especially efficient in Aα and Bβ chains, being completely efficient after 60 min of incubation. It is hoped that this enzyme can achieve the pharmaceutical industry, being a potential candidate for oral fibrinolytic therapy (Kim 1999).

A thermostable Lysine- specific metalloendopeptidase was also purified from higher fungi *Grifola frondosa* (Nonaka 1995), such as serine proteases were also already purified from *Hypsizigus marmoreus* (Zhang, Liu et al. 2010) and *Pholiota nameko* (Guan, Zhang et al. 2011).

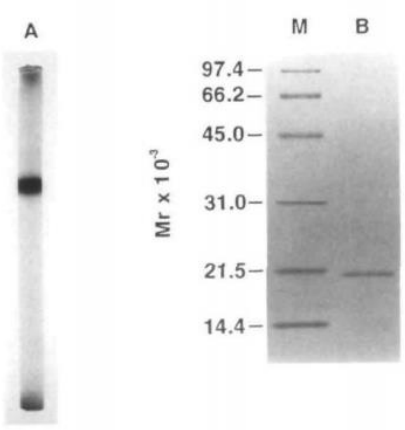

Figure 5 – PAGE and SDS-PAGE. Lane A) Purified MEP (15 µg) on PAGE using a 10% (w/v) ,pH4.3 gel; Lane B) Purified MEP on SDS-PAGE using a 12.5% (w/v) gel containing 0.1% (w/v) SDS; Lane M) Standard proteins for molecular mass determination (Nonaka 1995).

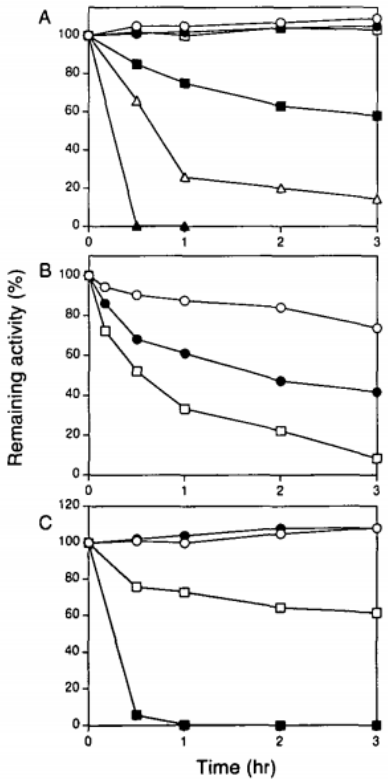

Figure 4 –Effects of various temperatures on MEP. A) The activity-temperature curves of MEP at pH7.2 at temperatures of 50-100°C for 3h. B) The activity-temperature curves of MEP at pH9.0. C) The activity-temperature curves of Mn^{2+}-MEP. The assays were carried out at (0) 50°C, (•) 60°C, (□) 70°C, (▪) 80°C, (Δ) 90°C and 100°C (Nonaka 1995).

Purified MEP of Grifola frondosa (Nonaka 1995) was readily inactivated with chelating agents such as EDTA and 1,10-Phenantroline. As shown in Figure 3, this MEP withstands heating at 70°C for 3h. Thereby, at high temperatures, this MEP appears to be more stable than thermolysin (Rein V. Ulijn 2000), which is stable at 60°C for 1h and is inactivated at 80°C in 1h.

The number and diversity of proteases found under specific conditions used in this study for basidiocarps, together with data on proteases characterized from different developmental stages described in literature, imply that the proteolytic potential of basidiomycetes is indeed immense. Therefore, basidiomycetes could prove to be a valuable source of proteases that could find use in biotechnological processes (Sabotic, Trcek et al. 2007).

1.1.2.1.2 – Other Hydrolytic Proteins

Fungi have already proven useful in many medical and biotechnological applications because of their unique metabolic activities, and as a source of degradative enzymes and secondary metabolites. One of the most investigated groups of proteins from higher fungi are enzymes involved in degradation of lignocellulose, where oxidative and hydrolytic enzymes cooperate, including laccases (Cohen, Persky et al. 2002, Hilden, Hakala et al. 2009), peroxidases and other oxidases, (hemi)cellulases, chitinases (Radjacomare 2004, Dahiya, Tewari et al. 2006) and different glycosidases (Ng 2004).

Recently, much research has been focused on the isolation of powerful thermostable xylanases (Sun, Zhang et al. 2004, Lee, Park et al. 2009, Lin, Wong et al. 2013) because of their potential applications in various industrial processes, such as bioconversion of lignocellulosic materials into fermentative products, improvement of digestibility of animal feedstock and clarification of juices, and facilitating the release of lignin from the pulp and reducing the amount of chlorine required for bleaching in the pulp and paper industry.

Researchers are especially interested in fungal xylanases because they are secreted extracellularly are their activity is higher than the xylanases from yeasts and bacteria purified until now (Lee, Park et al. 2009). Several recent studies are focused on the wood-rotting fungi as a source of xylanases because they effectively degrade wood biomass. However, only a few xylanases have been purified and characterized from the wood-rotting basidiomycetes fungi.

One of the current example of added value through cultivation of mushrooms on solid waste and residues is the production of enzymes of industrial importance. Laccases of white-rot fungi have caught the interest of the pulp and paper industry for developing environmentally acceptable technologies for pulp delignification and bleaching. Production of laccases in their host is not often economically feasible and natural laccases may not withstand the harsh conditions of industrial processes, therefore, much effort is put into developing more stable enzymes and heterologous expression systems for application in industrial processes (Hilden, Hakala et al. 2009).

Tyrosinases from higher fungi have been considered to produce, for example, protein – polysaccharide hydrogels, which could be useful for tissue engineering, matrices for drug delivery, adhesives, and skin substitutes(Faria 2007). Tyrosinase from *A. bisporus* has been used in several studies as the functional part of biosensor for detecting dopamine in diagnostics and phenolic environmental pollutants. Furthermore, tyrosinase from this higher fungi is also used as a model for the design and search of tyrosinase inhibitors and substrates, as it shows high homology to the mammalian enzymes (Erjavec, Kos et al. 2012).

Some basidiomycete's tyrosinases have the capacity to convert monophenols into diphenols, which can be used in the production of antioxidants for use as food aditives or pharmaceutical drugs (Fan and Flurkey 2004, Mauracher, Molitor et al. 2014).

Enzymes of higher fungi have a potential for use in the cosmetic industry, for example, laccases and peroxidases in hair dyeing and skin lightening preparations(Erjavec, Kos et al. 2012).

Another large consumer of enzymes is detergent industry, where proteases, lipases, amylases, mannanases and cellulases of microbial origin have been commercialized(Erjavec, Kos et al. 2012).

Peroxidases from higher fungi could offer superior biochemical properties in terms of stability, substrate specificity and catalytic mechanisms. These advantages, make higher fungi a source of potentially interesting peroxidases with many applications. They could be used, as plant peroxidases already are, in biosensors for *in vivo* monitoring of glucose for diabetes

mellitus, of lactate confirmation of hypoxia and ischemia, and total cholesterol in clinic diagnostics (Cohen, Persky et al. 2002, Wong, Ng et al. 2010, Erjavec, Kos et al. 2012).

Higher fungi or their enzymes can be used to improve the digestibility of animal feed. Lignocellulosic residues can be used as substrates for mushroom cultivation. The lignocellulosic substrate, provides a higher content as it's easier for ruminants to digest (Sun, Zhang et al. 2004, Karaman, Jovin et al. 2010).

Parasitic nematodes cause huge economic losses in crops and livestock production. Some lectins from different higher fungi have been shown to possess nematotoxic activity. They can eventually be used to identify suitable antigens for developing glycan-based vaccines to prevent parasitic infections (Zhao, Guo et al. 2009).

The properties of enzymes advantageous in bioremediation processes can also be exploited in the chemical and pharmaceutical industries as biocatalysts.

1.1.2.1.3 – Polysaccharides

Antitumor polysaccharides isolated from basidiomycetes include acidic and neutral ones with different types of glycosidic linkages, while some are bound to protein or peptide residues such as polysaccharide, protein or peptide complexes (Mizuno 1996, Zhang, Cui et al. 2007).

Numerous bioactive polysaccharides or polysaccharide–protein complexes from the considered medicinal higher fungi are described that appear to enhance innate and cell-mediated immune responses and exhibit antitumor activities in animals and humans. Many of these mushroom polymers were reported previously to have immunotherapeutic properties by causing growth inhibition and/or destruction of tumor cells (Wasser 2002, Pohleven, Obermajer et al. 2009, Hu, Zhang et al. 2011, Lung and Huang 2011).

Several of the mushroom polysaccharide compounds have proceeded through and passed phases I, II, and III clinical trials and are used extensively and successfully in Asia, but

not in western therapies, to treat various cancers and other diseases (Wasser 2002, Lindequist, Niedermeyer et al. 2005, Lung and Huang 2011).

1.2 - *Laetiporus sulphureus*

Laetiporus sulphureus is a polypore fungus (Wiater 2008, Wiater, Pleszczynska et al. 2012) that belongs to the Polyporaceae of Aphyllophorales and is a wood-rotting basidiomycete, a parasitic wood rotting, growing on several tree species and causing heart-rot disease in deciduous trees and conifers. *Laetiporus sulphureus* is found in Europe, Asia and North America. Concerning its ecological characteristics, *L. sulphureus* is thermophilic and creates large and strong fruiting bodies (Alquini, Carbonero et al. 2004).

This higher fungi produces shelf-shaped fruit-bodies of pink-orange colour, except for the fleshy margin which is bright yellow (Seo 2011). Due to its special fragrance and texture, *L. sulphureus* has been used for many years in oriental cultures as nutritional food.

Xylotrophic basidiomycete *Laetiporus sulphureus* is known under the common name “Sulphur Shelf” or “Chicken of the Woods”, since its texture is similar to the texture of cooked chicken (Klaus, Kozarski et al. 2013).

According to previous studies, fruiting bodies of this species contain polysaccharides, laetiporic acids, lanostane triterpenoids, N-methylated tyramine derivatives and other compounds (Lung and Huang 2011, Olennikov, Tankhaeva et al. 2011). Studies on the biological activity of *L. sulphureus* have identified the immunomodulation and antitumor (Seo, Kim et al. 2010, Lung and Huang 2011), hemagglutination, anticoagulation, cytotoxic (Rios, Andujar et al. 2012), apoptosis inducing , antioxidants (Olennikov, Tankhaeva et al. 2011), antimicrobial (Turkoglu, Duru et al. 2007), and insulinotropic activities (Hwang, Lee et al. 2008) of the total fractions and individual compounds. This macrofungus is also known as a producer of HIV-1 reverse transcriptase inhibitors (Mlinaric 2005, Lin, Wong et al. 2013). Many, if not

all, higher Basidiomycetes mushrooms contain biologically active polysaccharides in fruiting bodies, cultured mycelium, and cultured broth.

Moreover, *L. sulphureus*' basidiocarps are a rich source of α-(1-3)-D-glucan. Their cell wall contains up to 88 % of the dry matter of this glucan, whereas in other fungi it is present in amounts of 9–46 % (Pleszczynska, Wiater et al. 2013).

Xylotrophic basidiomycete *Laetiporus sulphureus* (Bull.: Fr.) Murr. (Polyporales family) causes brown cubical rot and is a perspective biotechnological object. According to ecological characteristics, *L. sulphureus* is thermophilic and, grown both in laboratory and nature conditions, creates fruiting bodies with great biomass in a short period.

Very little is known of the proteolytic fraction of *Laetiporus sulphureus*. However, interesting activities and molecules were already found and studied.

1.2.1 – Biotechnological Potential of *Laetiporus sulphureus*

1.2.1.1 - Antioxidant and Antimicrobial Activities

Higher fungi accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids (Klaus, Kozarski et al. 2013). Some common edible mushrooms, which are widely consumed in Asian culture, have currently been found to possess antioxidant activity, which is well correlated with their phenolic content (Olennikov, Tankhaeva et al. 2011). Recently, basidiomycetes were considered a good source of protein and phenolic compounds.

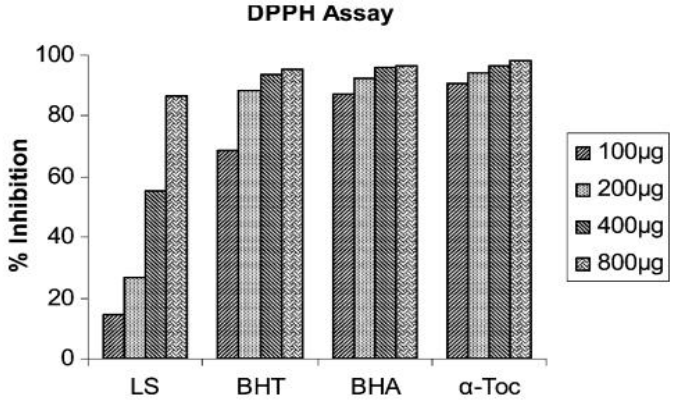

Figure 6- Free radical-scavenging capacities measure in DPPH assay (Turkoglu, Duru, Mercan, Kivrak, & Gezer, 2007).

In recent decades, the issue of multiple drug resistance in human pathogenic microorganisms, due, essentially, to the indiscriminate use of commercial antibiotics forced scientists to search for new substances with antimicrobial effect. Therefore, the study of novel sources of molecules takes an extreme importance.

Reseachers carried out a study to assess the potential of *Laetiporus sulphureus* as a source of antimicrobial and antioxidant agents in food industry (Turkoglu, Duru et al. 2007, Klaus, Kozarski et al. 2013).

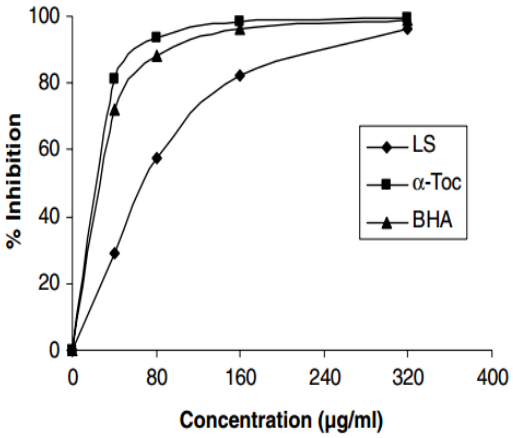

Figure 7 - Total antioxidants activities of BHA, α-tocopherol and extract of *L.sulphureus* in the linoleic acid emulsion (Turkoglu et al., 2007).

In this study, the ethanolic extracts of *L. sulphureus* were subjected to screening for their possible antioxidant activity through DPPH free radical-scavenging test and β-carotene/linoleic acid systems (Turkoglu, Duru et al. 2007).

As shown in Figure 6, free radical-scavenging capacity of the extract of *Laetiporus sulphureus*, to high concentrations, is equivalent to synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and α-tocopherol(Turkoglu, Duru et al. 2007).

The antioxidant activities of BHA, α-Tocopherol and *Laetiporus sulphureus* extract were compared, as represented in Figure 7. It was shown that 320 µg of extract had an inhibition capacity equivalent to 40 µg of α-Tocopherol. It is possible that this antioxidant activity of the *L. sulphureus* is due to the high concentration of chelating compounds or phenolic compounds (Turkoglu, Duru et al. 2007).

The antimicrobial effect of ethanol extracts of *L. sulphureus* were tested against six species of Gram-positive bacteria, seven Gram-negative and one species of yeast. As shown in

Table III, the extract strongly inhibits the growth of the Gram-positive bacteria tested, but had a narrow effect in the growth of the Gram-negative. However, its anticandidal activity is flagrant (Turkoglu, Duru et al. 2007).

Table III - Antimicrobial activity of ethyl alcohol extract of Laetiporus sulphureus and antibiotic sensitivity of microorganisms (zone size, mm) (Turkoglu et al., 2007).

Test bacteria	*L. sulphureus*	N	A	P	G	O	T
Pseudomonas aeruginosa NRRL B-23	6 ± 0	NT	NT	NT	16	NT	8
Salmonella enteritidis RSKK 171	5 ± 1	NT	–	NT	NT	NT	12
Escherichia coli ATCC 35218	10 ± 0	NT	10	11	NT	NT	8
Morganella morganii	4.5 ± 0.5	NT	NT	NT	–	NT	–
Yersinia enterecolitica RSKK 1501	6 ± 0	NT	20	18	NT	NT	7
Klebsiella pneumoniae ATCC 27736	–	NT	–	NT	NT	NT	5
Proteus vulgaris RSKK 96026	5.5 ± 0.5	NT	–	NT	NT	NT	16
Staphylococcus aureus ATCC 25923	9 ± 1	NT	NT	31	NT	21	20
Staphylococcus aureus Cowan I	7 ± 1	NT	NT	28	NT	18	21
Micrococcus luteus NRRL B-4375	15 ± 3	NT	30	31	NT	22	19
Micrococcus flavus	23 ± 1	NT	29	31	NT	24	20
Bacillus subtilis ATCC 6633	8 ± 0	NT	NT	12	NT	8	17
Bacillus cereus RSKK 863	12 ± 1	NT	NT	22	NT	14	19
Candida albicans	21 ± 1	19	NT	NT	NT	NT	NT

N, nystatin (100 U); A, ampicillin (10 µg); P, penicillin (10 U); G, gentamycin (10 µg); O, oxacillin (1 µg); T, tetracycline (30 µg); NT, not tested; (–) No inhibition.

In conclusion, it is thought that the strong antioxidant and radical scavenging activities of *Laetiporus sulphureus* extract may be due to the high concentration of phenolic a flavonoid-type compounds (Olennikov, Tankhaeva et al. 2011, Klaus, Kozarski et al. 2013, Petrovic, Glamoclija et al. 2013).

1.2.1.2 - HIV-1 Reverse Transcriptase inhibitors source

The search of novel inhibitors of the HIV-1 replication cycle is one of the main research fields of numerous investigators. HIV-1 reverse transcriptase (HIV-1 RT) is one of the main targets for inhibition of the replication of HIV-1. For the first time, researchers looked to higher fungi as a possible source of novel inhibitors to this enzyme.

As HIV exhibits a high ability to develop resistance to therapeutic agents, it is a priority to look and find new and promising substances. In order to find novel therapeutic agents, a study was carried out in order to find novel therapeutic agents.

Table IV - In vitro HIV-1 RT inhibitory activity of extracts obtained from wood-damaging fungi. (zone size, mm) (Adapted from (Mlinaric, 2005).

Subdivision	Species	Inhibition	
		Methanolic extract	Dichloro-methane extract
Basidiomycotina	*Heterobasidium annosum* (Fr.: Fr.) Bref.	–	–
Basidiomycotina	*Laetiporus sulphureus* (Bull.: Fr.) Murrill	+++	+
Basidiomycotina	*Lentinula edodes* (Berk.) Pegl.	–	–
Basidiomycotina	Marasmius stiptitarius	–	–
Basidiomycotina	*Peniophora gigantea* (Fr.) Massee	–	–
Basidiomycotina	*Pholiota adiposa* (Fr.) Kumm.	+	–
Basidiomycotina	*Pleurotus ostreatus* (Jacq.: Fr.) Kumm. (isolate 2)	–	–
Basidiomycotina	Pleurotus ostreatus (isolate 3)	–	–
Basidiomycotina	*Poria monticola* (isolate 2)	+++	+
Basidiomycotina	*Poria vaillanti* (DC. & Lamarck) Fries (isolate 1)	++	+
Basidiomycotina	*Poria vaillanti* (isolate 2)	++	+
Basidiomycotina	*Schizophyllum commune* (Fr.: Fr.) (isolate 2)	–	+
Basidiomycotina	*Serpula lacrymans* (Wulf.: Fr.) Schroet. (isolate 2)	+	+
Basidiomycotina	*Serpula lacrymans* (isolate 4)	+	–
Basidiomycotina	*Sistostrema brinkmannii* (Bres.) Erikss.	++	–

As shown in Tables IV and V, the higher fungi *Laetiporus sulphureus* has an outstanding activity, with an inhibition of 90.1% of reverse transcriptase of HIV-1. In a primary hypothesis, it is thought that this species may contain an acidic compound with an amino group in the most active fraction of the methanolic extract (Mlinaric 2005).

Table V - In vitro HIV-1 RT inhibitory activity of methanolic extracts (Mlinaric, 2005)

Species	Inhibition (%)[a]
Chondrostereum purpureum (isolate 1)	64.3 ± 4.8
Chondrostereum purpureum (isolate 4)	62.3 ± 5.0
Cladosporium herbarum	50.7 ± 4.3
Laetiporus sulphureus	90.1 ± 3.6
Leptographium lundbergii	54.7 ± 7.0
Pichia anomale	48.7 ± 3.9
Poria monticola (isolate 2)	86.1 ± 8.4
Poria vaillanti (isolate 2)	53.3 ± 2.1
Poria vaillanti (isolate 1)	68.7 ± 5.2

1.2.1.3 - Insulinotropic Effect

Many *L. sulphureus* polysaccharides have been isolated not only from fruiting bodies but also from submerged mycelial cultures (Alquini, Carbonero et al. 2004, Hwang, Lee et al.

2008, Lung and Huang 2011, Seo 2011, Li, Yan et al. 2013), and some therapeutic evidence has been reported such as a hypoglycemic effect in streptozotocin-induced Diabetic Rats, and an increased proliferation and insulin secretory function of rat insulinoma RINm5F cells (Hwang and Yun 2010).

As it is shown in Figure 8, the effect of the exopolyssacharide (EPS) on the plasma glucose level was investigated during 14 days. The results revealed that EPS, when given 48h after the treatment with STZ, exhibits hypoglycemic effect, lowering average plasma glucose levels

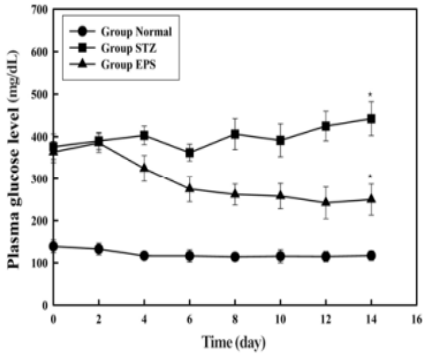

Figure 8 - Effect of extracelular polysaccharides (EPS) produced by submerged culture of L. sulphureus on the blood glucose levels in STZ-induced diabetic rat during 14 days. The EPS diabetic group was treated with 200 mg of EPS per Kg of body weight. STZ group was treated with 0.9% NaCl. The administration of EPS started 48h after the treatment with STZ (Hwang and Yun 2010).

Glucose tolerance was examined using the OGTT (Oral Glucose Tolerance Test) methodology. This showed that the EPS-treated diabetic group had a remarkable improvement in glucose response when compared with the STZ-induced diabetic group. As it is shown in Figure 9, the EPS group returned to baseline levels after approximately 120 minutes. Besides, the ΔAUC blood glucose response was reduced by almost 50% when compared with the STZ group, which is concurrent with the results in Figure 8 (Hwang and Yun 2010).

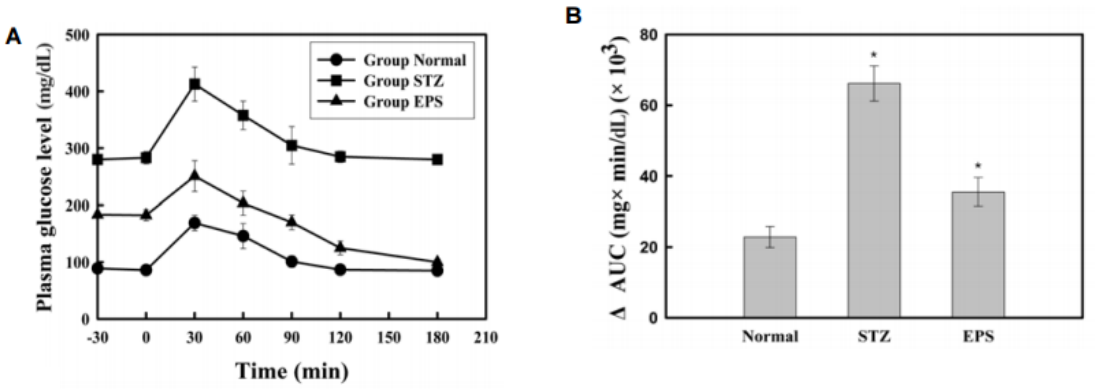

Figure 9 - (A) Blood glucose levels and (B) area under the curve (ΔAUC) during the oral glucose tolerance test (OGTT) in STZ-induced diabetic rats (2 g glucose/kg body weight). The EPS diabetic group was treated with 200 mg of EPS per Kg of body weight. ST STZ group was treated with 0.9% NaCl.(Hwang and Yun 2010)

As shown in Table VI, plasma levels of total cholesterol and triglyceride were significantly increased upon STZ treatment, and they were markedly reduced by oral administration of EPS to near-normal levels.

Table VI - Effect of extracelular polysaccharides (EPS) produced by submerged mycelial culture of L. sulphureus on the plasma total cholesterol and triglyceride in STZ-induced diabetic rats for 14 days (Hee Sun Hwang & Yun, 2010).

Group[a]	Total cholesterol (mg/dL)	Triglyceride (mg/dL)
Normal (N)	42.07 ± 3.15[b]	78.41 ± 5.18
N-EPS	41.35 ± 1.89**	73.83 ± 1.60**
STZ	72.74 ± 6.25*	125.74 ± 4.31*
EPS	45.76 ± 2.82*	86.44 ± 2.34**

[a] For detailed group division, see Materials and Methods section.
[b] Values are means ± S.E ($n = 6$).
*$p < 0.05$ and **$p < 0.01$, compared with that in control.

The immunohistochemical staining of the pancreatic tissues results showed a strong signal for insulin antigen in β-cells of the islets of healthy rats. On the other hand, low insulin immunoreactivity was detected in β-cells in Langerhans islet of the STZ group, as shown in Figure 10. As the results suggest, there was a considerable increase of insulin detection in β-cells in the EPS group. This suggest a possible β-cell proliferation or regeneration with EPS therapy (Hwang and Yun 2010).

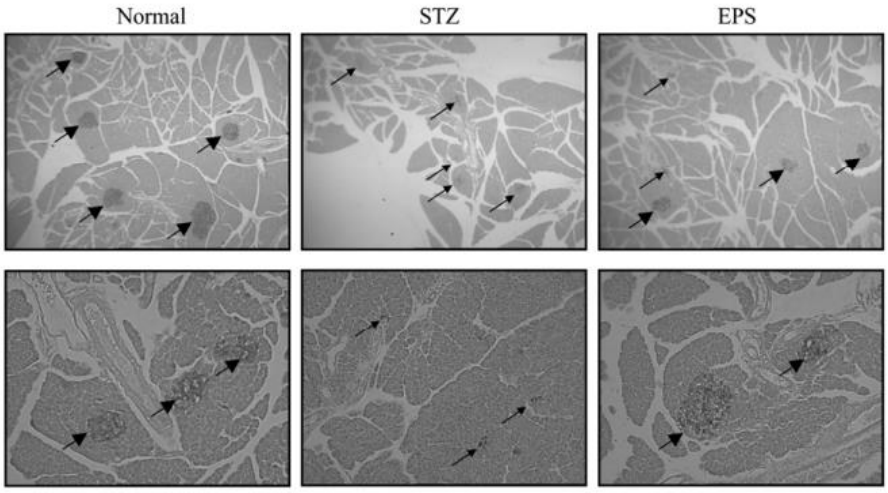

Figure 10 - Representative immunohistochemical staining images of insulin and diabetic rat pancreas on day 14 in response to EPS treatment. Large islets are indicated by arrow heads and small islets are indicated by arrows (Hee Sun Hwang & Yun, 2010).

According to previous studies (Hwang, Lee et al. 2008), the EPS were proven to be glucose-rich polysaccharides and were able to increase proliferation and insulin secretory function of rat insulinoma RINm5F cells, in a dose dependent manner, suggesting a cytoprotective role of EPS in cells.

1.2.1.4 - Capacity of Mutanase Induction

Mutanase (α-(1→3)-glucanase) is an enzyme important in dentistry and naturally expressed in *Trichoderma harzianum*, when stimulated with its substrate, α-(1→3)- glucan. This enzyme catalyses hydrolysis of α-(1→3)-glucoside linkages, cleaving the branched glucan Mutan, which is the great portion of the cariogenic biofilm and is secreted by the pathogenic and cariogenic microorganism, *Streptococcus mutans* (Wiater, Pleszczynska et al. 2012).

The mutanase reported until recently has been inducible in media containing S*treptococcus mutans* (Wiater, Szczodrak et al. 2004). Unfortunately, this biopolymer is not

available in bulk quantities due the pathogenicity of its producer, the necessity to use complex and expensive media (e.g., beef brain-heart infusion), the multistage production process, low product yields and high structural heterogeneity. Substitution of the glucan mutan by more available inducer could facilitate commercial-scale mutanase production. An alternative source of α-(1→3)-glucans is the fungal cell wall(Wiater, Pleszczynska et al. 2012).

It has already been shown that cell wall preparations from *L. sulphureus* effectively induced mutanase in *T. harzianum*. Moreover, the cell wall of this fungus is the richest source of α-(1→3)-glucans (up to 78% dry matter) (Wiater, Pleszczynska et al. 2012).

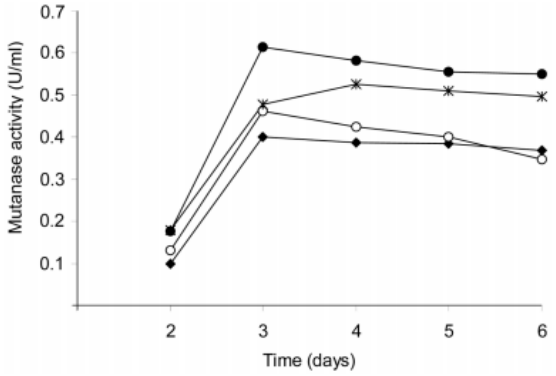

Figure 11 - Inductive effects of diferent α-(1-3)-Glucan preparations on the production of mutanase by T. harzianum in 6 days shaken flask cultures. Simbols: mutan preparation (◆), freeze-dried and milled preparation of fresh fruit bodies (○), cell wall preparati preparation (CWP) (●), α-(1→3)-glucan preparation (*) (Wiater 2008).

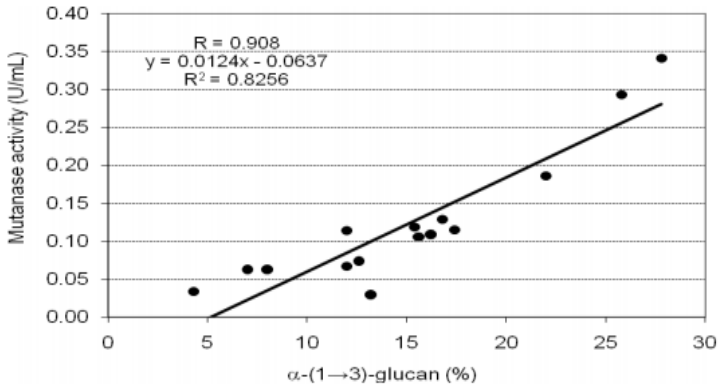

Figure 12 - Relationship between the mutanase activity obtained on particular CWP from mycelia of L.sulphureus used as enzyme inducers and the content of α-(1-3)-glucan in each of these preparations (A. Wiater, Pleszczynska, Szczodrak, & Janusz, 2012).

The results shown in Figures 11 and 12 indicate that *L. sulphureus* may be a valuable source of mutanase inducers. Moreover, the use of the mycelia can prove to be an efficient method of mutanase synthesis.

1.3 - Aims

Higher fungi are a potential unexplored source proteins with interesting hidden biotechnological applications in the most diverse areas. *Laetiporus sulphureus* shows itself as a pool of unexplored hydrolytic potential since almost nothing is known about its protein profile and characterization. Furthermore, due to its large size and easy large scale production, it is possible to have a high portion of material to research. Also, as proteins from higher fungi have already been proven to have stability at a wide range of temperatures, *Laetiporus sulphureus* do not have high maintenance difficulty and special need, reducing the costs of upkeep.

To explore the biotechnological potential and partial characterization of *Laetiporus sulphureus*, we define

1. Enzymatic characterization of fruiting body of *Laetiporus sulphureus* in order to access its biotechnological potential and interesting protein contents. Once very little is known of this basidiomycete, it is important to have a wide knowledge of its protein profile and characterization.
2. Isolation and characterization of hydrolases of fruiting body of *Laetiporus sulphureus* with biotechnological relevance.
3. Access biotechnological relevance and applications of purified hydrolases.

MATERIALS AND METHODS

2.1 – Sampling

The fruiting body of *Laetiporus sulphureus* was obtained by Doctor Marisa Azul from Centre for Functional Ecology (Faculty of Sciences and Technology of the University of Coimbra).

2.2 – Extracts of Laetiporus sulphureus

Protein extracts of *Laetiporus sulphureus* were prepared as reported previously (Ertunga, Cakmak et al. 2009). Mushroom samples were treated with liquid nitrogen for about 5 min to decompose the cell membranes. The frozen samples will be macerated until they become fine powder and homogenised in a proportion of 1g in 2mL of 30mM sodium phosphate buffer (pH 7.0) using a mortar.

The homogenates were centrifuged at 6200 g for 10 min at 4^{o}C. The supernatants were filtered and stored. The pellets were dismissed.

2.3 – Protein quantification of LS extracts

Total proteins concentrations were determined by Bradford Microassay Procedure, using 1 mg/ml bovine serum albumin (BSA) as standard. The protein concentration were determined at 595 nm after incubation. The linear range of this assay is 8µg/ml to 80µg/ml.

2.4 – SDS-Page

2.4.1 – Protein Electrophoresis under denaturing conditions (SDS-Page)

The samples were electrophoretically separated in polyacrylamide gels (PAGE) under denaturating conditions, in the presence of 0.2% SDS, using Biorad's *Mini-Protean*

II system (Laemmli 1970). Samples were denatured previously to application for 15 min at 98°C with denaturating solution [125mM Tris (w/v), 100mM Glycine (w/v), 4% SDS (w/v), 8% β-mercaptoethanol(v/v), 8M Urea (w/v)] and Brilliant Blue R250. As a molecular weight reference, it was applied to the gel a protein standard with molecular weights between 18.5 and 96 KDa (Low Molecular Weight from Nzytech). Samples were subjected to electrophoresis at 140 V for approximately 1h on 12.5% (w/v) polyacrylamide gels in electrophoresis buffer [25mM Tris (w/v), 192 mM Glycine (w/v), 0.1% SDS (w/v)].

2.4.2 – Staining with Comassie Brilliant Blue R250

For the purpose of fixing and staining the proteins separated by electrophoresis, gels were incubated with staining solution [0.25% (w/v) Brilliant Blue R250, 45% (v/v) Methanol, and 10 % (v/v) Acetic Acid] for 15 min approximately and then distained with successive rinsing with distaining solution [25% (v/v) Methanol, 5% Acetic Acid (v/v)].

2.4.3 – Staining with Silver Nitrate

Due to the low protein concentration, in several cases it is difficult to see the corresponding bands in the gel stained through Brilliant Blue method. Therefore, we proceeded with silver nitrate staining of the gels.

Protein staining was carried out, starting washing the gel for 10 minutes in 50% ethanol, followed by a 10 minutes wash with 30% ethanol. After these two steps, gels were sensitized by a 1 minute incubation in 0.02% Sodium Thiosulphate (w/v) and subsequently incubated in 0.2% Silver Nitrate (w/v) solution. The revelation was accomplished by submerging the gel in revealing solution [0.03% (v/v) Formaldehyde, 3% (w/v) Sodium Carbonate and 0.001% (w/v) Sodium Thiosulphate]

2.5 – Characterization of Enzymatic Activity

2.5.1 – Zymography

For the detection of Proteases, Xylanases, Endoglucanases and Chitinases activities, were carried out several specific zymographies. This was achieved co-polymerizing substrate in a 12.5% polyacrylamide gel in the presence of 0,2% SDS (SDS-PAGE). Samples were incubated with non-denaturating buffer [125mM Tris-HCl pH 6.8, 4% (w/v) SDS and 20% (v/v) glycerol], 10 minutes at room temperature. Proteins were separated electrophoretically in electrophoresis buffer, according as previously described in 2.4. For all zymographies except proteases zymography, it was compared boiled samples at 80°C for 10 min, with not boiled.

Table VII - Zymographies specifications according the target enzyme.

Target Enzyme	Substrate	Renaturing Buffer	Time/ T	Devolving Buffer/incubation time/ T	Running T	Visualisation
Protease	Gelatin (1mg/mL)	30 mM Sodium Phosphate(pH7) with 0.25% Triton X-100	30 min at RT	30 mM Sodium Phosphate(pH7), ON at 37°C	4°C	Comassie Blue
Xylanase	Beechwood Xylan (0.1% (w/v))	30 mM Sodium Phosphate(pH7) with 2.5% Triton X-100	1h at RT	50mM Citrate (pH5.5), 3h at RT	RT	Congo Red
Endoglucanase	Carboxy-methyl-cellulose (0.1% (w/v))	30 mM Sodium Phosphate(pH7) with 2.5% Triton X-100	1h at RT	50mM Citrate (pH5.5), 3h at RT	RT	Congo Red
Chitinase	Glycol Chitosan (0.1% (w/v)	30 mM Sodium Phosphate(pH7) with 1% Triton X-100	3h at RT	-	RT	Calcofluor White and Comassie

After electrophoresis, the gels were washed at RT with renaturating buffer for sample renaturation. Gels were then incubated overnight at 37°C with 30 mM Sodium Phosphate (pH 7) and rinsed two times with miliQ water and then stained as listed in Table VII.

2.5.1.1 - Congo Red Staining

For the purpose staining the zymographies separated by electrophoresis, gels were incubated with staining solution (0.1% Congo-Red (w/v)) for 30 min approximately and then distained with 1M NaCl. Samples with activity will show destained bands.

2.5.1.2 - Calcofluor White staining

For the purpose staining the Chitinase zymography separated by electrophoresis, gel was incubated with staining solution (0.01% Calcofluor White (w/v), 500mM Tris-HCl pH 8.9) for 2h in the dark and then images were captured in Gel Doc XR system (BioRad)(238-242 nm).

2.5.2 – Native PAGE

Samples were electrophoretically separated in polyacrylamide (PAGE) gels, similarly to SDS-PAGE technique described in 4.1, in absence of SDS. Samples were incubated with non-denaturating buffer (125mM Tris-HCl pH 6.8, 30% (v/v) glycerol and Bromophenol Blue), 10 minutes at room temperature. As a molecular weight reference, it was applied to the gel a pre-stained protein standard with molecular weights between 18.5 and 96 KDa (Low Molecular Weight from Nzytech). After electrophoretical separation, gels were incubated in 50 mM Tris-HCl pH 7.4 with 100µM Phe-AMC during 1h at 37°C. After incubation, gel was briefly washed with 30 mM Sodium Phosphate (pH 7) and revealed in VersaDoc™ Imaging System (BioRad).

2.5.3 – Enzymatic Assays

The hydrolytic activity of the *L. sulphureus* extract was assessed using peptidic substrates coupled with a fluorescent molecule , 7-amino-4-methylcoumarin (AMC) and glycosidic substrates coupled with 4-methylumbellipherone (MU).

The enzymatic assays were carried out in a 96 multi well plaque, with a final volume of 202µL. Fluorescent substrates were added in concentrations shown in Tables VIII and IX. The cleavage of fluorescent substrates causes the release of the AMC or MU groups, which is visible through the increase of fluorescence. The assays were performed in 30mM Sodium Phosphate buffer (pH7). The fluorescent peptides were added immediately before readings that were performed in a SpectraMAX-GeminiEM fluorimeter for 10 to 15 minutes, at the conditions shown in Tables VIII and IX:

Table VIII - Fluorescent AMC substrates used in enzymatic assays, as well its effective concentration in the assay.

Fluorescent Peptide	Effective Concentration	λ_{exc} / λ_{em}
Ala - AMC	0.1 mM	380nm / 460 nm
Met – AMC	0.1 mM	380nm / 460 nm
Leu – AMC	0.1 mM	380nm / 460 nm
Arg – AMC	0.1 mM	380nm / 460 nm
Bz-Arg – AMC	0.1 mM	380nm / 460 nm
Lys - AMC	0.1 mM	380nm / 460 nm
Ala-Ala-Phe-AMC	0.1 mM	380nm / 460 nm
Phe-Arg-AMC	0.1 mM	380nm / 460 nm
Gly-Pro-AMC	0.1 mM	380nm / 460 nm
Suc-Leu-Leu-Val-Tyr-AMC	0.1 mM	380nm / 460 nm
Gly-Pro-Arg-AMC	0.1 mM	380nm / 460 nm
Suc-Ala-Ala-Pro-Phe-AMC	0.1 mM	380nm / 460 nm

Table IX - Fluorescent MU substrates used in enzymatic assays, as well its effective concentration in the assay and specificity.

Fluorescent Peptide	Effective Concentration	λ_{exc} / λ_{em}	Specificity
4- MU-Phosphate	0.05 mM	365nm / 465 nm	Phosphatases
4-MU-β-Cellobiose	0.05 mM	365nm / 465 nm	Cellulases
4-MU-β-D-Glucopyranoside	0.05 mM	365nm / 465 nm	B-Glucosidases
4-MU-N-Acetyl-Glucosaminide	0.05 mM	365nm / 465 nm	B-N-Acetylhexosaminidase (Chitinase)

2.5.4 – Identification of the protease class through inhibition of Enzymatic Activity

In order to assess the class of purified or partial purified proteases, along with the total range of proteases present in the extract, we proceeded to the specific inhibition of enzymatic activity assays. The inhibitors were specific to all protein classes known, as shown in Table X.

Enzymatic activity inhibition was assayed accordingly to enzymatic activity assays described in 5.1, with a previous 10 minute incubation of the sample and 30 mM Sodium Phosphate pH 7 buffer with the inhibitor. The fluorescence was monitored for 10 minutes in a SpectraMAX-GeminiEM.

Tabela X - Inhibitors used in enzymatic assays for inhibition of proteolytic activity, respective effective concentration and specificity of those inhibitors.

Inhibitor	Protease Classes	Efective Concentration (mM)
Pepstatin	Aspartic	0.05
E-64	Cysteine	0.001
PFblock	Serine	1
TPCK	Serine like Chymotrypsin	0.1
TLCK	Serine like Trypsin	0.1
Bestatin	Aminopeptidases	0.001
Amastatin	Aminopeptidases	0.001
EDTA	Metalloproteases	5
DTT	Cysteine	2
Leupectin	Serine, Cysteine and Threonine	
Phenantroline	Metalloproteases	0.05
Divalent Cations		
Ca^{2+}		0.1
Mg^{2+}		0.1
Mn^{2+}		0.1
Zn^{2+}		0.1

2.6 – Protein purification of *Laetiporus sulphureus* extract

2.6.1 – Ammonium Sulphate Precipitation

In order to concentrate the sample a total of 50 mL of extract will be precipitated with 85% Ammonium sulphate and centrifuged at 12 000 rpm for 30 min. The supernatant will be discard and the pellet ressuspended in 30 mM Sodium Phosphate pH7.

2.6.2 – Gel filtration chromatography

Gel filtration chromatography is a technique that manages to separate proteins according to its molecular weight. This separation is accomplished in a porous matrix where the molecules, according to their size, have different degrees of access.

This chromatographic process was carried out in a Superdex 200 *pg* Hiload 16/600 column in a FPLC (Fast Flow Liquid Chromatography) with an AKTA prime (Amersham Pharmacia Biotech), previously equilibrated in 30mM Sodium Phosphate pH 7 at a flow rate of 1mL/min. According manufacturer recommendations, all buffers and samples applied in the system were previously subjected to filtration with 0.45µm filters and degassed with helium.

2.6.3– Ion Exchange Chromatography

Ion exchange chromatography is a separation technique which rests on a principle than a charged groups on the surface of a protein interact with opposite charged groups immobilized in the matrix. The pH at which the protein has net charge zero is known as pI. Therefore, buffer pH can be modified to improve the protein binding to the matrix.

In this line of thought, cation and anion exchange chromatographies were carried out. The anion exchange chromatography was carried out in a Bio-Scale™ Macro-Prep® High Q (5mL, Bio-Rad) column. This chromatographic technique was performed in a FPLC (Fast Flow Liquid Chromatography) with an AKTA prime (Amersham Pharmacia Biotech), previously equilibrated with the respective buffer at a flow rate of 1.5 mL/min. According manufacturer recommendations, all buffers and samples applied in the system were previously subjected to filtration with 0.45 µm Minisart syringe filters and degassed with helium.

2.7 – Statistical Analysis

The results of this project are shown in terms of average ± standard error bar of the number of replicates performed. The statistical analysis was carried out in Graphpad, using One-way ANOVA followed by Bonferroni's post-test.

RESULTS AND DISCUSSION

4.1 - *Laetiporus sulphureus* extract characterization

4.1.1 – Protein Profile of *Laetiporus sulphureus* extract

The contents of 24 g of fruiting body of higher fungi *Laetiporus sulphureus* were macerated in liquid nitrogen until it became a fine powder and homogenised in 70 mL of 30 mM Sodium Phosphate buffer pH 7. After centrifugation, supernatant was filtered in 0.45 µm syringe filters, yielding 52.4 mL of total extract with a protein concentration of 0.595 mg/mL.

The protein fraction of extract was analysed electrophoretically by SDS-Page as described in Section 2.4.

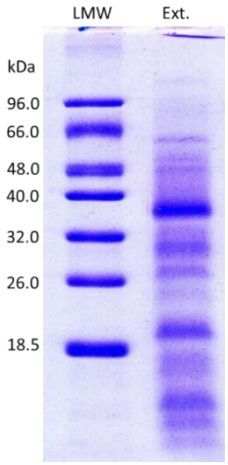

Figure 13 - Proteic Profile of *Laetiporus sulphureus* extract obtained by 12.5% SDS-Page stained with Comassie Blue R-250. LMW) Low Molecular Weight standard; Ext) 5.95 µg of *Laetiporus sulphureus* extract.

As shown in Figure 13, extract holds a wide range of molecular weights, with a band at approximately 38 kDa with greater intensity. Two other intense bands are also notorious at approximately 20 kDa and 10 kDa.

4.1.2 - Enzymatic Profile

To obtain a more accurate enzymatic characterization of the extract of *Laetiporus sulphureus*, a wide range of enzymatic assays were carried out, from a variety of zymographies, as described in section 2.5.1, to enzymatic assays with synthetic fluorogenic substrates, as described in section 2.5.3.

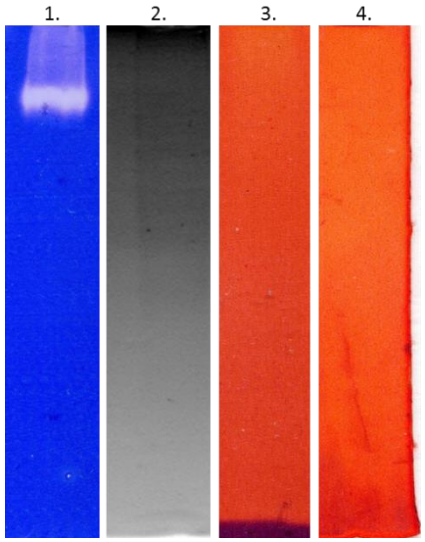

Figure 14 - Protein Profile of *Laetiporus sulphureus* extract obtained by 12.5% SDS-Page in non denaturing conditions, stained with Comassie Blue R-250, calcofluor white and congo red, respectively. 1) Proteases zymography tested with 1.3 µg of *Laetiporus sulphureus* extract; 2) Chitinases zymography tested with 5.95 µg of *Laetiporus sulphureus* extract; 3) Endoglucanases zymography tested with 5.95 µg of *Laetiporus sulphureus* extract; 4) Xylanases zymography tested with 5.95 µg of *Laetiporus sulphureus* extract.

We proceeded to proteases, chitinases, endoglucanases and xylanases zymographies. As shown in Figure 14, the tested extract showed great proteolytic activity. On the other hand no activity was detected in the specific zymographies for chitinases, xylanases and endoglucanases. This registered lack of activity can be due to the specificity of the substrate used.

Furthermore, activities were also tested with fluorescent glycosides (MU substrates) and peptides (AMC peptides), as described in section 2.5.3. This assay is more sensitive and specific than zymographies.

As shown in Figure 15 and Table XI, it is clear that MU-NAG (4-MU-N-Acetyl-Glucosaminide) has a prevalence of activity when compared with the other MU substrates. MU-NAG has an activity of 6724.8 nmol MU/min/mg, more than ten times superior to MUC (4-MU-β-Cellobiose) with 188.1 nmol MU/min/mg. Moreover, MUGlc (4-MU-Glucopyranose) has 753.4 nmol MU/min/mg and 4-MUP (4-MU-Phosphate) has 218.9 nmol MU/min/mg.

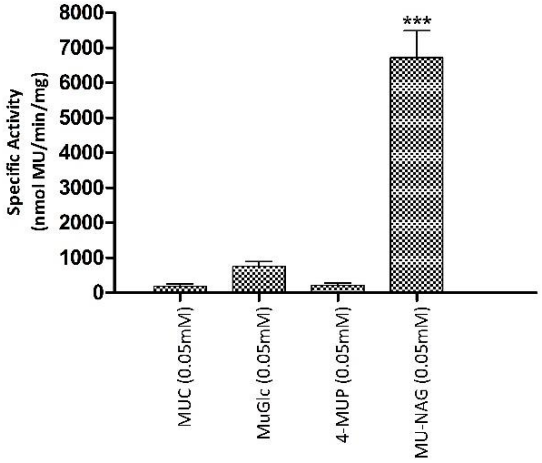

Figure 15 - Glycosidic activities determination. *Laetiporus sulphureus* extract was assayed with the aid of MU-conjugated fluorescent peptide substrates. To 2.9 µg of extract total protein, 2µL of fluorescent peptide were added. The final volume was 200 µL, adjusted with sample buffer (n=3).

Table XI - Glycosidic activities list, obtained by the assay of *Laetiporus sulphureus* extract with MU fluorescent peptide substrates.

Fluorescent Peptide	Specific activity (nmol MU/min/mg)
MUC (0.05mM)	188.1 ± 67.3
MUGlc (0.05mM)	753.4 ± 145.6
4-MUP(0.05mM)	218.9 ± 61.6
MU-NAG(0.05mM)	6724.8 ± 761.9

As it was already described in section 2.5.3, MU-NAG is a chitinase specific substrate, showing that *Laetiporus sulphureus* possess great chitinase activity when compared with the other MU-conjugated fluorescent substrates.

Thereby it is possible that the substrates used in chitinase, endoglucanase and xylanase zymographies were not as specific as the fluorescent MU substrates used. Zymography was carried out with Glycol Chitosan substrate instead of Glycol Chitin, its

specific substrate (Asselin 1989, Krishnaveni 1999, Radjacomare 2004). On the other hand, N-Acetyl-Glucosaminide is the monomer that constitutes chitin polymer, so it's highly specific. Furthermore, that is the possible reason to the activity differences seen in chitinase zymography and fluorescent assays.

In order to identify and characterize proteolytic activity in extract, preferential substrate and inhibition assays were carried out with AMC conjugated fluorescent substrates, as described in section 2.5.3. (Figure 16).

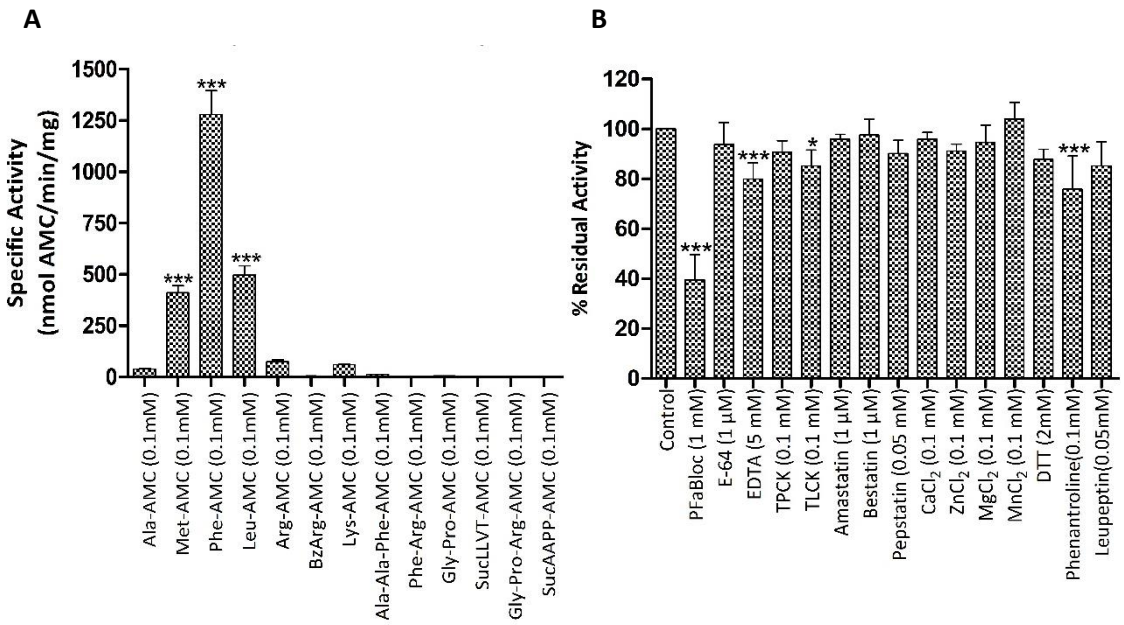

Figure 16- Proteolitic activity and inhibition assays. A) *Laetiporus sulphureus* extract was assayed with the aid of AMC-conjugated fluorescent peptide substrates. To 0.3 µg of extract were added 2 µL of fluorescent peptide. The final volume was 200 µL, adjusted with sample buffer (n=5); B) *Laetiporus sulphureus* extract was assayed with the preferential substrate Phe-AMC (0.1mM). Inhibitors were added to 0.3 µg of protein and 0.1 mM of Phe-AMC. The final volume was 200 µL, adjusted with sample buffer. The control was carried out exactly like the inhibition assays, with a 10 minutes incubation with only the extract and the sample buffer, and subsequent addition of 0.1mM Phe-AMC (n=4).

As shown in Figure 16A, it is clear that Phe-AMC is the preferential substrate with an activity of 1279.7 nmol AMC/min/mg, immediately followed by Leu-AMC and Met-AMC, with 498.6 nmol AMG/min/mg and 410.9 nmol AMC/min/mg, respectively. Also, as it clear

in Figure 16 and complemented with Table XII, the other tested AMC fluorescent substrates do not show significant or relevant activities.

Table XII - All proteolytic activities obtained by the assay of *Laetiporus sulphureus* extract with AMC fluorescent peptide substrates.

Fluorescent Peptide	Specific activity (nmol AMC/min/mg)
Ala – AMC (0.1mM)	38.8 ± 2.9
Met – AMC (0.1mM)	410.9 ± 36.8
Phe – AMC (0.1mM)	1279.7 ± 116.1
Leu – AMC (0.1mM)	498.6 ± 42.8
Arg – AMC (0.1mM)	74.9 ± 10.3
Bz-Arg – AMC (0.1mM)	4.4 ± 1.3
Lys – AMC (0.1 mM)	60.2 ± 3.4
Ala-Ala-Phe-AMC (0.1mM)	12.6 ± 0.6
Phe-Arg-AMC (0.1mM)	0.00
Gly-Pro-AMC (0.1mM)	6.1 ± 0.1
SucLLVT-AMC (0.1mM)	0.3 ± 0.1
Gly-Pro-Arg-AMC (0.1mM)	0.3 ± 0.1
SucAAPP-AMC (0.1mM)	1.3 ± 1.0

Inhibition assays were carried out as described in Section 2.5.4 with 0.3 µg of total protein. These assays were performed with the previously established preferential substrate, Phe-AMC (0.1mM). Samples were incubated for 10 minutes with the specific inhibitor, previously to the addition of Phe-AMC.

As it is shown in Figure 16B and complemented with Table XIII, extract of *Laetiporus sulphureus* shown a significant inhibition, of approximately 60%, with PFabloc, a serinic protease irreversible inhibitor. It also shown about 25% inhibition with Phenantroline and 20% inhibition with EDTA, metalloproteinase inhibitors. Both TLCK and Leupeptin showed about 15% of inhibition, as DTT showed about 13% of inhibition.

Table XIII - Proteolytic activity inhibition list. *Laetiporus sulphureus* extract was assayed with Phe-AMC (0.1mM).

Inhibitors	% Residual Activity
Control	100
PFaBloc (1 mM)	39.6 ± 10.1
E-64 (1 µM)	93.9 ± 8.7
EDTA (5 mM)	79.9 ± 6.6
TPCK (0,1 mM)	90.7 ± 4.6
TLCK (0.1 mM)	85.3 ± 6.3
Amastatin (1 µM)	95.9 ± 2.0
Bestatin (1 µM)	97.6 ± 6.3
Pepstatin (0.05 mM)	90.1 ± 5.3
CaCl2 (0.1 mM)	95.9 ± 2.8
ZnCl2 (0.1 mM)	91.4 ± 2.9
MgCl2 (0,1 mM)	94.7 ± 6.9
MnCl2 (0.1 mM)	104.0 ± 6.7
DTT (2 mM)	87.792 ± 4.034
Phenantroline (0.1 mM)	75.823 ± 13.333
Leupeptin (0.05 mM)	85.236 ± 9.648

Therefore, it is clear that *Laetiporus sulphureus* extract possesses serine proteases and metalloproteases activities considering its inhibition profile. This conclusion is confirmed by the inhibition with PFaBlock and TLCK, both irreversible serine protease inhibitors. The inhibition achieved with EDTA and Phenantroline, both divalent metals chelating agents, is also statistically relevant and confirms the existence of metalloproteinases in the extract.

4.2 – Hydrolases Purification

In order to proceed to the purification of hydrolases of the extract in study, a serie of separation processes were carried out, including: ammonium sulphate precipitation, gel filtration chromatography and ion exchange chromatography. In this way, several interesting activities were found. All fractions were analysed with all fluorogenic substrates according to the enzymatic assay described in Section 2.5.3. The preferential substrates were Phe-AMC and MU-NAG, the same as the extract.

4.2.1 – Proteases Purification

Thereby, in order to isolate the proteolytic and chitinolytic activity we proceeded with the fractionation of *Laetiporus sulphureus* extract. We precipitated 50 mL (29.75 mg of total protein) of extract with 85% Ammonium Sulphate in order to concentrate the extract. The pellet was ressuspended in 7 mL of 30 mM Sodium Phosphate pH7 buffer. Four millilitres of the precipitate was filtered through 0.45 µm syringe filters and applied to a gel filtration *Superdex 200* column (section 2.6.2) previously equilibrated in 30 mM Sodium Phosphate pH 7 buffer, at the flow rate of 1 mL/min (Figure 17) with the purpose of sort proteins according their molecular weight.

Fractions with a 4 mL volume were collected and the ones that showed a higher absorbance at 280 nm were assayed for enzymatic activity with the preferential substrates, Phe-AMC and MU-NAG, as described in section 2.5.3. All fractions showing higher activity with MU-NAG were pooled together, such as fractions showing higher activity with Phe-AMC. Nevertheless, there were fractions which had residual activity with both Phe-AMC and MU-NAG (Figure 17).

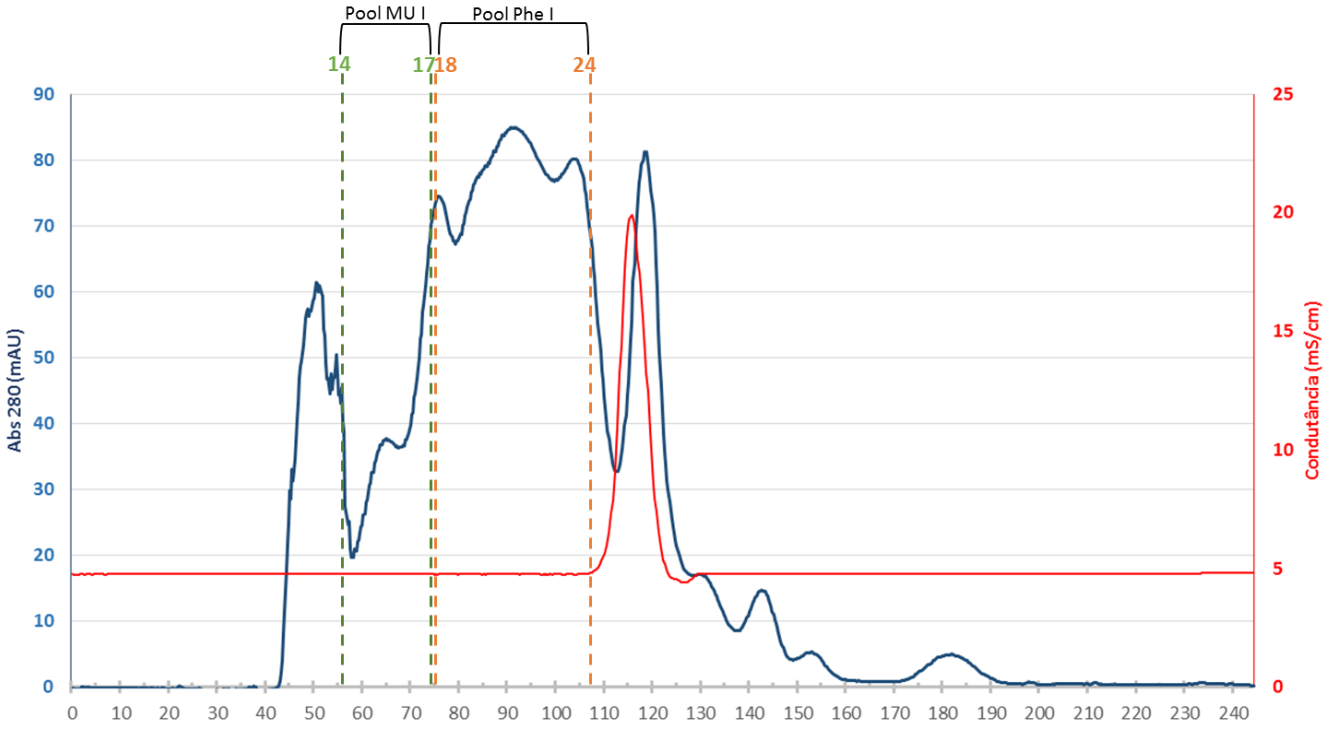

Figure 17 – Chromatogram obtained after gel filtration chromatography in an AKTA FPLC system. To HiLoad 16/600 Superdex 200 pg (16x600 mm) column, 4mL of *Laetiporus sulphureus* extract previously precipitated with 85% Ammonium Sulphate and ressuspended with 30mM Sodium Phosphate pH7 (sample buffer) were applied. The column was previously equilibrated with sample buffer (30 mM Sodium Phosphate pH7). The elution was carried out at the flow of 1 mL/min in the same buffer. Fractions of interest are identified.

As it was stated before, a great variety of activities were found. According to the higher activity found, two different pools were taken in consideration, Pool MU I (F14 to F17) and Pool Phe I (F18 to F24).

In order to determine the proteolytic profile of the fractions poolled together, they were submitted to electrophoretical analysis by SDS-Page, as described in Section 2.4.

It is clear that in pool MU we can see the prevalence of band at high molecular weights while in pool Phe I we can see a prevalence of lower molecular weights bands. Once the pools have different elution volumes, it was expected this result (Figure 18).

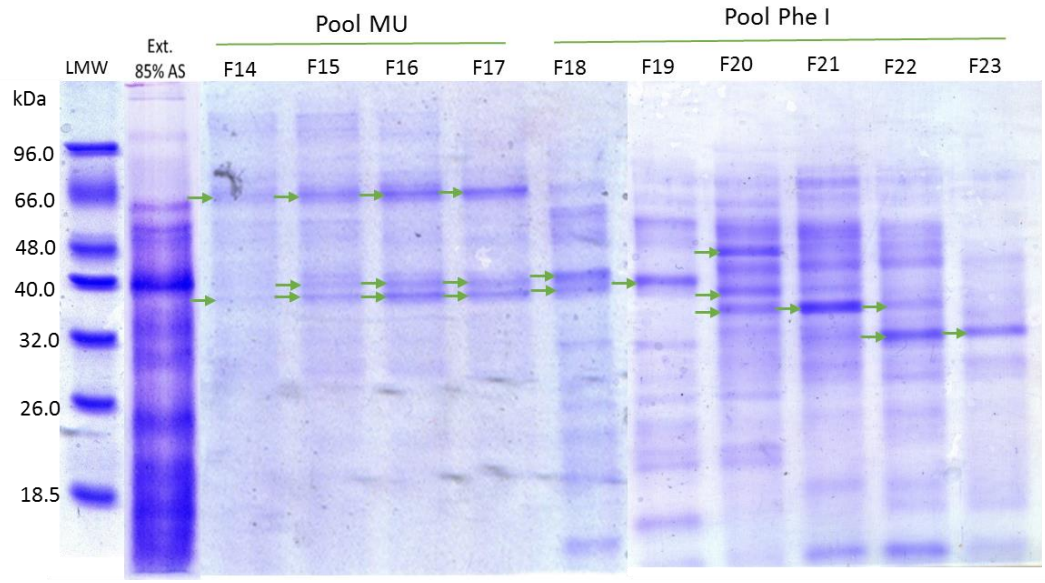

Figure 18 - Proteic Profile Analysis of Gel Filtration Chromatography fractions obtained in a HiLoad 16/600 Superdex 200 pg (16x600 mm) column in an FPLC by 12.5% SDS-Page stained with Comassie Blue R. LMW) Low Molecular Weight standard; Ext 85%AS) 20µL of *Laetiporus sulphureus* extract precipated with 85% Ammonium Sulphate; Pool MU) 20 µL of each fraction of *Superdex 200* put together to for Pool MU; Pool Phe I) 20 µL of each fraction of *Superdex 200* put together to for Pool Phe-AMC.

In fractions constituting pool MU, there are three constant and more noticeable bands, one of approximately 38 kDa, another of 40 kDa and a third one of 66 kDa approximately (Figure 18).

Meanwhile, pool Phe I revealed a greater diversity of bands. We can see a band with higher intensity at approximately 36 kDa appearing in F20 and intensifies from there, until F22, where it is clearly visible. In F20 it is also visible a band at approximately 47 kDa. In F22 and 23 it is visible a band at approximately 33 kDa. Also, it is visible a band at approximately 38 kDa in F19 and F20 (Figure 18).

In order to achieve a higher level of separation, a next purification strategy is needed. The next purification step is an ion exchange chromatography, as described in section 2.6.3. Thereby, 32 mL of Pool Phe I were filtered through 0.45 µm syringe filter and applied in two

5 mL *High Q* columns tandem together, previously equilibrated with 30 mM Sodium Phosphate pH6.5, at a flow of 1.5 mL/min (Figure 19).

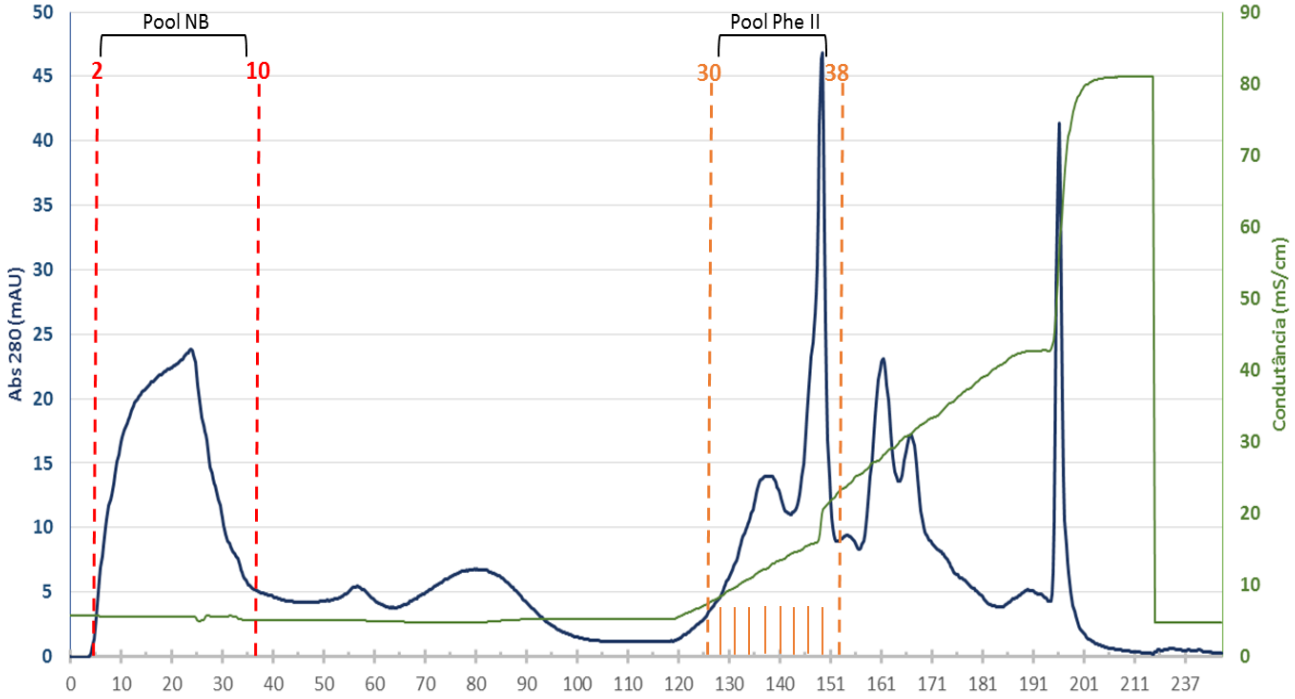

Figure 19 - Anion exchange chromatogram. To two High Q (5mL) column were injected 32mL of pool Phe-AMC. The column was previously equilibrated with sample buffer (30mM Sodium Phosphate pH6.5). The elution was carried out with a salt gradient of 0% to 45% of elution buffer [30mM Sodium Phosphate, 1M NaCl, pH6.5] at the flow of 1.5mL/min in the same buffer. Fractions of interest are identified.

The column was eluted with a continuum salt gradient of 0-45% of NaCl in 30 mM Sodium Phosphate pH 6.5 buffer.

All fractions with absorbance at 280nm were tested with Phe-AMC, as described in Section 2.5.3, verifying that the fractions with Phe-AMC activity are the identified in Figure 19.

All the non-binding fractions were also assayed with Lys-AMC, Arg-AMC and Met-AMC. An interesting activity with Arg-AMC was found, being this substrate the preferential in non-binding fractions (Table XIV).

Table XIV - Proteolytic activity of nonbinding fractions of pool Phe I submitted to anion exchange chromatography.

AMC Substrate	pmol AMC/min
Arg – AMC (0.1 mM)	66.7
Leu – AMC (0.1 mM)	25.2
Lys – AMC (0.1 mM)	5.7
Met – AMC (0.1 mM)	49.8
Phe – AMC (0.1 mM)	25.4

All fractions were analysed electrophorectically by SDS-Page, as described in section 2.4 (Figure 20).

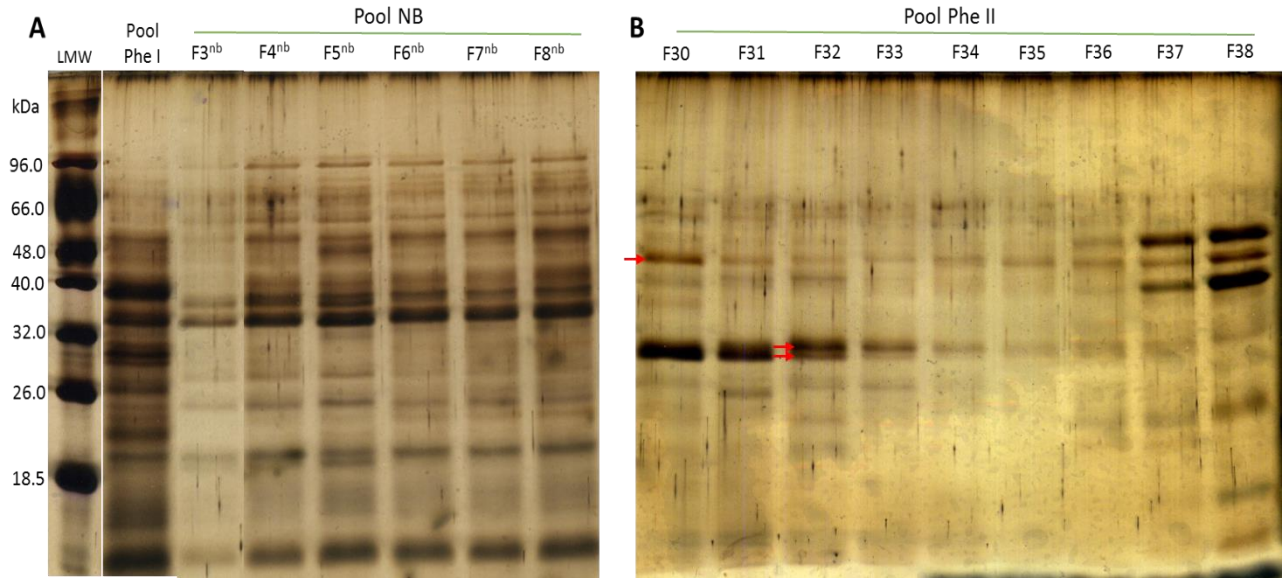

Figure 20 - Proteic Profile Analysis of Anion Exchange Chromatography fractions obtained in two High Q columns in an FPLC by 12.5% SDS-Page stained with silver nitrate. Gel A) **LMW - Low Molecular Weight standard; Pool Phe I) 20µL of Pool Phe which was resultant of the fractions put together in gel filtration chromatography with activity with Phe-AMC; Pool NB) Non-binding fractions (20 µL);** Gel B) **Pool Phe. Fractions with activity with Phe-AMC, bounded to the column (20 µL) (F30 to 38).**

As we can see, the nonbinding pool hold a great variety of proteins, with a large range of molecular weights, unlike to the fractions which bounded to the column (Figure 20A).

In pool Phe II, F32 and F33, we see that the intense band that we thought to be only one is, in fact, two bands with very similar molecular weights, between 30 and 32 kDa. Also, it is clear the presence of bands with 50 kDa, with 38 kDa (Figure 20B).

Although, it was observed that the nonbinding pool to the anion exchange chromatography with two 5 mL *High Q* columns did not possessed relevant activity with Phe-AMC but rather with Arg-AMC. In order to access the class of protease present in the nonbinding fractions, inhibitor assays were carried out with this nonbinding pool, Figure 21, with the appropriate substrate, Arg-AMC, as described in section 2.5.4.

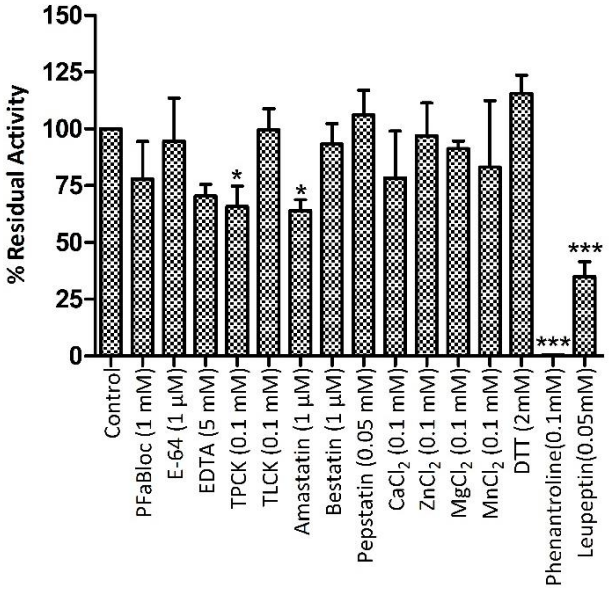

Figure 21 - Proteolytic activity inhibition assay. Nonbinding Pool of the anion exchange chromatography in two High Q columns were assayed with Arg-AMC (0.1mM). Is was added 50 µL of protein and 0.1mM of Arg AMC. The control was carried out exactly like the inhibition assays, with a 10 minutes incubation with only the extract and the sample buffer, and after the addition of 0.1mM Arg-AMC (n=3).

As it is clear in the inhibition assays, nonbinding fractions showed an inhibition of approximately 100 % with Phenantroline. It is also clear the 35 % inhibition with Amastatin, which indicates the presence of aminopeptidase. So, as we know, aminopeptidases can also be metalloproteinases, so it is possible that we are facing of this cases.

Consequently, it is possible to assert that this nonbinding pool has metalloproteinase characteristics, yet, as it is possible to see in Figure 20A, we have an extended range of proteins, with different molecular weights.

Table XV - Proteolytic activity inhibition of nonbinding pool assyed with Arg-AMC (0.1mM).

Inhibitors	% Residual Activity
Control	100
PFaBloc (1 mM)	78.1 ± 16.4
E-64 (1 µM)	94.7 ± 16.4
EDTA (5 mM)	70.4 ± 5.2
TPCK (0,1 mM)	65.7 ± 9.1
TLCK (0,1 mM)	99.6 ± 9.2
Amastatin (1 µM)	64.1 ± 4.7
Bestatin (1 µM)	93.3 ± 9.0
Pepstatin (0.05 mM)	106.2 ± 10.9
CaCl2 (0,1 mM)	78.4 ± 20.6
ZnCl2 (0,1 mM)	96.9 ± 14.5
MgCl2 (0,1 mM)	91.3 ± 3.4
MnCl2 (0,1 mM)	83.1 ± 29.3
DTT (2mM)	115.7 ± 8.0
Phenantroline (0,1mM)	0.4 ± 0.1
Leupeptin (0,05mM)	34.9 ± 6.6

It is clear the great purification level that these two chromatographic steps achieve, however it's imperative reach a higher separation ration. However, as it was explained before, pool MU that were pooled together in the gel filtration chromatography step had a residual activity with the substrate Phe-AMC.

In order to process all the fractions put together in gel filtration chromatography, another anion exchange chromatography was carried out now with pool MU, as reported in section 2.5.3.Thereby, 12mL of Pool MU were filtered through 0.45 µm syringe filters and applied in two 5 mL *High Q* columns, tandem in serie, previously equilibrated with 30mM Sodium Phosphate pH6.5, at a flow rate of 1.5 mL/min (Figure 22).

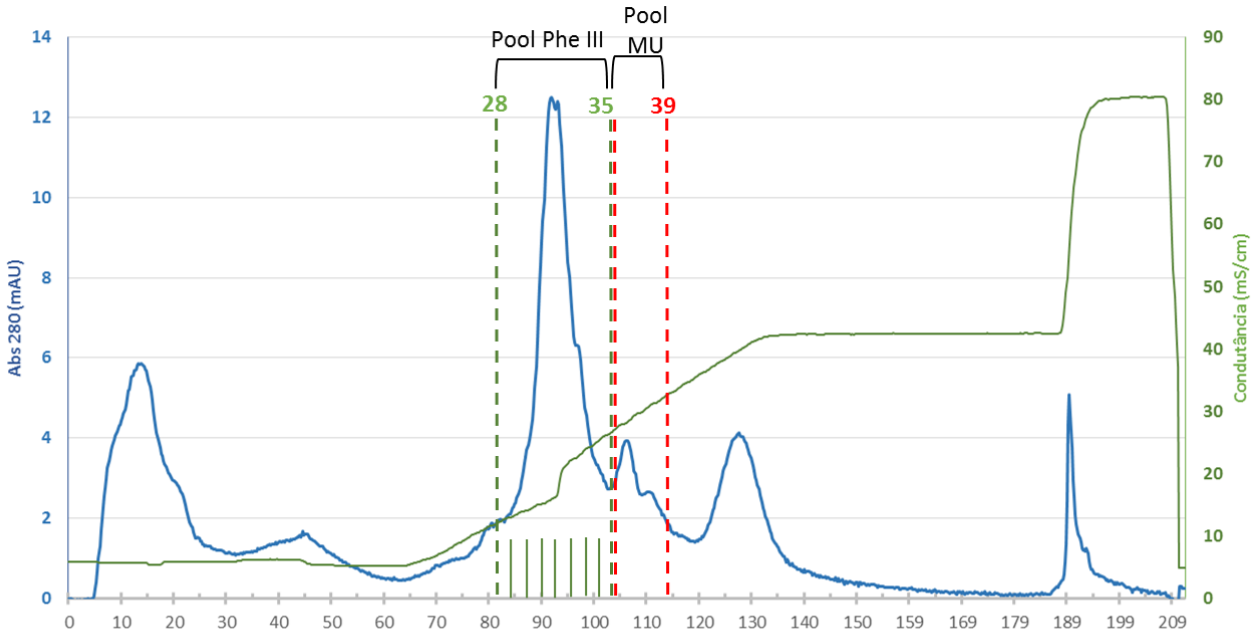

Figure 22 - Anion exchange chromatogram. To two High Q (5 mL) columns were injected 12 mL of pool MU. The column was previously equilibrated with sample buffer (30 mM Sodium Phosphate pH6.5). The elution was carried out with a salt gradient of 0% to 45% of elution buffer [30mM Sodium Phosphate, 1 M NaCl, pH6.5] at the flow of 1.5 mL/min in the same buffer. Fractions of interest are identified.

The column was eluted with a continuum salt gradient of 0-45% of NaCl in 30 mM Sodium Phosphate pH 6.5 buffer. All fractions with absorbance at 280 nm were tested with Phe-AMC and MU-NAG, as described in section 2.5.2, observing a distinct peak with activity only with Phe-AMC.

All fractions with Phe-AMC activity resulting of anion exchange chromatography were subjected to SDS-Page, as described in section 2.4 (Figure 19).

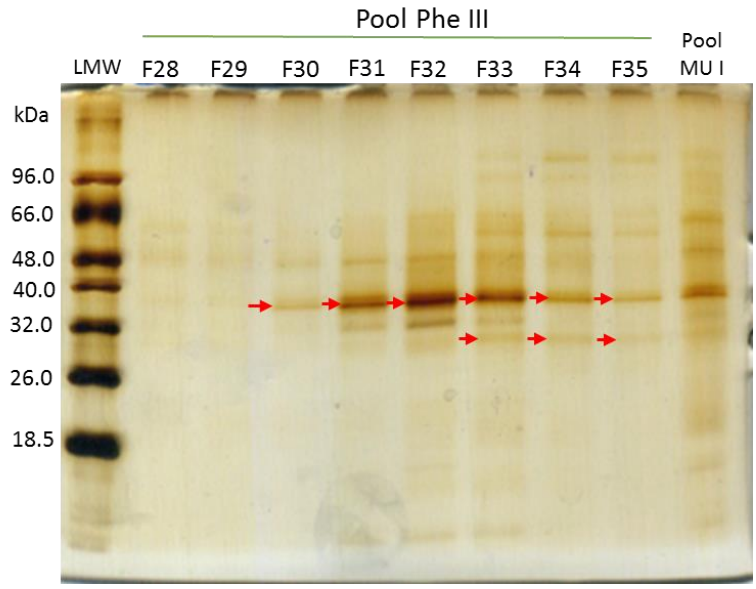

Figure 23 - Proteic Profile Analysis of Anion Exchange Chromatography fractions obtained in two 5 mL High Q columns in an FPLC by 12.5% SDS-Page stained with silver nitrate. LMW) Low Molecular Weight standard; Pool MU I) 20µL of Pool MU which was resultant of the fractions put together in gel filtration chromatography with activity with MU-NAG; Pool Phe III) Fractions of Pool MU with activity with Phe-AMC.

As we can see, the band with greater intensity is at approximately 38 kDa. It is clear more bands in the gel, such as approximately 32 kDa (Figure 23).

All fractions with Phe-AMC activity of both ion exchange chromatografies were pooled totalling a volume of 51 mL. This pool was concentrated with an Amicon of 10 kDa cutoff until achieving a final volume of approximately 5mL, forming pool Phe IV.

Three millilitres of pool Phe IV were applied in a *Superdex 200* in an FPLC system. This column was previously equilibrated with 30mM Sodium Phosphate pH7 buffer, at a flow rate of 1 mL/min (Figure 24).

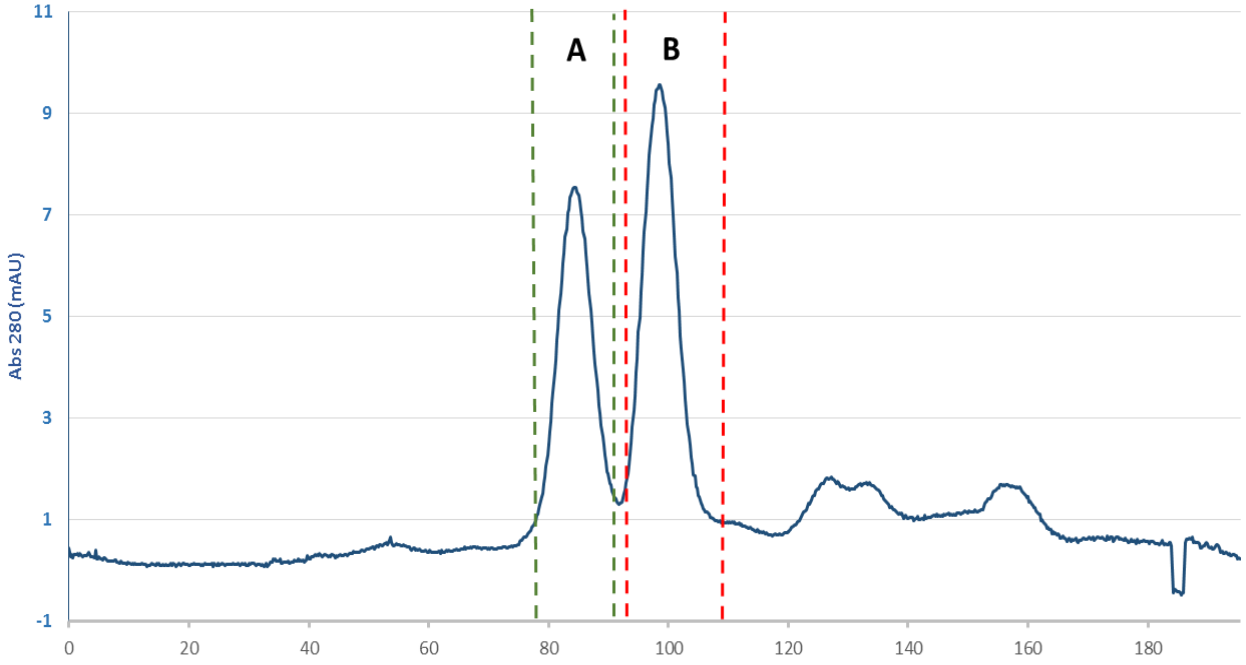

Figure 24 - Gel filtration chromatography chromatogram. To a HiLoad 16/600 Superdex 200 pg (16x600 mm) were applied in a FPLC system 3 mL of Pool Phe IV, resulting of the two anion exchange chromatographies, concentrated. The column was previously equilibrated with sample buffer (30 mM Sodium Phosphate pH7). The elution was carried out at the flow of 1mL/min in the same buffer. Fractions of interest are identified.

After the elution, there are two clear peaks in chromatogram, as we can see in Figure 24. Thereby, these peaks of interest were analysed with Phe-AMC, as described in section 2.5.3.

It is possible that each peak represent a different protein. However, more data is needed to confirm this hypothesis. So peak A and B were subjected to SDS-Page, as described in section 2.4.1, as shown in Figure 25.

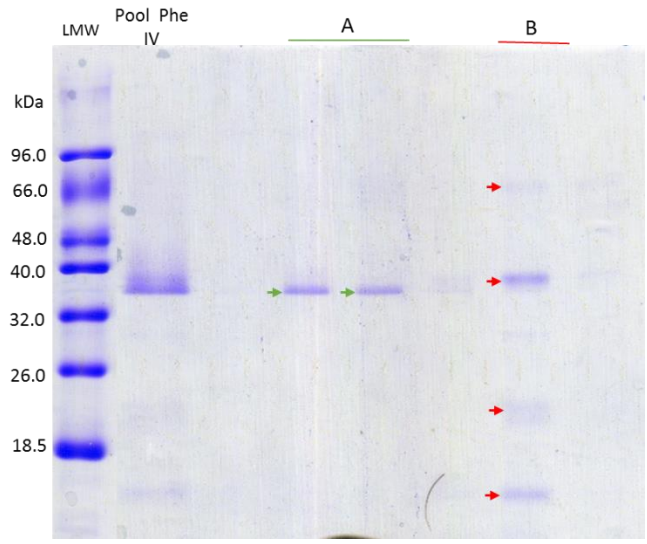

Figure 25 - Protein Profile Analysis of Gel Filtration Chromatography peaks obtained in a HiLoad 16/600 Superdex 200 (16x600 mm) column in an FPLC by 12.5% SDS-Page stained with Comassie Blue R-250. LMW) Low Molecular Weight standard; Pool Phe Conc.) Pool Phe IV.Fractions put together resulting of both ion exchange chromatographies with activity with Phe-AMC (20µL); A) Fractions of the first peak (Green) of Gel filtration chromatography in Figure 20 (20 µL); B) Fractions of the second peak (red) of Gel filtration chromatography in Figure 20 (20 µL).

As we can see, it is clear a difference of protein profile of A to B. Peak A showed a single band at approximately 38.5 kDa, whereas B holds more than one band: one at approximately 39 kDa, with greater intensity, other at 10 kDa, and yet another two at approximately 70 kDa and 22 kDa.

In order to know the molecular weights of A and B, two Native-Pages were carried out, as described in section 2.5.2, as it is shown in Figure 26. To enforce the data supported by Native-Page, one of the gels will be revealed in Phe-AMC (Figure 26 – 2) and the other will be stained with Silver Nitrate, as described in section 2.4.3 (Figure 26 – 1). In Native- Page, proteins will not be separated considering only its molecular weight but also its net charge.

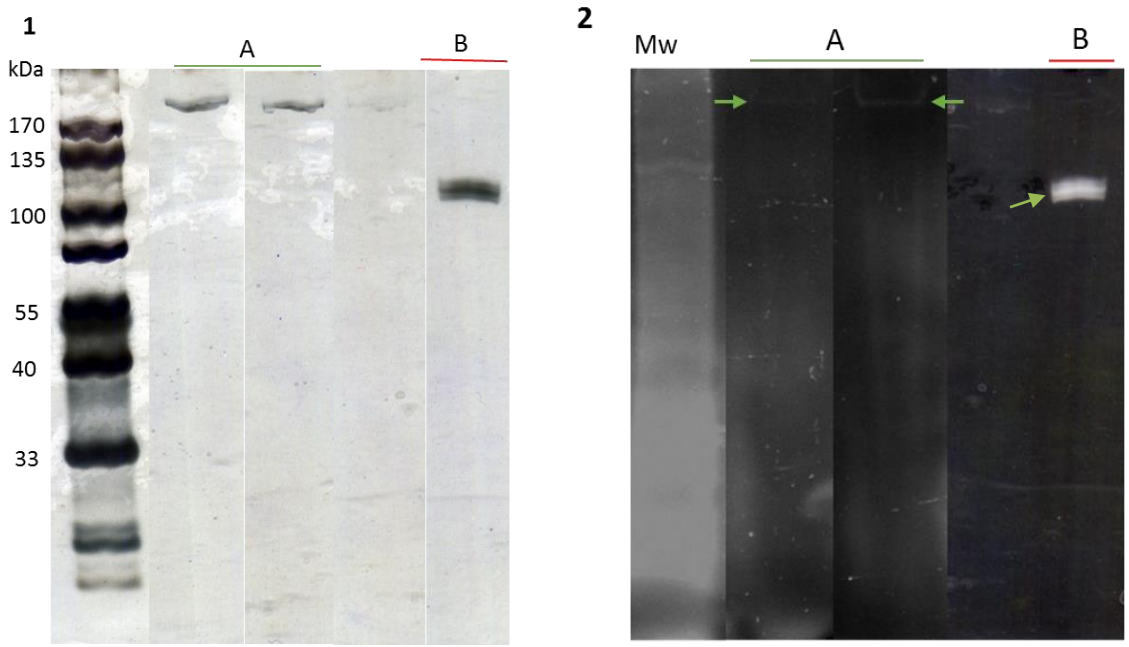

Figure 26 - Proteic Profile Analysis of Gel Filtration Chromatography fractions obtained in a Superdex 200 column in an FPLC by Native-Page with 12.5% acrylamide/bisacrylamide in non denaturing conditions. 1) Copy of the native page stained with silver nitrate. 2) Native page incubated with Phe-AMC and revealed in Versadoc.

It is possible to see the corresponding bands in Gel 1 and 2 (Figure 26). In peak A we can see a band above 170 kDa, meanwhile in peak B, it is possible to see a band above 100 kDa. So, when we relate the results obtained in Native-Pages (Figure 26) with the results obtained in SDS-Page (Figure 25), we can say that is possible that we are dealing with multimeric proteins. Although, as it was already stated above, in Native-Page, proteins are not separated according the molecular weight only, but also accordingly to the protein net charge, so it is important other confirmation methods.

In order to determine the exact molecular weights, a calibration curve of the column was needed. Accordingly, A and B were applied separately in a Superdex 200 Increase 5/150 GL (GE Healthcare Lifesciences) at a flow of 0.3 mL/min. This analytical gel filtration chromatography was carried out in Molecular Biotechnology Laboratory in UC-Biotech in Biocant.

A calibration curve was deduced from a wide range of molecular weight proteins: Aprotinin (6.5 kDa), Ribonuclease (13.7 kDa), Carbonic Anhydrase (29 kDa), Ovalbumin (43 kDa), Conalbumin (75 kDa), Aldolase (158 kDa), Ferritin (440 kDa), Thyroglobulin (669 kDa). With the equation obtained from calibration curve, we were able to assess the molecular weights of our proteins of interest.

Furthermore, protein A has a molecular weight of 232.2 kDa, with an elution time of 5.178 min and an elution volume of 1.55 mL. As regards B, it was estimated a molecular weight of 86.7 kDa, with an elution time of 6.025 min and an elution volume of 1.81 mL.

So, taking in consideration all data, we raise the possibility that protein in A exists in hexameric form. Accordingly to calibration curve, protein in A has a molecular weight of 232 kDa, therefore, a hexameric protein with six subunits with 38.5 kDa owned approximately that molecular weight.

As it is shown in Figures 17, 18 and 19, the peaks present in figure are in fact two distinct proteins with different molecular weights. The first peak, corresponding to A, hold a protein with approximately 232.2 kDa, and the second peak corresponding to B, hold a protein of approximately 86.7 kDa.

This data is supported with gels, both native gel as SDS-Page, and for a gel filtration chromatography column calibration, as shown above.

With these new information, it is possible that the band at approximately 70 kDa seen in Figure 25 was protein aggregated.

After the purification process, we proceeded to an enzymatic characterization of the protease. The protease of the peak A, with 232.2 kDa has, as preferential substrate Phe-AMC, with a specific activity of 17.2 nmol AMC/min/mg, soon followed by Leu-AMC with 16.0 nmol AMC/min/mg, as shown in Figure 27 and Table XVI.

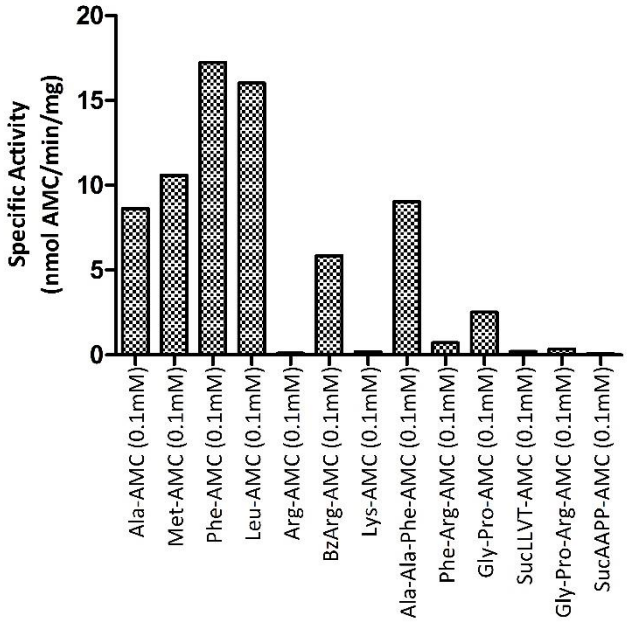

Figure 27 - Determination of Proteolitic activities of the protease with approximately 232.2 kDa (A). Protease activity was assayed with the aid of AMC-conjugated fluorescent peptide substrates. To 50 µL of the sample F15, 2µL of fluorescent peptide were added. The final volume was 200 µL, adjusted with sample buffer (n=1).

Table XVI - All proteolytic activities obtained by the assay of the protease with approximately 232.2 kDa (A) with AMC fluorescent peptide substrates.

Fluorescent Peptide	Specific activity (nmol AMC/min.mg)
Ala – AMC (0.1mM)	8.6
Met – AMC (0.1mM)	10.6
Phe – AMC (0.1mM)	17.2
Leu – AMC (0.1mM)	16.0
Arg – AMC (0.1mM)	0.1
Bz-Arg – AMC (0.1mM)	5.9
Lys – AMC (0.1 mM)	0.2
Ala-Ala-Phe-AMC (0.1mM)	9.0
Phe-Arg-AMC (0.1mM)	0.7
Gly-Pro-AMC (0.1mM)	2.5
Suc-Leu-Leu-Val-Tyr-AMC (0.1mM)	0.2
Gly-Pro-Arg-AMC (0.1mM)	0.4
Suc-Ala-Ala-Pro-Phe-AMC (0.1mM)	0.1

An inhibition assay was carried out in order to determine the protease class in study. As shown in Figure 28, the protease present in A is a metalloproteinase, since it is inhibited by 1,10-Phenantroline, a metalloproteinase inhibitor, showing an inhibition of approximately 60%. Moreover, A is strongly inhibited by Leupeptin, with an inhibition of approximately 70%. It is known that Leupeptin is a serine, cysteine and threonine proteases

inhibitor. Although, Leupeptin is a compound of the aldehyde class that can also act as metalloproteinases inhibitors (Westerik JO 1972).

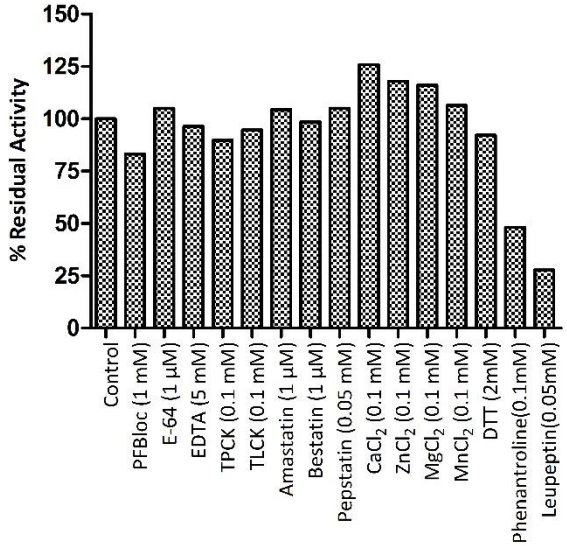

Figure 28 - Proteolytic activity inhibition assay. A was assayed with Phe-AMC (0.1mM). It was added 50 µL of protein and 0.1 mM of Phe-AMC. The final volume was 200 µL, adjusted with sample buffer. The control was carried out exactly like the inhibition assays, with a 10 minutes incubation with only the extract and the sample buffer, and after the addition of 0.1 mM Phe-AMC.

Table XVII - Proteolytic activity inhibition of A assayed with Phe-AMC (0.1 mM).

Inhibitors	% Residual Activity
Control	100
PFaBloc (1 mM)	83.2
E-64 (1 µM)	105.1
EDTA (5 mM)	96.5
TPCK (0,1 mM)	89.8
TLCK (0,1 mM)	94.7
Amastatin (1 µM)	104.4
Bestatin (1 µM)	98.6
Pepstatin (0.05 mM)	105.1
CaCl2 (0,1 mM)	125.9
ZnCl2 (0,1 mM)	118.0
MgCl2 (0,1 mM)	116.0
MnCl2 (0,1 mM)	106.4
DTT (2mM)	92.2
Phenantroline (0,1mM)	48.2
Leupeptin (0,05mM)	27.8

The protease of the fraction B (86.7 kDa), has as preferential substrate Phe-AMC, with a specific activity of 31.8 nmol AMC/min/mg, soon followed by Leu-AMC with 24.9 nmol AMC/min/mg, as shown in Figure 29 and Table XVIII.

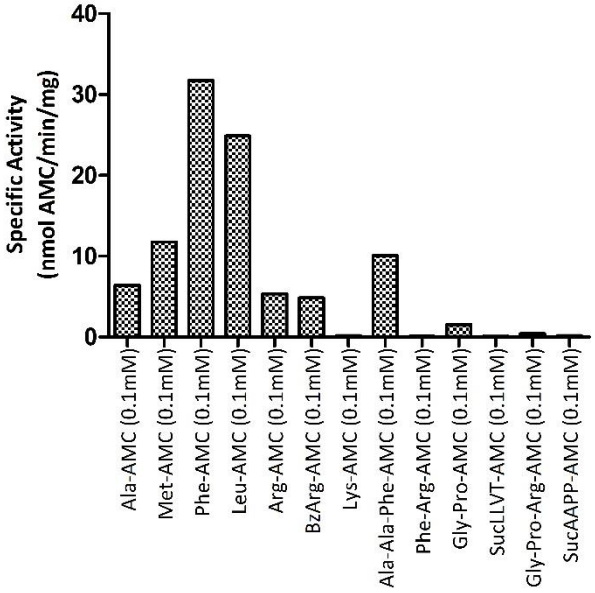

Figure 29 - Proteolitic activities determination of the protease with 86.7 kDa (B). Protease activity was assayed with the aid of AMC-bound fluorescent peptide substrates. Is was added 50 µL of the sample pool and 2µL of fluorescent peptide. The final volume was 200 µL, adjusted with the sample buffer.

Table XVIII - All proteolytic activities obtained by the assay of the purified protease of 86.7 kDa (B) with AMC fluorescent peptide substrates.

Fluorescent Peptide	Specific activity (nmol AMC/min.mg)
Ala – AMC (0.1mM)	6.4
Met – AMC (0.1mM)	11.7
Phe – AMC (0.1mM)	31.8
Leu – AMC (0.1mM)	24.9
Arg – AMC (0.1mM)	5.4
Bz-Arg – AMC (0.1mM)	4.8
Lys – AMC (0.1 mM)	0.2
Ala-Ala-Phe-AMC (0.1mM)	10.1
Phe-Arg-AMC (0.1mM)	0.1
Gly-Pro-AMC (0.1mM)	1.5
Suc-Leu-Leu-Val-Tyr-AMC (0.1mM)	0.1
Gly-Pro-Arg-AMC (0.1mM)	0.4
Suc-Ala-Ala-Pro-Phe-AMC (0.1mM)	0.2

An inhibition assay was carried out in order to determine the protease class in study. As shown in Figure 30, the protease present in B is a metalloproteinase, since it is

inhibited by Phenantroline, showing an inhibition of approximately 45%. Furthermore, B is also inhibited by Leupeptin, with an inhibition of approximately 75% (Table XIX).

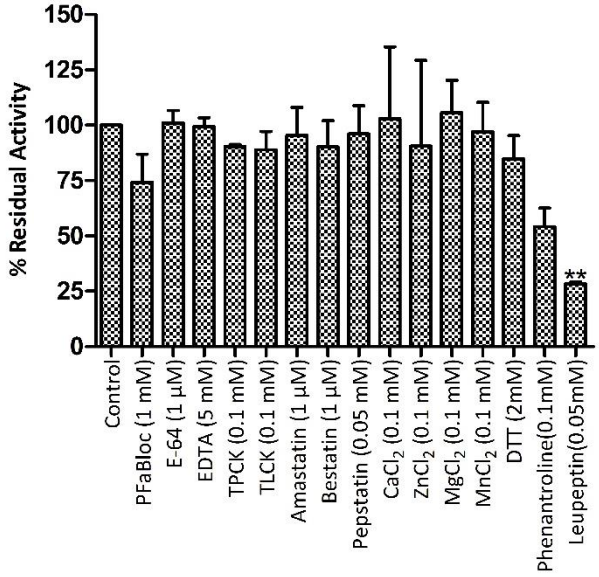

Figure 30 - Proteolytic activity inhibition assay. Fraction B was assayed with Phe-AMC (0.1mM). Is was added 50µL of protein and 0.1mM of Phe AMC. The final volume was 200 µL, adjusted with the sample buffer. The control was carried out exactly like the inhibition assays, with a 10 minutes incubation with only the extract and the sample buffer, and after the addition of 0.1mM Phe-AMC (n=2).

Table XIX - Proteolytic activity inhibition of B assayed with Phe-AMC (0.1mM).

Inhibitors	% Residual Activity
Control	100
PFaBloc (1 mM)	74.2 ± 12.7
E-64 (1 µM)	101.1 ± 5.6
EDTA (5 mM)	99.3 ± 4.1
TPCK (0,1 mM)	90.4 ± 0.9
TLCK (0,1 mM)	88.9 ± 8.2
Amastatin (1 µM)	95.4 ± 12.7
Bestatin (1 µM)	90.2 ± 11.9
Pepstatin (0.05 mM)	96.1 ± 12.7
CaCl2 (0,1 mM)	103.0 ± 32.4
ZnCl2 (0,1 mM)	90.5 ± 38.8
MgCl2 (0,1 mM)	105.6 ± 14.7
MnCl2 (0,1 mM)	97.1 ± 13.2
DTT (2mM)	84,862 ± 10,42
Phenantroline (0,1mM)	54,162 ± 8,45
Leupeptin (0,05mM)	24,832 ± 0,80

According to all the data presented, we can say that is possible that we ha isolated two different metalloproteinases.

Figure 31 presents protein profile of all metalloproteinases purification process.

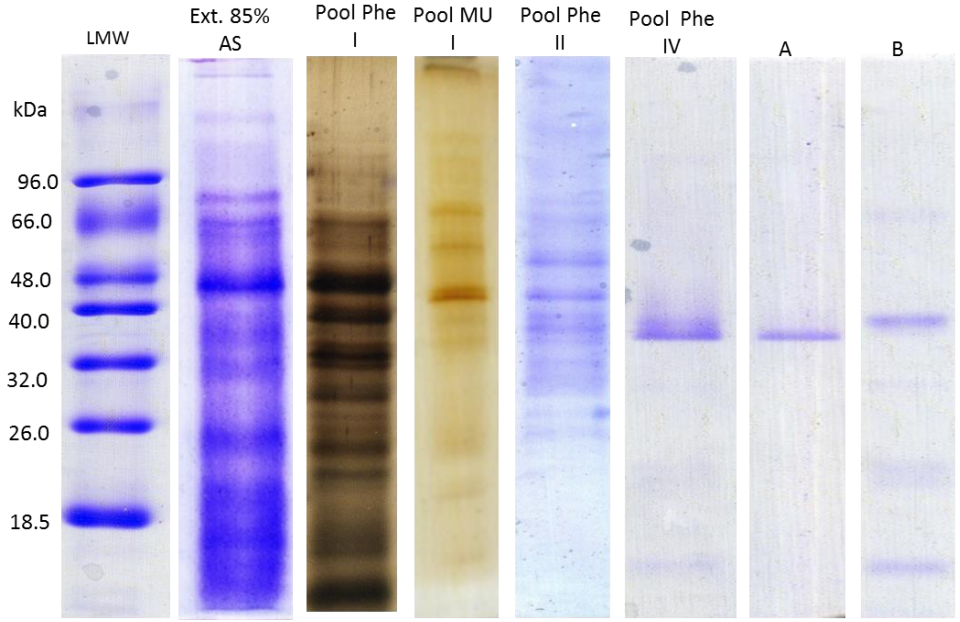

Figure 31 - Protein Profile Analysis of all purification process obtained by the concentration of the extract with 85% Ammonium sulphate, a gel filtration chromatography in an Superdex 200 column, a anion exchange chromatography in an 5 mL *High Q* column, and another gel filtration chromatography in an *Superdex 200* column, in an FPLC by SDS-Page with 12.5% acrylamide/bisacrylamide in denaturing conditions, stained with Comassie Blue R-250 and Silver Nitrate. LMW) Low Molecular Weight standard; Ext 85% AS) 20µL of *Laetiporus sulphureus* extract precipitated with 85% Ammonium Sulphate. Pool Phe I) 20µL of Pool Phe which was resultant of the fractions put together in gel filtration chromatography with activity with Phe-AMC; Pool MU I) 20µL of Pool MU I which was resultant of the fractions put together in gel filtration chromatography with activity with MU-NAG; Pool Phe II) Fractions put together resulting of anion exchange chromatography with activity with Phe-AMC (20µL); Pool Phe IV) Fractions concentrated in an Amicon with 10 kDa cutoff (20 µL); A) Metalloproteinase of 232.2 kDa present in peak A of Gel filtration chromatography (20µL); B) Metalloproteinase of 86.7 kDa present in peak B of Gel filtration chromatography (20µL).

Table XX - Purification table of the metalloproteases purified from Laetiporus sulphureus.

	Total Protein (mg)	Total Activity (nmol MU/min)	Specific Activity (µmol MU/min/mg)	Fold Purification	Yeld (%)
Extract	29.75	38 070.0	1279.7	1	100
Ammonium Sulphate Precipitation (85 %)	38.71 [(a)]	3771.8 [(a)]	97.4 [(a)]	0.1	9.9
Superdex 200 (Pool Phe I)	4.24	2826.3	666.1	0.5	7.4
High Q (Pool Phe IV)	3.85	1590,4	413.1	0.3	4,2
Superdex 200 (A)	0.06	1.1	17.3	0.03	0.02
Superdex 200 (B)	0.26	8.3	31.8	0.01	$2,9 \times 10^{-5}$

([a])The high quantity of Ammonium Sulphate present in the precipitated extract of Laetiporus sulphureus interfered with protein quantification, described in section 2.3, and with the enzymatic activity.

Thereby, it is well known, essentially from plants (BRZIN 1995, van der Hoorn 2008) and animals (Lopez-Otin and Bond 2008) that proteases are a key regulator in many physiological processes. Furthermore, proteases, in comparison with animals and plants, also play a major role in physiology, morphogenesis and metabolism of higher fungi, so called mushrooms (Zhang, Liu et al. 2010). Several proteases with interesting properties have already been characterized and purified in higher fungi such as *Pleurotus eryngii* (Wang and Ng 2001), *Agaricus bisporus* (Burton 1993), *Flammulina velutipes* (Shin 1998), *Cordyceps sobolífera* (Wang, Liu et al. 2012), *Armillarella mellea* (Kim 1999), *Cordyceps militaris* (Choi, Cha et al. 2011), *Lentinus edodes*(Terashita 1985), *Grifola frondosa*(Nonaka 1995).

Proteases are enzymes with important applications in industry and have been used for centuries in diversified fields such as the manufacture of cheese and milk-clotting, among others (Sumantha 2006). Moreover, is important to understand that proteases beyond nonspecific degradative functions, proteases can act as sharp scissors and, in that way, catalyse highly specific reactions of proteolytic processing, producing new protein products (Lopez-Otin and Bond 2008).

Thereby, it is important to recognize the biotechnological progress in the purification of new proteases, coming for higher fungi, *Laetiporus sulphureus*, not only for the deeper knowledge of the properties of this higher fungi and their capabilities, but also to find response to some problematics of our society.

Through the inhibition patterns shown in Figure 28 and Figure 30, where we can see inhibition with 1,10-Phenantroline (metalloproteases) and Leupeptin (serine, cysteine, threonine and metallproteases), we can say that both purified proteases are classified as metalloproteases. As we know, Phenantroline inhibits essentially Zi^{2+} metalloproteases, contrary to EDTA, that being a metalloprotease inhibitor, has more affinity for Ca^{2+}. So it is possible that we are dealing with a metalloprotease with zinc in the active center.

Most of the studies carried out in higher fungi shown that most of them are rich in metalloproteases and serine proteases (Choi, Cha et al. 2011). A chymotrypsin-like serine metalloprotease was already purified from higher fungi *Cordyceps militaris* (Choi, Cha et al. 2011). This metalloprotease was inhibited by PMSF (phenylmethylsulfonyl fluoride), an analog of PFabloc, inhibitor of serine proteinases, TPCK, inhibitor of serine-type chymotrypsin and 1,10-Phenantroline, competitive inhibitor of metalloproteinases, exactly like the me metalloproteinases purified from *Laetiporus sulphureus*, as described above, in which we can see the same inhibition profile.

However, references were not found in relation of molecular weights. It is usual molecular weights such as 16.5 kDa, as purified in *Armillarella mellea (Lewis 1978)*, 20 kDa, as a Lysine-specific purified in *Grifola frondosa* (Nonaka 1995), or the fibrinolytic enzyme, identified as a serine protease of 14kDa, purified from *Paecilomyces tenuipes* (Kim, Choi et al. 2011). So, it is most common the purification of proteases with low molecular weights in higher fungi. Thereby, the majority of those molecular weights are established considering the bands on SDS-PAGE and the aminoacids sequence (Nonaka, Dohmae et al. 1997, Cui, Dong et al. 2007). Nevertheless molecular weights observed in the purified metalloproteinases of 232.2 kDa and 86.7 kDa, while not usual for higher fungi, are considered plausible for an active metalloproteinase

Thus, it is possible that the purified metalloprotease of 232.2 kDa corresponding to A exist in a hexametric form of approximately 38.5 kDa subunits. This is supported by the results shown in Figures 25, 26 and 31.

Regarding the purification table (Table XX), it is important to notice the loss of activity in the second purification step, precipitation with 85% ammonium sulphate. The high concentration of salt interfere with sample quantification and with the enzymatic assays, once it is possible that there was a inhibition of enzymatic activity due to proteins denaturation resulting from the high salt concentration.

While several sequenced proteins are already found in basidiomycetes (Nonaka, Dohmae et al. 1997, Choi, Cha et al. 2011), very little is known from Laetiporus sulphureus, being a potentially strong study element.

4.2.2 – Chitinase Purification

In order to proceed to the purification of the Chitinase of the extract in study, a serie of chromatographic processes were carried out, as descrived in section 4.2.1. In this way, and with the previous knowledge of the preferential substrate, 4-MU-NAG, all fractions of interest were analysed according to the enzymatic assay described in section 2.5.3.

Thereby, in order to isolate the chitinolytic activity we proceeded with the fractionation of *Laetiporus sulphureus* extract, as described in section 4.2.1. So 4 mL of the extract precipitated with 85% Ammonium Sulphate were applied to a gel filtration *Superdex 200* column, previously equilibrated in 30 mM Sodium Phosphate pH 7 buffer, at the flow of 1 mL/min, Figure 32.

Fractions that showed a higher absorbance at 280 nm were assayed for enzymatic activity with the extract preferential substrate MU-NAG. All fractions showing activity with MU-NAG were pooled together. Nevertheless, there were fractions which had residual activity with Phe-AMC, as described in section 4.2.1. (Figure 32).

The pool MU resulted from the first Gel Filtration chromatography described in 4.2.1.

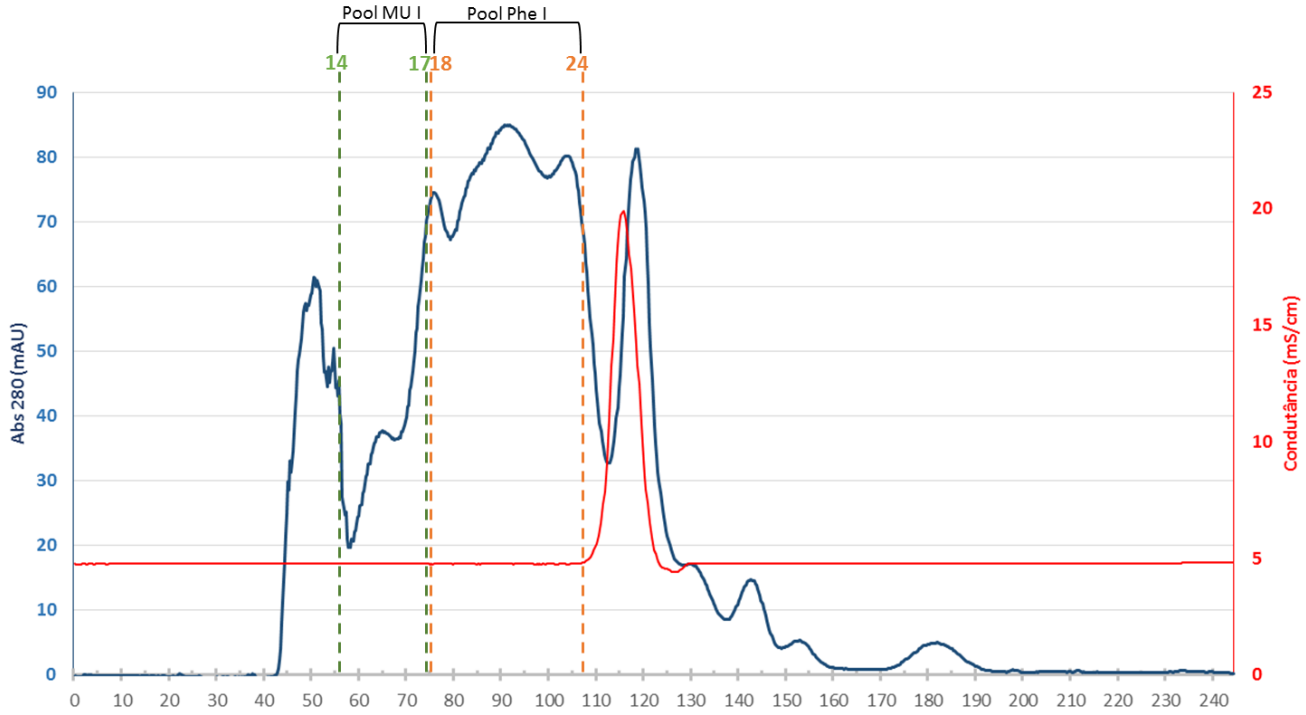

Figure 32 - Chromatogram obtained after gel filtration chromatography in an AKTA FPLC system. To a HiLoad 16/600 Superdex 200 pg (1.6x60cm, 120 mL) column, 4mL of *Laetiporus sulphureus* extract previously precipitated with 85% Ammonium Sulphate and ressuspended with 30mM Sodium Phosphate pH7 (sample buffer) were applied. The column was previously equilibrated with sample buffer (30 mM Sodium Phosphate pH7). The elution was carried out at the flow of 1 mL/min in the same buffer. Fractions of interest are identified.

Furthermore, after filtration, 12mL of this pool MU I was applied to two 5 mL High Q, as described in section 4.2.1.

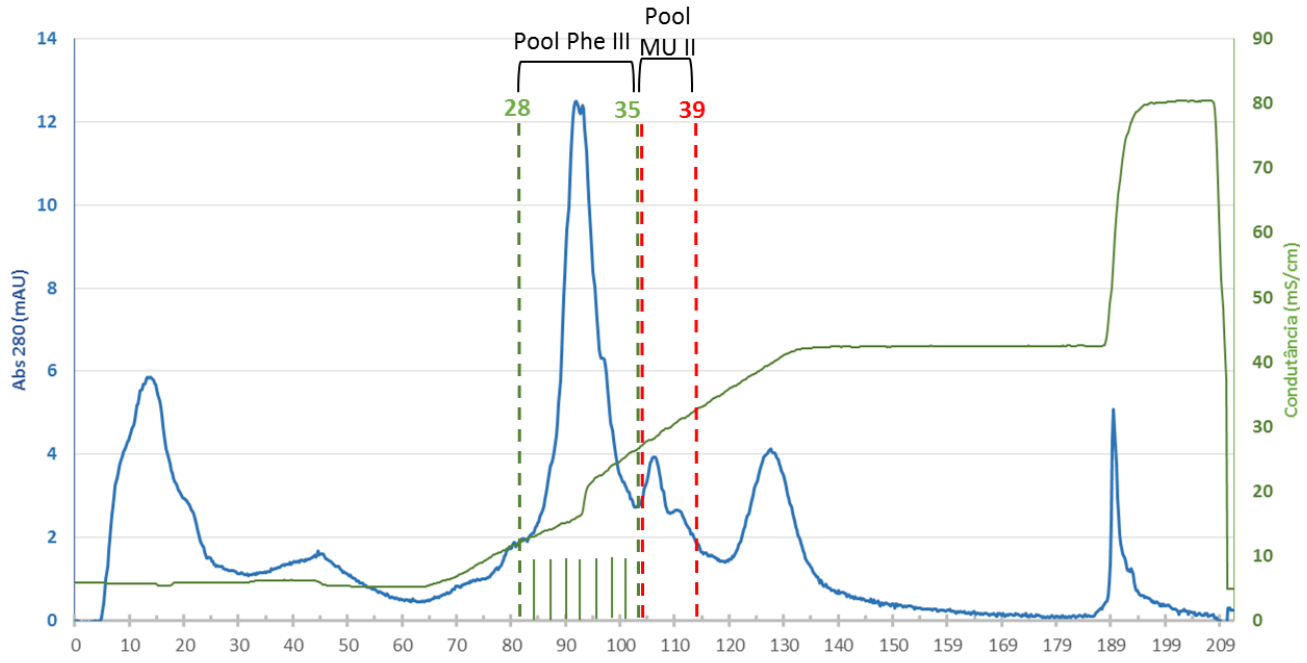

Figure 33 - Anion exchange chromatogram. To two High Q (5mL) column were injected 12 mL of pool MU. The column was previously equilibrated with sample buffer (30 mM Sodium Phosphate pH6.5). The elution was carried out with a salt gradient of 0% to 45% of elution buffer [30mM Sodium Phosphate, 1 M NaCl, pH6.5] at the flow of 1 mL/min in the same buffer. Fractions of interest are identified.

The zone delimited in red correspond to the fractions with activity with MU-NAG. This showns the presence of a chitinase possible or partially purified once this peak only shown activity with MU-NAG. These assays were carried out with 0.044 µg of protein and 0.05 mM of MU-NAG, for a final volume of 200 µL, achieving a specific activity of 69.0 µmol MU/min/mg. These assays were carried out as described in section 2.5.3.

This chitinase, despite showing it's presence with fluorimetric assays, we yet have to seen it in gel, in spite of the efforts.

Table XXI – Purification table of a chitinase purified from Laetiporus sulphureus.

	Total Protein (mg)	Total Activity (µmol MU/min)	Specific Activity (µmol MU/min/mg)	Fold Purification	Yeld (%)
Extract	29.75	200.06	6.73	1	100
Ammonium Sulphate Precipitation (85%)	38.71 [(a)]	5.84 [(a)]	0.15 [(a)]	0.02	2.92
Superdex 200	2.92	25.52	8.75	1.30	12.76
High Q	0.12	8.50	69.01	10.26	4.25

([a])The high quantity of Ammonium Sulphate present in the precipitated extract of Laetiporus sulphureus interfered with protein quantification, described in section 2.3, and with the enzymatic activity.

Chitinases (EC 3.2.1.14) hydrolyze the β-1,4-glycosidic linkages of the N-acetylglucosamine polymer, chitin (Flach 1992, Muzzarelli, Boudrant et al. 2012). It is known that chitin(Shivakumar, Karmali et al. 2014) is one of the most abundant polymers on earth and its recycling is extremely important once a vast amount of chitin waste material is released into the environment.

Furthermore, the fungi are organisms with a chitin-containing cell-wall that has the function of providing rigidity and shape (Vetter 2007). The physiological role of the great variety of chitinolytic enzymes produced by diverse organisms involve parasitism, nutrition, chitin recycling, morphogenesis and defense(Gortari and Hours 2008).

Moreover, the eagerness of the discover of chitinolytic enzymes for biological control arises once it is possible its involvement in the battle against chitin containing organisms such as nematodes (Jansson H-B. 1997, Gortari and Hours 2008).

It known that fungal chitinases are active at a pH range of 4.0 to 7.0 with optimal temperatures of 20-40°C. It has been estimated that chitinases are stable enzymes with molecular weights between 27 to 190 kDa(Dahiya, Tewari et al. 2006).

These information is consistent with the obtained result since in gel filtration chromatography (Figure 32) the named Pool MU has a lower elution volume than Pool Phe. This means that it is possible that we are handling a protein with a higher molecular weight than 232 kDa, corresponding to the first purified metalloprotease, or at least a molecular weight of the same magnitude.

It is a big step to consider this possibility since it was not possible to observe band in SDS gel, although the activity with MU-NAG is specific and incontestable. At the pH 6.5, this protein has negatively charged, indicating an acidic pI.

Although, it is possible that this results were enhanced if we tested the mycelium once we are studying a brown rot fungi. So, it is known that hydrolytic enzymes in this fungus are expressed in greater concentrations in mycelium (Sun, Zhang et al. 2004).

CONCLUSION AND

FUTURE PERSPECTIVES

In the course of this work we were able to provide a enzymatic characterization of fruiting body of Laetiporus sulphureus. We reveal the presence of different proteases such as serine proteases and metallloproteinases. Also, we access the presence of other hydrolytic proteins, verifying essentially the presence of chitinases.

Furthermore, we were able to isolate two proteases inhibited by 1,10-Phenantroline, Leupeptin and EDTA. Thus, they are characterized as metalloproteinases due to its inhibition profile.

One of the metalloproteinases possibly is hexameric since its SDS-Page profile shows only one band at approximately 38.5 kDa and its Native Page and gel filtration column calibration suggests a molecular weight of 232.2 kDa.

Other metalloproteinase shows a molecular weight of 86.7 kDa, suggesting a tetrameric protein, with two subunits of approximately 39 kDa and two of approximately 10 kDa.

Also, a chitinase was partially purified from fruiting body of *Laetiporus sulphureus*. This protein, presented a high level of specificity and activity with the substrate MU-NAG. However the total protein contained in the last purification step, possible was not enough to see this protein in gel.

This work opens new opportunities in hidrolases discovery. So, in future studies it will be relevant deepen the knowledge of this metalloproteinases isolated. Thus, maybe it will be significant study the fibrinolytic potential of this proteins, since several proteases of higher fungi already shown this activity (Kim 1999, Kim, Choi et al. 2011).

Moreover, a characterization of thermostability of this metalloproteases and the pH range resistance is needed in order to access its commercialization viability. Also, protein sequencing will be a relevant step in order to make real changes in macrofungus proteomic. Once when we have this proteins sequence, we will be able to induce recombinant expression.

Furthermore, extracellular expression of hydrolytic proteins would be a pertinent study. Wood-rotting basidiomycetes have to secrete proteins such as xylanases, chitinases, cellulases, and others, to the medium in order to degrade wood. So with the right medium we possible will be able to have a higher protein concentration, easier to purify and with low cost production (Terashita 1995, Kjoller 2006).

In addition, it would be interasting to test the nematotoxic activity of the partially isolated chitinase. Probably chitinase would demonstrate a significant nematotoxic activity cutting through nematode egg shells and avoiding the plague (Gortari and Hours 2008).

Concluding, it is possible to say that the main goals initially proposed were achieved, resulting in major changes in the knowledge of hydrolytic potential of *Laetiporus sulphureus*. As a whole, this work opened a new door in biotechnological research presenting a new source of protein production with low maintenance costs and great potential.

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