Secretome Analysis in Higher Basidiomycetes - Freely Secreted and Cell Wall Proteins from *Coprinopsis cinerea*

Dissertation

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Zusammenfassung

Basidiomyceten sekretieren eine Vielzahl an Proteinen nicht nur in ihre weitere Umgebung, sondern auch in extrazelluläre Strukturen wie der Zellwand und der damit assoziierten äußeren Polysaccharid-Schicht. Diese Arbeit hatte das Ziel, einen Überblick über die extrazellulären Proteine (das Sekretom) des Modellorganismus *Coprinopsis cinerea* zu schaffen. Um einen detaillierten Überblick zu erhalten, wurde das Sekretom fraktioniert: in frei sekretierte Proteine, in Proteine der äußeren Polysaccharid-Schicht, welche die Hyphen umgibt, und in Proteine der pilzlichen Zellwand. Letztere können auf verschiedene Arten an das Netzwerk der Zellwandpolysaccharide gebunden sein. Aus diesem Grund wurden die Zellwandproteine in folgende Fraktionen unterteilt: in ionisch gebundene Zellwandproteine (durch Salz extrahierbar), in andere durch nicht kovalente Bindungen assoziierte Zellwandproteine sowie über Disulfidbrücken gebundene Proteine (beide durch SDS extrahierbar) und in kovalent gebundene Zellwandproteine (Pir-Proteine; extrahierbar unter milden, alkalischen Bedingungen). Zuletzt wurde versucht, die durch Glykosylphosphatidylinositol-Anker (GPI-Anker) gebundenen Zellwandproteine mittels Cyanogenbromid (CNBr)/Trypsin Verdau der Zellwände zu isolieren. In dieser Arbeit konnten jedoch keine GPI-gebundenen Zellwandproteine isoliert werden. Die einzelnen anderen extrahierbaren Fraktionen wurden mit proteomischen Methoden untersucht, um ihre Proteine mittels Massenspektrometrie (LC-MS^2) zu identifiziert.

Etablierte Methoden zur Präparation von extrazellulären Proteomen von Ascomyceten zeigten sich als unzureichend für die Untersuchung des Sekretoms des Basidiomyceten *C. cinerea*. Dies traf besonders in der frühen exponentiellen Phase des Wachstums zu, da möglicherweise der Pilz in dieser Wachstumsphase eine Vielzahl an sekundären Metaboliten ausscheidet und vor allem große Mengen an löslichen Polysacchariden produziert. Diese Substanzen störten die Probenaufarbeitung und beeinflussten die Gelelektrophorese stark, was zu unverwertbaren zweidimensionalen Elektrophoresegelen (2-DE) führte. Aus diesem Grund wurde zu Beginn der hier vorliegenden Arbeit ein optimiertes Protokoll zur Aufarbeitung von frei sekretierten Proteinen von *C. cinerea* und anderen höheren Basidiomyceten etabliert.

Zur Analyse des fraktionierten Sekretoms in der frühen exponentiellen Wachstumsphase [Tag 3 der Kultivierung in flüssigem Vollmedium (YMG) bei 37 °C] wurden zwei proteomische Techniken angewandt: zweidimensionale Gelelektrophorese in Kombination mit LC-MS^2 zur Identifizierung von definierten Proteinspots, sowie eine eindimensionale Gelelektrophorese als Shotgun-Untersuchung zur Identifizierung von Proteinen in Proteingemischen mittels LC-MS^2. Aus beiden Experimenten wurde deutlich, dass sich das Proteom außerhalb der Zellwand (frei sekretierte Proteine sowie Proteine der Polysaccharid-Schicht) und das Zellwandproteom signifikant voneinander unterscheiden. Nur wenige der identifizierten Proteine waren in der frei sekretierten Proteinfraktion und der Proteinfraktion der Polysaccharidschicht einerseits und den Zellwandproteinfraktionen andererseits vorhanden. Eine deutliche räumliche Trennung dieser Subproteome zeichnete sich also ab. Insgesamt wurden im Zuge dieser Versuchsreihe 162 Proteine (mit Überlappungen) identifiziert: 41 waren frei sekretiert, 61 waren aus der zellwandassoziierten Polysaccharid-Schicht, 59 waren NaCl-extrahierbare Zellwandproteine, 50 waren SDS-extrahierbare Zellwandproteine und 6 waren NaOH-extrahierbare Zellwandproteine. In der frei sekretierten Fraktion und in der Proteinfraktion aus der Polysaccharid-Schicht wurden hauptsächlich Glykosid-Hydrolasen, Proteasen und Oxidoreductasen isoliert. Diese sind höchst wahrscheinlich der Substrataufnahme dienlich. In der Zellwand konnten einige Proteine mit möglichen Funktionen im Zellwandaufbau, wie zum Beispiel Glykosyltransferasen, Mannosidasen und Chitinasen, bekannt von Ascomyceten, identifiziert werden. Des Weiteren wurden aber auch Enzyme identifiziert, welche normalerweise in intrazelluläre Prozesse involviert sind, zum Beispiel Malat-Dehydrogenase oder Enolase. Obwohl die Existenz dieser Enzyme in den Zellwänden von Pilzen in mehreren Fällen eindeutig nachgewiesen werden konnte, ist ihre Funktion in der Zellwand umstritten.

Es konnte gezeigt werden, dass sich alle Fraktionen des Sekretoms von *C. cinerea* dynamisch mit der Zeit veränderten, möglicherweise als eine Anpassung an die sich ändernden Umweltbedingungen während des Wachstums. Es zeigte sich, dass das fraktionierte Sekretom im Laufe des Wachstums an Komplexität verliert. Im Gegensatz dazu variierten einzelne Proteine wie z.B. Peptidasen oder Glykosidhydrolasen signifikant in ihrer Konzentration sichtbar in der Intensität der zugehörigen Proteinspots auf den 2-DE Gelen. Abschließend entstand das Bild eines hoch diversen, dynamischen Sekretoms in Hinsicht auf die Konzentration einzelner Proteine von *C. cinerea*, welches signifikante Unterschiede zwischen dem frei sekretierten und dem Zellwandproteom aufweist.

Die 2-DE Untersuchung des fraktionierten Sekretoms von *C. cinerea* zeigte weiters, dass viele extrazelluläre Proteine von *C. cinerea* stark post-translatorisch modifiziert sind. Eine extensive Glykosylierung des freien Sekretoms (etwa 70% der Proteine) konnte nachgewiesen werden. Des Weiteren lieferten Versuche mit radioaktiv markiertem Phosphat Hinweise auf spezifische, phosphorylierte Proteine im Sekretom vom *C. cinerea*. Insgesamt konnten sieben Proteine mit gebundenen Phosphatgruppen identifiziert werden; drei Oxidasen, zwei Glykosid-Hydrolasen und zwei bislang uncharakterisierte Proteine. Bei dreien dieser Proteine (einer Oxidase und den beiden uncharakterisierten Proteinen) konnte mittels einem Verdau mit PNGaseF (Endoglykosidase) festgestellt werden, dass die Phosphorylierung auf der N-Glykosilierung des Proteins lokalisiert ist. Bei den anderen vier Proteinen mit Phosphatgruppen könnte das Phosphat an einer prosthetischen Gruppen gebunden sein (Oxidoreduktasen), an einem GPI-Anker (Glykosid-Hydrolase der Familie 72), an den Aminosäuren des Proteins oder an den Zuckerresten einer putativen O-Glykosilierung, welche durch die verwendete PNGaseF nicht angegriffen werden (Glykosid-Hydrolase der Familie 37 und ein uncharakterisiertes Protein).

Die Experimente zur Untersuchung des Sekretoms von *C. cinerea* bei Kultivierung in Flüssigmedium konnten einen Überblick bezüglich der Kompartimentierung, der Beschaffenheit und der Dynamik (in Bezug auf die Proteinkonzentration einzelner Proteine) der sekretierten Proteine von *C. cinerea* in flüssigem Medium schaffen. Um einen tieferen Einblick in Substratabbau und Zellwandaufbau zu erhalten, ist jedoch eine Untersuchung der Pilze auf naturnahem Substrat (Pferdemist bei *C. cinerea*) unumgänglich. Im Zuge dieser Arbeit konnte für einen holzabbauenden Pilz (*Pleurotus ostreatus* Stamm PC9) gezeigt werden, dass die zuvor an *C. cinerea* in Flüssigmedium entwickelten Methoden bedingt auch auf naturnahem Substrat (Weizenstroh) angewendet werden können. Während der Beobachtung von 31 Tagen wurden im Sekretom mittels 2-DE Untersuchung keine wesentlichen Veränderungen im Proteinmuster beobachtet. Im Gegensatz dazu konnten bei der Messung von Enzymaktivitäten Lignozellulose abbauender Enzyme aber Fluktuationen der Enzymaktivitäten über die Zeit festgestellt werden. Die Identifizierung von Proteinen aus diesem Versuch war nur bedingt erfolgreich. Nur 5 von 30 Proteinspots (insgesamt drei verschiedene Proteine) konnten positiv identifiziert werden. Dies lag u.a. höchst wahrscheinlich daran, dass das annotierte Genom des *P. ostreatus* Stammes PC9 zum Zeitpunkt des Versuches noch nicht zur Verfügung stand und auf das annotierte Genom eines anderen *P. ostreatus* Stammes (PC15) zurückgegriffen werden musste.

Summary

Basidiomycetous fungi secrete a multitude of proteins into their environment and as well to the extracellular fungal structures, i.e. the cell wall and the associated extracellular glycan structure (hyphal sheath). The aim of this thesis was to study the extracellular proteins from the model basidiomycete *Coprinopsis cinerea*, including the freely secreted proteins, the hyphal sheath proteins, and the cell-wall-linked proteins. The cell wall proteins can be linked to the polysaccharide network of the cell wall in different ways. Therefore, the cell wall proteins were further fractionated into non-covalently-bound cell wall proteins (CWPs) extractable by first NaCl and further by SDS (sodium dodecyl sulfate) containing buffers; into covalently bound cell wall proteins, extractable under alkaline conditions (NaOH; proteins with internal repeats, Pir) and into glycosylphosphatidylinositol- (GPI) anchored CWPs (extractable by digestion with CNBr and trypsin of the cell wall). In this work, no putative GPI anchored proteins were isolated. The other fractions of the secretome were individually analyzed by proteomic methods and proteins were identified by mass spectrometry (LC-MS^2).

Two dimensional gel electrophoresis (2-DE) for the analysis of the secretome, as already established for yeasts and other ascomycetes, had to be optimized for the study of the *C. cinerea* secretome. High amounts of extracellular soluble polysaccharides and extracellular metabolites were effecting the quality of the sample preparation and the resolution of the 2-DE gels leading to unusable 2-DE gels, especially in the early exponential phase. Therefore at the beginning of this work, an optimized protein preparation protocol applicable for various higher basidiomyctes was established.

For the analysis of the secretome from the early exponential growth phase of *C. cinerea* [day 3 of cultivation in liquid full medium (YMG) at 37 °C] two proteomic techniques were applied: a 2-DE gel approach in combination with protein identification by LC-MS^2 and in parallel a one-dimensional (1-DE) shotgun approach for the identification of proteins by LC-MS^2. Both methods showed a clear compartmentation between the cell wall proteome on the one hand and the freely secreted and the hyphal sheath proteome

on the other hand. Only few of the identified proteins were overlapping between the free secretome and the hyphal sheath proteome on the one hand and the fractions of the cell wall proteome on the other hand. This analysis revealed a clear separation of the subproteomes. In total, 162 proteins in five different fractions (with overlappings) were identified in this experimental setup: 41 from the free secretome, 61 from the hyphal sheath and 59, 50 and 6 NaCl-, SDS- and NaOH-extractable cell wall proteins, respectively. The identified proteins from the free secretome and the hyphal sheath included mainly glycoside hydrolases (e.g. glycosyl-transferases, cellulases, glucanases), peptidases and oxidoreductases, all putative enzymes involved in nutrient supply. Within the cell wall, proteins with putative functions in the cell wall formation and restructuring (e.g. chitinases, mannosidases), homologous to cell wall proteins identified in ascomycetes, were detected. Also enzymes known from intracellular processes (e.g. malate dehydrogenase, enolase) were identified and thus could possibly be located in the cell wall of *C. cinerea*. Although the existence of such typically intracellular proteins in the cell wall of fungi was already shown previously, the extracellular function of these proteins is controversial.

Further analysis of the fractionated secretome of *C. cinerea* over the time of cultivation revealed a dynamic secretome, possibly an adaption to the changing environmental conditions. The secretome showed a reduced complexity over the time. Contrary, single proteins such as peptidases and glycoside hydrolases changed significantly in their concentration over the time of cultivation, as visible in the 2-DE gels. In conclusion, these experimental setups revealed a dynamic and strictly compartmented secretome of *C. cinerea*.

The analysis of the *C. cinerea* secretome by 2-DE showed that many of the extracellular proteins have extensive posttranslational modifications (PTM). The freely secreted proteins of *C. cinerea* were found to be highly glycosylated (about 70% of the proteins). In addition, growth of *C. cinerea* in the presence of ^{33}P labeled phosphate revealed seven proteins with linked phosphate groups in the free secretome, the hyphal sheath and the cell wall. Analysis of the nature of linked phosphate groups by a digest of the whole secretome with PNGaseF (removing the N-glycosylations of proteins) showed that three proteins had a linked phosphate group on the sugars of the proteins' N-glycosylation. On the four other proteins, the phosphate group might be either part of a prosthetic group (oxidoreductases), or part of a GPI anchor (glycoside hydrolase from family 72), or linked to the amino acids, or linked to the sugars of a putative O-glycosylation in

a manner not removable with PNGaseF (glycoside hydrolase from family 37 and an unknown protein).

The analysis of the fractionated secretome from *C. cinerea* grown in liquid medium gave an overview concerning the nature and the dynamics of the secretome (concerning protein concentrations of specific proteins) over the time and gave an overview of the secretome from *C. cinerea* in liquid cultures. However, for a deeper insight into substrate degradation and the cell wall formation of fungi, an analysis on natural substrate (horse dung for *C. cinerea*) is crucial. In course of this work, it could be shown that the methods developed for *C. cinerea* in liquid culture are as well applicable to fungi growing on natural substrate, as demonstrated for *Pleurotus ostreatus* strain PC9 grown on wheat straw for a period of 31 days. The extracted secretome was analyzed by 2-DE and revealed similar protein profiles over the time. In contrast, the biochemical activities of lignocellulose degrading enzymes showed a fluctuation of enzyme activities over the time. The identification of protein spots from the 2-DE gels revealed only 3 positively identified proteins (present in 5 different spots) from in total 30 picked spots. This was most likely due to the fact that an annotated genome of *P. ostreatus* strain PC9 was not available at the time when this study was performed and that the annotated genome of another *P. ostreatus* strain (PC15) had to be used.

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Protein precipitations and the SDS-PAGE shown in Figure 1 in chapter 2 were performed by Mojtaba Zomorrodi. Figure 4B in chapter 2 was taken from the thesis of Sudhakar Peddireddi (Peddireddi, 2008; Hydrophobins In Wood Biology and Biotechnology. PhD-thesis Göttingen).

The Mascot database for the identification of proteins obtained by LC-MS^2, as well as the SQL database for the organization and filtering of the obtained LC-MS data also used for the average peptide scoring (APS) was constructed by Dr. Andrzej Majcherczyk. These databases were used for the experimental work described in the chapters 3 to 5.

Radioactive labeling of *C. cinerea* proteins and autoradiography of the 2-DE gels was performed with the help of Mr. Thomas Klein and Mrs. Gabriele Lehmann. The measurement of the phosphorus content in the YMG cultivation medium of *C. cinerea* was measured by KOSI (Center for Stable Isotope Research and Analysis) at the faculty of Forest Sciences and Forest Ecology (University of Göttingen).

Contents

1 General Introduction

1.1 The Fungal Cell Wall Architecture

Members of the fungal kingdom appear in all parts of the world living, e.g. in soil, and on dead matter as saprotrophs, or as symbionts or pathogens of plants, animals, or humans. Fungi play an essential role in the ecosystem by decomposing organic matter recirculating it back into the nutrient cycle. A structure crucial for this global process is the fungal cell wall, as it is for example essential for maintaining the osmotic balance, providing mechanical strength and creating the shape of the cell. Providing the contact to the fungal's environment makes the cell wall an interesting research field and led to extensive studies of the cell wall components. Until the 1950ties, the examination of the distinct cell structures, such as the cell wall, was comparably difficult due to the lack of preparation methods for single cell structures free from cytoplasmic contaminations. The bakers' yeast *Saccharomyces cerevisiae* was one of the first fungi used for investigations of the cell wall, summarized in a review on yeast cell wall structures provided by Phaff (1963). Also first investigations on the amount of the fungal cell wall proteins were made during this time (reviewed by Gander (1974)). Starting with *S. cerevisiae* and other ascomycetous yeasts, bit by bit the cell wall structures of several other fungal species were also extensively studied. *Schizophyllum commune* as the first well studied model for wood degrading basidiomycetes was one of the first in its taxonomic group with a morphologically studied cell wall (Wessels & Sietsma, 1979). In addition, also for another basidiomycetous model organism, *Coprinopsis cinerea* [formerly known under various different names, including *Coprinus cinereus* (Kües, 2000)], studies concerning the fungal cell wall structure were performed (Bottom & Siehr, 1979, 1980; Schaefer, 1977).

However, up to now ongoing investigations of the cell wall structure are mainly focused on the composition of the polysaccharide network and the protein content of cell

walls from the ascomycetous model yeast *S. cerevisiae* (Klis et al., 2006; Kollár et al., 1995, 1997; Lipke & Ovalle, 1998) and the opportunistic human pathogens *Candida albicans* (Chaffin et al., 1998), *Candida glabrata* (De Groot et al., 2008) and *Aspergillus fumigatus* (Bernard & Latgé, 2001; Fontaine et al., 2000). Targeting at the development of antifungal drugs the cell wall proteins involved in the adhesion, cell wall composition and biofilm formation are studied extensively for those human pathogens (Chaffin et al., 1998; De Groot et al., 2008). Though the basic concept of the fungal cell wall seems to be alike for all the up to now analyzed fungal species, a closer look into the architecture of the cell wall reveals great differences in the composition. The early studies of the cell wall structures pointed out that the composition of wall polysaccharides is very diverse between the fungal species analyzed (Bottom & Siehr, 1979). Evolutionary diversification of the fungal cell wall is not only demonstrated by the composition of the polysaccharides but was also shown by a phylogenetic comparison of the products of cell wall related genes in 18 fungal genomes (Coronado et al., 2007).

Cell wall proteomes from ascomycetous yeasts and basidiomycetes are highly divergent sharing only proteins critical for the wall biogenesis. Other cell wall proteins seem to have evolved so fast that homologies even in closely related taxonomic groups are often hardly recognized. This rapid evolution is possibly due to the highly diverse habitats of the single species. The outer cell surface is, not only in fungi, definitely at the front of natural selective pressure. This increases the demand for a fast adapting structure (Coronado et al., 2007). Indeed, innovations of the cell surface structures and its proteins may be responsible for the success of major evolutionary lineages (Cavalier-Smith, 2006). Concerning this, comparisons of the biochemical structure and proteins of the fungal cell wall between different fungal species have to be done cautiously.

1.1.1 The Fungal Cell Wall Structure - State of the Art

The fungal cell is surrounded by a polysaccharide cell wall providing not only the shape of the cell but also protecting the cell from osmotic, and physical stresses (Klis et al., 2002), and it constitutes a barrier against chemicals harmful to the fungal cell (Klis et al., 2006). The cell wall is defined as the structure enveloping the fungal cell external to the plasma membrane. After Arnold (1991), this would as well include the periplasmic space. However, the periplasmic space can also be defined as a dedicated structure of the cell, separating the plasma membrane from the cell wall structure. The

dynamic structure of the cell wall allows the fungus to adapt to various situations such as vegetative growth, substrate colonization, host penetration or reproduction (Bartnicki-García, 1968). In this work the cell wall is defined as the polysaccharide structure occurring on the outer surface of the cell membrane, thus including the periplasmic space.

The cell wall as the outer layer of the cell is a complex network of polysaccharides, glycoproteins, lipids and minor components such as inorganic salts and pigments (Ruiz-Herrera, 1992). The structure of the fungal cell wall differs significantly from the cellulose-based cell wall of plant cells or the peptidoglycan-based cell envelope of bacteria. However, also the fungal cell walls mainly consist of carbohydrates. Glucose as a basic monosaccharide (Bowman & Free, 2006) forms together with other highly abundant sugars such as glucosamine (mainly in its acetylated form N-acetylglucosamine), galactose, and mannose, the highly complex structure of the fungal cell wall. The glucan component of the cell wall makes up about 45 to 80% of its dry weight (Klis, 1994; Ruiz-Herrera, 1992), depending on the fungal species and glycan can be regarded as the main component of the cell wall (Table 1.1). The glucose molecules form mainly β-1,3-linked linear chains that are additionally branched by β-1,6-glucans. In *A. fumigatus* also α-1,4-glucans and galactomannans were found in addition to β-1,3- and β-1,6-glucans (Bernard & Latgé, 2001). Highly branched polysaccharides consisting of α-1,4, β-1,3 and β-1,6 linkages were found to be present in the cell wall of *C. cinerea*. Besides glucose, mannose and glucosamine as well amino acids were identified from hydrolysates of the cell walls from *C. cinerea* (Bottom & Siehr, 1979, 1980; Schaefer, 1977). The occurrence of β-1,4-linked glucan in the cell wall of *C. cinerea* was unexpected, as this structure is only known from one other fungus [basidiomycete QM 806 (Bush & Horisber, 1972)]. The cell wall polysaccharide composition is based on glucose as basic sugar in all examined cases, but the linkages between the sugar molecules differ depending on the fungal species (Table 1.1). In the well studied ascomycetous yeasts, mainly β-1,3 and β-1,6 glucans were found, while the sugar composition is becoming more diverse in the group of the true filamentous fungi such as *A. fumigatus* (Bernard & Latgé, 2001).

A characteristic and crucial but by content minor polysaccharide of the fungal cell wall is chitin, an unbranched polysaccharide made of N-acetylglucosamine linked by α-1,4-bonds (Bowman & Free, 2006). Regardless that chitin accounts for only 1-3 % of the dry weight in yeast cell walls (Klis, 1994; Klis et al., 2002) and about 10 - 20 % in

Table 1.1: The cell wall composition of investigated fungal species.

Fungus	Cell wall (% dry weight)	Polysaccharide composition	Cell wall composition Glucans	Chitin	Protein	Reference
Ascomycetes						
Candida albicans	n.d.	1,3 β-; 1,6 β-glucans	47-60%	1-9%	6-25%	Chaffin et al. (1998)
Candida glabrata	19%	1,3 β-; 1,6 β-glucans	n.d.	2%	6%	De Groot et al. (2008)
Saccharomyces cerevisiae	30%	1,3 β-; 1,6 β-glucans	60%	1-3%	30%	Lipke & Ovalle (1998) Lesage & Bussey (2006) Kollár et al. (1997) Kollár et al. (1995)
Aspergillus fumigatus	n.d.	1,3 α-; 1,3 β-; 1,4 β-glucans, galactomannan	n.d.	n.d.	3.5%	Bernard & Latgé (2001) Fontaine et al. (2000)
Fusarium oxysporum	n.d.	1,3 α-; 1,3 β-glucan	75%	11%	7%	Schoffelmeer et al. (1999)
Basidiomycetes						
Ustilago maydis (yeast form)	n.d.	1,3 β-; 1,6 β-glucans	73%	14%	12%	Ruiz-Herrera et al. (1996)
Ustilago maydis (filamentous form)	n.d.	1,3 β-; 1,6 β-glucans	62%	16%	16%	Ruiz-Herrera et al. (1996)
Coprinopsis cinerea	15%*	1,4 α-; 1,3 β-; 1,6 β-glucans	76%	n.d.	2%**	Bottom & Siehr (1979)
Schizophyllum commune	n.d.	1,3 α-; 1,6 β-glucan	71%	10%	7.2%**	Wessels & Sietsma (1979)

* this work Chapter 5

** refers to values for the protein content determined by total amino acid analysis or Kjedahl, and these may not be comparable with the other values for the protein content of other species. In these other species protein amounts were determined by protein assays (e.g. Bradford)

filamentous fungi [(Bartnicki-García, 1968; De Nobel et al., 2000) Table 1.1], it seems to play a structural and protective role. When chitin synthesis is disrupted, the fungal cell is sensitive to osmotic pressure, the structure of the wall becomes disordered and the cells lose their normal shape (Bago et al., 1996; Specht et al., 1996).

In addition to the cell wall, many fungal species assemble an extracellular polysaccharide layer outside of the cell wall but nevertheless closely connected to the wall polysaccharides. This outer layer is in various terms referred to in the literature: e.g. hyphal sheath, extracellular mucilagous material, extracellular matrix (ECM), mucin or extracellular membranous structures (Ali et al., 1999). In this work, the term hyphal sheath will be used whenever referring to this outer polysaccharide layer. The occurrence of a hyphal sheath has been reported for many fungi including wood-degrading species as well as pathogenic fungi (Ali et al., 1999; Asiegbu, 2000; Dubourdieu et al., 1981; Gutiérrez et al., 1995; Ruel & Joseleau, 1991). The hyphal sheath is described as a fine, water-based, fibrillar and granular structure (Arnold, 1991). The composition of this extracellular mucilagous layer depends on the fungal species but the main component is β-1,3-glucan branched with β-1,6-glucans (Gutiérrez et al., 1995) as it is found in the cell wall itself. The hyphal sheath is distributed around the fungal hyphae (Gutiérrez et al., 1995) and covalently linked to the cell wall glucans and chitin (Sietsma & Wessels, 1981). Though the role of the hyphal sheath is not completely understood, various functions have been proposed in previous studies. The hyphal sheath can act as an additional protective layer against environmental threats such as toxic molecules or radicals (Vesentini et al., 2007). Storing of nutrients in the form of extracellular glucans, used by the fungus under starvation conditions, was shown by Stahmann et al. (1992). Daniel (1994) suggested that the hyphal sheath and the associated extracellular matrix of polysaccharides may play an important role in wood degradation and might explain lignin and cellulose degradation in distance of hyphae. As this structure forms the contact between the fungus and its substrate, it could act as a reaction space for extracellular degradation enzymes (Gutiérrez et al., 1995) which indicates that studying the proteome of the hyphal sheath of wood-degrading fungi could reveal a number of interesting degradation enzymes.

1.1.2 Fungal Cell Wall Proteins

During the early structural studies of the cell wall (CW) also cell wall proteins (CWPs) were already examined to certain extends (Gander, 1974). Especially, CWPs released by mild alkali treatments of the cell wall were intensively studied and found to be heavily glycosylated proteins attached to the cell wall. Invertase, glycoside hydrolases, acid phosphatases and proteases were already in the early 1970ties known to be attached to the cell wall (Gander, 1974). These specific cell wall proteins were suggested to be involved in mating type recognition (Crandall & Brock, 1968) or the flocculation of cells (Mill, 1966). Thus, harboring of enzymes of either synthetic or hydrolytic functions within the cell wall was already known in the early stage of the fungal cell wall research (Burnett, 1979). Though these early studies of the fungal cell wall were restricted mainly to extensive structural analysis of the cell wall, the basic methods for the isolation and examination of cell wall proteins were established (Fleet, 1991; Hunsley & Burnett, 1970). However, the long living myth that cell wall proteins are either *en route* for secretion or artifacts of cell wall preparations may have hampered for long extensive research in this field (Ruiz-Herrera, 1992). Consistently, typically cytoplastic enzyme activities were measured in samples of isolated cell walls which supported the hypothesis of intracellular contaminations of the cell wall fractions (Taylor & Cameron, 1973). Research started to intensively address proteins in the fungal cell wall as a research target only in the last years and for a few organisms great progress was done in the research on CWP. As above stated for the architecture of the fungal cell wall, also the CWP are mainly studied in model organisms such as *S. cerevisiae*, *C. albicans* and *Aspergillus* species (Kapteyn et al., 2000; Lesage & Bussey, 2006).

Depending on the fungal species, about 2% to 20% of the wall dry weight consists of protein in filamentous fungi (Table 1.1). In *S. cerevisiae*, the percentage is even higher with 30% of the wall dry weight (Bowman & Free, 2006). Proteins of the fungal cell wall were considered to be glycoproteins with extensive N- and O-linked glycosylations, which link these proteins tightly to the cell wall polysaccharides. Proteins are modified with mannans or mannans and galactans (Bowman & Free, 2006), depending on the fungal species. Additionally, some CWPs are attached to the cell wall or the cell membrane with a glycosylphosphatidylinositol (GPI) anchor (De Groot et al., 2005). This C-terminal structure, composed of a phosphatidylinositol group linked through a carbohydrate containing linker to an amino acid , directs the proteins to the cell wall or

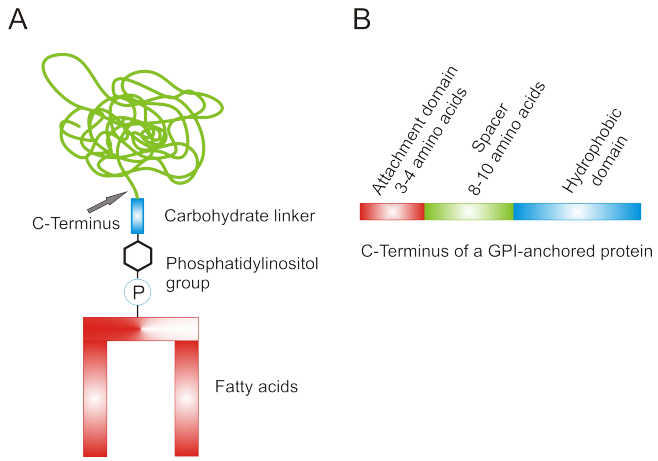

Figure 1.1: A: Composition of a typical GPI-anchor consisting of fatty acids, a phosphatidylinositol group and a carbohydrate linker linked to the C-terminal end of the protein (ω-site), modified after Paulick & Bertozzi (2008). B: Composition of the ω-site at the C-terminus of a GPI-anchored protein (Coyne et al., 1993).

membrane (Figure 1.1). Various techniques were used to extract proteins from the cell wall, including the treatment of crude cell wall fractions or whole cells with detergents [such as sodium dodecyl sulfate (SDS)], reducing agents [such as β-mercaptoethanol or dithiothreitol (DTT)], mild alkali or hydrolyzing enzymes (such as zymolase, β-glucanase or chitinase) (López-Ribot et al., 1991). These various methods have been used alone or in combination. In general, proteins attached to the fungal cell wall can be divided into non-covalently and covalently bound proteins. Non-covalently bound proteins are most likely attached by hydrogen bonds, van der Waals forces, electrostatic forces or disulphide bonds. A high number of the covalently bound proteins are linked to the cell wall by a glycosylphosphatidylinositol (GPI) anchor attached to the C-terminal end of the proteins [Figure 1.1 and (Kapteyn et al., 1996)]. Other proteins bind covalently by mild-alkali-sensitive linkages to the glucan network of the fungal cell wall (Kapteyn et al., 1999; Mrsa et al., 1997).

1.1.2.1 Non-covalently Bound Cell Wall Proteins

Non-covalently bound CWPs may be associated with the cell wall by electrostatic or van-der-Waals forces, linking them to the cell wall polysaccharides. Otherwise, such proteins could also be directed to the external milieu, being on their way through the

cell wall. Proteins found in the external milieu often have hydrolytic or oxidative functions acting either in providing nutrients, in self-defence or in host infection (Chaffin, 2008). While enzymes involved in nutrient supply differ significantly between fungal species depending on their environmental divergence and their natural habitat, enzymes involved in the formation and rebuilding of the cell wall structure are conserved to a certain extend over all fungal species (Mouyna et al., 2000a,b).

By the measurement of direct cell-wall-linked catalytic activity in the cell wall (pure cell wall fraction or whole cells) several enzymes were detected [for an extensive review see Rast et al. (2003)]. Most of these enzymes appear to be involved in the synthesis and rebuilding of the cell wall (Rast et al., 2003), being characterized as glucanases, transglycosylases and chitinases (Adams, 2004). Such cell wall-synthesizing enzymes are up to now best studied in the bakers' yeast *S. cerevisiae* [reviewed in Lesage & Bussey (2006)] and in the human pathogen *C. albicans* (Chaffin, 2008). Fungal glucan synthases, responsible for the synthesis of β-1,3-glucan, were described for several fungal species from the phylum ascomycota such as e.g. *Yarrowia lipolytica* (Kellner et al., 2005), *Aspergillus nidulans* (Beauvais et al., 2001) and *A. fumigatus* (Ibrahim et al., 2005). Also enzymes involved in the synthesis of β-1,6-glucan and several chitinases were already examined in *C. albicans* and *S. cerevisiae*. For comprehensive reviews on the enzymes involved in the cell wall synthesis in these two yeasts see references Lesage & Bussey (2006) and Chaffin (2008). The cell wall synthesis of filamentous fungi is possibly best understood in the human pathogen *A. fumigatus* (Bernard & Latgé, 2001). Though not as comprehensively studied as the yeast cell walls, many enzymes involved in the wall biogenesis were characterized at the molecular level. Proteins being homologues to the glucan synthase complex of *S. cerevisiae* and chitinases are known from *A. fumigatus*. Also two proteins involved in the synthesis of α-1,3-glucan, a polysaccharide specific for *Aspergillus spp.*, were identified as putative hydrolases with synthase domains (Bernard & Latgé, 2001).

An extensively studied family of cell wall remodeling enzymes is the Gas-protein-family (Arroyo et al., 2007). The Gas-protein family is a family of GPI-anchored β-1,3-glucanosyl-transferases involved in the cell wall biogenesis, acting as β-1,3-glucan processing enzymes (Arroyo et al., 2007; Hartland et al., 1991; Mouyna et al., 2000b). Members of this family are extremely well conserved in *S. cerevisiae*, *C. albicans* and *Aspergillus* species (Arroyo et al., 2007). These enzymes catalyze the cleavage of an internal glycosidic linkage of a β-1,3-glucan chain, releasing a reducing end and finally

transferring it to a non-reducing end of another β-1,3-glucan (Hartland et al., 1991; Mouyna et al., 2000b). Thus, the members of the Gas-protein family act similar to glycoside hydrolases. The Gas-proteins were grouped into glycoside hydrolase family 72 (GH72) in the carbohydrate active enzyme database (CAZy; http://www.cazy.org/).

Hardly anything is known about the CWPs of higher basidiomycetes (Agaricomycotina). However, many of the fungi from this subphylum are involved in degradation of complex substrates such as wood and other lignocellulosic substrates and are responsible for the mineralization of wooden biomass and decomposing of organic materials. For this purpose, many basidiomycetes produce numerous enzymes responsible for degradation of plant cell wall material, such as laccases, manganese-dependent (or -independent) peroxidases, cellulases, and xylanases (Bouws et al., 2008; Cohen et al., 2002; Conesa et al., 2002; Morozova et al., 2007; Ng, 2004). Certain individual enzymes have already been detected within the fungal cell wall or the associated hyphal sheath (Barrasa et al., 1998; Ruel & Joseleau, 1991). For example, Barrasa et al. (1998) showed the association of aryl-alcohol oxidase in *Pleurotus eryngii* with the cell wall glycans of the fungus during the degradation of wheat straw.

There is ample of evidence that also typically intracellular proteins are attached to the fungal cell wall, such as glycolytic enzymes and other high abundant cytosolic proteins (Chaffin et al., 1998; Delgado et al., 2003; Edwards et al., 1999; Motshwene et al., 2003; Urban et al., 2003). They are generally released by extraction of the pure cell wall fraction with β-mercaptoethanol. In the ascomycetous yeasts *S. cerevisiae* and *C. albicans*, glycolytic enzymes and chaperones (proteins assisting unfolded proteins to fold correctly) were shown to be attached to the cell wall (Alloush et al., 1997; Eroles et al., 1997; Gil-Navarro et al., 1997; López-Ribot & Chaffin, 1996; López-Ribot et al., 1996). Enzymes known to be involved in glycolytic processes in the intracellular space such as 3-phosphoglycerate kinase (Alloush et al., 1997) and glyceraldehyde-3-phosphate dehydrogenase (Gil-Navarro et al., 1997) are shown to be located in the cell wall of *C. albicans*. Enolase, another enzyme involved in glycolysis, and several proteins of the heat shock protein family were also detected (Edwards et al., 1999; Eroles et al., 1997; Russo et al., 1992). All these enzymes lack the well described classical N-terminal secretion signal and it remains unknown how they reach the cell surface. However, it has been speculated that these proteins are transported to the cell surface by a non-conventional export pathway (De Groot et al., 2005). Another assumption claims that these proteins hitch-hick in small amounts to the cell surface by leaking into vesicles

during the formation of transport vesicles (De Groot et al., 2005). This theory would be in agreement with the fact that until now only high abundant proteins such as heath shock proteins and glycolytic enzymes were detected in the cell wall. However, another explanation for the occurrence of these typically intracellular proteins in the cell wall could be the following: proteins may indeed derive from aging cells or cells damaged by shear stress. The cell walls of fungi are mostly negatively charged because of large numbers of phosphate groups, forming phosphodiester bridges. Thus, it might be possible that normally intracellular proteins with a relatively high isoelectric point (IP) bind to the mostly negatively charged cell wall (De Groot et al., 2005). Nevertheless, it seems to be unlikely that those typically intracellular proteins are only contaminations since there is evidence from *C. albicans* that they play a role during infection of the host which was proven by immunoblotting and indirect immunofluorescence detection (Eroles et al., 1997). Further, Pardo et al. (1999) showed that also regenerating protoplasts secrete glycolytic enzymes. Nombela et al. (2006) suggested that these unconventional cell wall proteins are so called moonlighting proteins, performing multiple functions depending on their location.

1.1.2.2 Covalently Bound Cell Wall Proteins

Many covalently linked cell wall proteins were identified and studied in ascomycetous yeasts such as *S. cerevisiae* and *C. albicans* (for a review see De Groot et al. (2005)) but also in the filamentous ascomycete *Trichoderma reesei* (Lim et al., 2001), and the yeasts *Y. lipolytica* (Jaafar et al., 2003) and *Schizosaccharomyces pombe* (De Groot et al., 2007). Covalently bound cell wall proteins were studied either experimentally or potential enzymes were detected by *in silico* studies (Caro et al., 1997; Weig et al., 2004). Within the covalently linked CWPs, one can distinguish between the proteins bound by alkali sensitive linkages to the cell wall polysaccharides and the Glycosylphosphatidylinositol (GPI)-anchored proteins (Figures 1.1 and 1.2). The latter are either linked to the cell membrane or the cell wall polysaccharides. At least in ascomycetous yeasts, the GPI-proteins represent the most abundant covalently bound CWP (De Groot et al., 2005). They often show a common organization with a serine-threonine-rich spacer at the C-terminus and a functional domain at the N-terminus. Kopecká et al. (1974) were the first to discover that the treatment of *S. cerevisiae* cell walls with 1,6-β-glucanase reveals a tightly interwoven layer of glucans sensitive to 1,3-

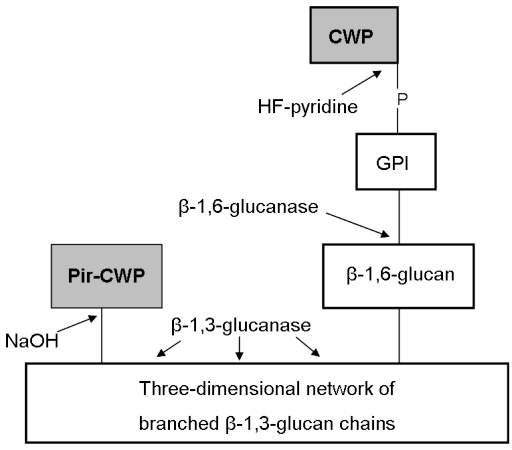

Figure 1.2: *C. albicans* and *S. cerevisiae* contain two different classes of cell wall proteins. GPI-dependent CWPs are linked with their C-terminus to a GPI anchor through a phosphodiester bond, while the GPI anchor is attached either to the cell wall polysaccharides or to the cell membrane; Pir-CWPs are CWPs with internal repeats, linked to β-1,3-glucan through an alkali-sensitive linkage. Possible treatments for the release of the CWPs are indicated by arrows, after De Groot et al. (2004)

β-glucanase. This work gave the basics for further biochemical work leading to the discovery that many CWPs are covalently linked to the β-1,6-glucans of the cell wall polysaccharides through GPI anchors (Kapteyn et al., 1996, 1997; Kollár et al., 1997). This organization of GPI-anchored proteins had been later confirmed for *C. albicans* and *C. glabrata* and in a more indirect way also for other ascomycetous yeasts such as *Y. lipolytica* (Frieman et al., 2002; Jaafar & Zueco, 2004; Kandasamy et al., 2000; Kapteyn et al., 2000; Weig et al., 2004). GPI-anchored CWPs were until now only analyzed in ascomycetous yeasts and only to a certain extend. It is not known how they are linked within the cell wall in filamentous ascomycetes or how common GPI-anchored CWP are throughout the fungal kingdom. However, the available studies can be taken as a rough model for other fungal species and give a first insight into the world of GPI-anchored fungal CWP. In *S. cerevisiae*, GPI CWPs are linked to the β-1,6 glucan through the GPI anchor, which includes a phosphodiester bridge connecting ethanolamine to a mannosyl residue of the glycan core [(De Groot et al., 2005) Figure 1.1]. This linkage can therefore easily be broken by either phosphodiesterase, aqueous hydrogen fluoride (HF) or HF-pyridine (De Groot et al., 2004; Kapteyn et al., 1996). The first GPI-anchored Cell wall protein (CWP) was released from *Fusarium oxyspo-*

rum by Schoffelmeer et al. (1999) and lead to the cloning of a gene called *Fusarium* extracellular matrix protein (FEM1p) (Schoffelmeer et al., 2001). Deletion of a homologous gene in *M. grisea* showed that this GPI protein helps to withstand the enormous turgor pressure during leaf penetration (Ahn et al., 2004).

Most research concerning GPI-anchored CWP was done for ascomycetous fungi, whereas data for basidiomycetes is rare. Ruiz-Herrera et al. (1996) found that proteins from SDS-treated cell walls of the basidiomycete *Ustilago maydis* can be released with either a chitinase or a 1,3-β-glucanase. This suggests a possible linkage with chitin or with β-1,3 glucan, either direct or indirect. However, it is still unknown whether cell walls of basidiomycetes contain as well GPI-anchored proteins and in which way these would be linked to the polysaccharide network.

Since the extraction of GPI-anchored CWP is laborious and only few of those proteins were identified in this way, *in silico* identification of GPI-modified proteins can give a first insight whether extraction steps will possibly be successful in identifying such proteins in a species. Already in 1997, the whole genome of *S. cerevisiae* was searched for genes for possible GPI-modified proteins (Caro et al., 1997). All potential proteins with a N-terminal signal sequence were screened for the presence of a typical hydrophobic domain at the C-terminus, the attachment site of the GPI anchor [(Benghezal et al., 1996; Hamburger et al., 1995) Figure 1.1]. The GPI attachment site, or so called ω-site, is composed of a short attachment domain, requiring small amino acids, a spacer domain (8 - 10 amino acids), and a C-terminal hydrophobic domain containing at least 11 amino acids [Figure 1.1 (Coyne et al., 1993; Nuoffer et al., 1993)]. These structural requirements can thus be used for an *in silico* identification of putative GPI-anchored proteins. The algorithm, used for the identification, was updated and optimized for fungal genomes (Eisenhaber et al., 2004) providing a more reliable prediction of fungal GPI-anchored proteins (http://mendel.imp.univie.ac.at/gpi/fungi_server.html). This more refined algorithms revealed 66, 104, 33, 97, and 106 putative GPI proteins in *S. cerevisiae*, *C. albicans*, *S. pombe*, *N. crassa*, and *C. glabrata*, respectively (De Groot et al., 2003).

In addition to the GPI-anchored CWP, another group of CWP was found to be covalently linked to the fungal cell wall, the proteins with internal repeats [Pir (Table 1.2)]. The Proteins with internal repeats (Pir) protein family was first described in *S. cerevisiae* by Russo et al. (1992) and Tohe et al. (1993) and consists of proteins directly connected to the β-1,3 glucan in the cell wall through alkali-sensitive linkages

Table 1.2: Proteins and potential proteins with internal repeats (Pir) identified from different fungal species, either experimentally or by *in silico* analysis of whole genome sequences.

Fungus	Number	Name (alternative name)	Method of identification	Reference
Candida glabrata	5	Pir1-5p	*In silico*	Weig et al. (2004)
Saccharomyces cerevisiae	4	Pir1p (Ccw6p) Pir2p (Ccw7p, Hsp150) Pir3p(Ccw8p) Pir4p(Ccw5p, Cis3)	Experimental	Moukadiri et al. (1999) Tohe et al. (1993) Castillo et al. (2003) Ecker et al. (2006) Mrsa & Tanner (1999) Kapteyn et al. (1999)
Candida albicans	2	Pir1p Pir32p	*In silico*, experimental	Kandasamy et al. (2000) Kapteyn et al. (2000)
Yarrowia lipolytica	1	YlPIR1	Experimental	Jaafar et al. (2003)

(Kapteyn et al., 1999). It is suggested that they form a novel alkali-sensitive ester linkage between the γ-carboxyl group of a glutamine residue from the protein and hydroxyl groups from glucose residues of β-1,3 glucan (Ecker et al., 2006). Typical Pir proteins contain a signal peptide for secretion, a Kex2 peptidase cleavage site, a glutamine-rich domain with a variable number of internal repeats and a C-terminal domain with four cysteine residues [Figure 1.3 (Ruiz-Herrera et al., 2008)]. They seem to be involved in the recovery of cells from heat shocks (Tohe et al., 1993) and in the maintenance of the cell wall structure (Mrsa & Tanner, 1999). Individual members of the Pir family are extractable by the action of reducing agents [Table 1.2 (Cappellaro et al., 1998; Moukadiri & Zueco, 2001; Moukadiri et al., 1999)]. This is the case for Pir2 (Russo et al., 1992) and Pir4 (Moukadiri & Zueco, 2001) from *S. cerevisiae*, for YlPIR1 from *Y. lipolytica* and for Pir homologues from *C. albicans* (Jaafar et al., 2003). The internal repeats of the Pir family were shown to be involved in the linkage of the protein to the β-1,3 glucan (Castillo et al., 2003). Deletion of the repetitive sequence resulted in a failure to link Pir4p to β-1,3 glucan in *S. cerevisiae* (Castillo et al., 2003). Pir proteins are uniformly distributed all over the cell wall structure of yeasts and their production is strongly up-regulated in the case of cell wall damage (Boorsma et al., 2004; García et al., 2004; Kapteyn et al., 2000; Lagorce et al., 2003).

The recently published *in silico* analysis of the genome from the basidiomycete *U. maydis* predicted the absence of Pir proteins in this organism (Ruiz-Herrera et al.,

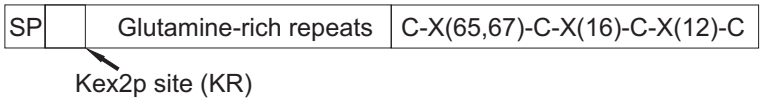

Figure 1.3: Schematic structure of Pir proteins (proteins with internal repeats). The C-terminal motive is in Prosite formate, as used for the FUZZPRO analysis to identify Pir proteins from the genome of *C. cinerea*; SP: signal peptide; after Ruiz-Herrera et al. (2008)

2008). Ruiz-Herrera et al. (2008) also stated that an *in silico* analysis of other genomes of basidiomycetes and zygomycetes revealed no Pir homologues as well, concluding that Pir proteins are a special feature of ascomycetes. Though this assumption was not confirmed until now, Pir-like proteins from basidiomycetes could instead have totally different internal repetitive sequences compared to ascomycetous Pir-proteins. Candidates for cell wall-bound proteins with new types of repeat-motives are certain class I hydrophobins (Velagapudi, 2006) and *U. maydis* repellent protein Rep1-1 (Kämper et al., 2006).

1.2 Secreted Proteins from Higher Basidiomycetes

Various higher basidiomyctes from the subphylum Agaricomycotina are adapted to growth on complex substrates such as lignocellulosic material. For this reason, they produce an arsenal of extracellular enzymes necessary for the degradation of these complex biopolymeres. A large number of extracellular peroxidases and H_2O_2-generating enzymes has been identified from basidiomycetous fungi within the last years. Lignin peroxidase and manganese-dependent peroxidase (MnP) were first discovered from *P. chrysosporium* (Glenn et al., 1983) and versatile peroxidases from members of the genus *Pleurotus* (Ruiz-Dueñas et al., 1999). The peroxidases require for their activities hydrogen peroxide typically provided by glyoxal oxidases (Vares et al., 1995), superoxide dismutases (Guillén et al., 1997) and alcohol oxidases (Marzullo et al., 1995). The possibly best characterized enzymes from basidiomycetes are the laccases. These are phenol oxidases reducing oxygen to water and at the same time performing an one-electron-oxidation of various aromatic substrates (Hoegger et al., 2006; Leonowicz et al., 1999). A broad range of other oxidative as well as hydrolytic enzymes was also detected e.g. in the secretome of *Pleurotus sapidus*, including a high number of metallopeptidases and serine peptidases (Zorn et al., 2005b). Also for *Trametes versicolor*, a well stud-

ied basidiomycete in lignocellulose degradation, several extracellular enzymes involved in lignin degradation are known and characterized (Bertrand et al., 2002; Xiao et al., 2003). For the two fungi mentioned above, the identification of proteins from large scale proteomic approaches has its challenges as their genomes are so far not sequenced.

Besides the oxidoreductases, a broad range of hydrolytic enzymes have been found in the secretomes from basidiomycetes. From *P. sapidus* an extracellular esterase, specified as a carboxy esterase, was characterized on the molecular level (Zorn et al., 2005a). In *P. chrysosporium* various peptidases were detected on media supplemented with cellulose or wood chips as a carbon source (Sato et al., 2007). Numerous carbohydrate degrading enzymes are usually found in the culture supernatants of basidiomycetes [e.g. (Hamada et al., 1999; Kawai et al., 2006; Shu et al., 2006; Valásková & Baldrian, 2006)]. A computational analysis of the sequenced and annotated genome from *P. chrysosporium* revealed multiple genes for (potential) oxidoreductases, esterases, glycoside hydrolases and peptidases having secretion signals (Vanden Wymelenberg et al., 2006). Experimental approaches revealed that protein patterns from ligninolytic cultures and cellulolytic cultures of *P. chrysosporium* differ significantly from each other (Kersten & Cullen, 2007; Ravalason et al., 2008; Sato et al., 2007; Shary et al., 2008; Vanden Wymelenberg et al., 2006). Depending on the media composition different glycoside hydrolases, peroxidases and peptidases were identified (Sato et al., 2007).

C. cinerea is a model organism for homobasidiomycetous fungi (Agaricomycotina), commonly used for developmental studies (Kües, 2000) because of its comparably short life-cycle of two weeks which can easily be performed under laboratory conditions (Moore & Pukkila, 1985). Besides classical genetic methods for the study of sexual development and recombination (Kamada, 2002), molecular methods such as DNA-mediated transformation (Binninger et al., 1987) and insertional mutagenesis (Granado et al., 1997) make the fungus an interesting research organism. Though easy to cultivate on artificial media, the natural substrate of *C. cinerea* is horse dung (Kües, 2000). Therefore, the fungus can not be classified as a typical wood degrader but nevertheless, it still acts as a lignocellulose degrading fungus (Chaisaena, 2009; Navarro-González, 2008). The best studied secreted enzyme of *C. cinerea* is the well known *Coprinus cinereus* peroxidase Cip (Baunsgaard et al., 1993). Cip has a high sequence homology to manganese-dependent peroxidases from white rot fungi, while in the substrate specificity Cip has more in common with the horseradish peroxidase (Baunsgaard et al., 1993). Other extensively studied secreted enzymes in *C. cinerea* are laccases. There

are 17 different laccase genes in *C. cinerea* which were analyzed at the molecular level (Kilaru et al., 2006a) as well as overexpressed for individual biochemical studies of the different laccase isoenzymes of *C. cinerea* (Kilaru et al., 2006b). Release of the genome sequence from *C. cinerea* in 2003 by the Broad Institute made proteomic and *in silico* gene and protein studies for this fungus more interesting and feasible (Stajich et al., 2010). A family of fungalysin extracellular metallopeptidases was for example investigated by a computational approach, predicting genes for eight different fungalysin metalloproteases in the genome of *C. cinerea* (Lilly et al., 2008). Also recently, genes for members of the glycoside hydrolase family 6 were identified in the fungal's genome, subcloned and transcript levels for the genes were determined on glucose- and cellulose-based medium (Yoshida et al., 2009).

1.3 Fungal Proteomics - State of the Art

A proteomic study in general is the global assessment of proteins in a particular biological state either of a whole cell or a specific cell compartment. Such proteomic studies are a powerful tool for the understanding of events on a molecular level and can reveal new insights into many known and unknown processes on the protein level. Proteomic studies had their start around 1975 with the application of two dimensional gel electrophoresis (2-DE) enabling the investigation of proteins in complex mixtures (Klose, 1975; O'Farrell, 1975). 2-DE changed the way of investigating biological systems drastically. However, at first only well studied organisms could profit from these new strategies (Lee & Lee, 2003). Though fungi, especially filamentous fungi are of great interest for industrial applications and many of them occur as human and animal or as plant pathogens, proteomic studies on studies on a broader scale were only established within the last few years (Kim et al., 2007). Some of the first proteomic studies in a filamentous fungus were done on cell envelope proteins of the industrial interesting ascomycete *T. reesei* (Lim et al., 2001) and further on GPI-anchored proteins in the opportunistic human pathogen *A. fumigatus* (Bruneau et al., 2001). Since then, numerous publications followed concerning proteomic approaches in filamentous fungi; many of which involved ascomycetous fungi (Kim et al., 2007). Also a few proteomic studies of higher basidiomycetes were already performed, though the total number is still comparably small. The secretome of *P. sapidus* grown on different substrates in submerged

cultures was analyzed by means of 2D-gel-electrophoresis (Zorn et al., 2005b). Proteomic approaches were as well used to study the intracellular iron-regulated proteins of *P. chrysosporium* (Hernandez-Macedo et al., 2002) and the intracellular response of the fungus on the exposure to vanillin (Shimizu et al., 2005). Secretome studies on various substrates, natural and artificial media, such as softwood, wheat straw and cellulose, were also performed for *P. chrysosporium* (Kersten & Cullen, 2007; Ravalason et al., 2008; Sato et al., 2007; Shary et al., 2008; Vanden Wymelenberg et al., 2006).

The secretome consist not only of the freely secreted proteins isolated from the culture medium, but is defined as the combination of secreted proteins and the cellular machinery involved in their secretion (Tjalsma et al., 2000). This includes as well the proteins of the periplasmic space, the fungal cell wall and the hyphal sheath. Different chemical and/or enzymatic strategies were already applied for comprehensive proteomic analysis of the cell wall proteome mostly in ascomycetous yeasts in order to provide an overall overview of this subproteome. A very detailed fractionation of *C. albicans* cell wall proteins from the yeast and the hyphal form was performed by Pitarch et al. (2002). The cell wall proteins were separated into four different fractions, obtained by SDS/DTT extraction, subsequent NaOH-extraction and further two enzymatic digestions of the cell wall polysaccharides with Quantazyme (a commercially available, recombinant β-1,3-glucanase from Thomas Scientific, USA) and an exo-chitinase. This study revealed similarities between the yeast and the hyphal form of the fungus. In a similar study concerning the two distinct cell forms of *C. albicans*, the cell wall fraction, containing the covalently bound cell wall proteins, was digested with cyanogen bromide (CNBr) and trypsin, revealing more than 100 identified proteins (Ebanks et al., 2006). Besides other comprehensive proteomic analysis of the cell wall proteome from *S. cerevisiae* (e.g. (Yin et al., 2005)), proteins secreted from regenerating protoplasts were analyzed by 2-DE (Pardo et al., 1999). This interesting approach revealed many proteins potentially involved in cell wall construction, several heat shock proteins and some glycolytic enzymes. One of the first proteomic studies concerning the cell wall of filamentous fungi was done for *T. reesei*, giving an overview of the cell wall proteins in this industrially used fungus (Lim et al., 2001). For this study, the cell wall proteins were isolated by sonication of the pure cell wall fraction in buffer and subsequent analysis by 2-DE.

These early global studies of the fungal cell wall proteome were followed by more specific studies concerning single protein classes such as GPI-anchored proteins or Pir proteins in different fungal species, especially *S. cerevisiae* and *C. albicans* [e.g. (De Groot

et al., 2004, 2007, 2008; Iranzo et al., 2002; Jaafar & Zueco, 2004; Yin et al., 2007)].

The strategies used before on the cell wall proteins of ascomycetes might be taken as a guideline for research focussing on the so far little studied basidiomycetous fungi. With the increasing number of fungal genomes becoming available, including numerous basidiomycetes such as *P. chrysosporium*, *Postia placenta*, *S. commune*, *C. cinerea* and many others, also the proteomic studies of further ascomycetous and particularly also basidiomycetous fungi become more feasible.

1.4 Genomes of Higher Basidiomycetes

The NCBI GenBank database has lately doubled its size by the time of 18 month (Benson et al., 2008) and contained by April 2009 around 103 million sequences (http://www.ncbi.nlm.nih.gov/Genbank/). Whole genome sequences represent a rapidly growing part of this database. Already 532 eukaryotic genome sequences are completed or in progress, 165 of these are fungal genomes which mainly belong to the phylum Ascomycota (April 2009; http://www.ncbi.nlm.nih.gov/Genbank/index.html). The first fungal genome sequenced was the one of *S. cerevisiae* published in 1996 and, with it, a milestone for fungal genomics was laid (Goffeau et al., 1996). However, further genome sequencing of the first filamentous fungus *Neurospora crassa* (Galagan et al., 2003) showed how limited the genome of *S. cerevisiae* is in total functional genes, as the predicted gene number in *N. crassa* is almost double.

Genomes from filamentous fungi were released one after another in 2005 such as the ones from *A. nidulans* (Galagan et al., 2005a), a model organism and industrial relevant fungus, *A. fumigatus* (Nierman et al., 2005), a human pathogen, and *Aspergillus oryzae* (Machida et al., 2005), an industrial fungus. Basidiomycetous fungi, especially Agaricomycotina play a crucial role in the environment by degrading complex substrates such as lignocellulose and wood (Baldrian & Valásková, 2008), decomposing organic materials, and forming symbiotic relationships with plants (Martin et al., 2001). Although the fungal genome sequencing started already more than 10 years ago comparably, few genomes from the group of higher basidiomycetes (subphylum Agaricomycotina) are available until now: the genomes of the dung fungus *C. cinerea* (Stajich et al., 2010), of the ectomycorrhizal fungus *Laccaria bicolor* (Martin et al., 2008), of the white rots *P. chrysosporium* (Martínez et al., 2004) and *S. commune* and the brown rot *Postia*

placenta (Martinez et al., 2009). Others such as the genomes of the white rot conifer pathogen *Heterobasidion annosum*, the white rot *Pleurotus ostreatus*, the edible saprotroph *Agaricus bisporus*, the pathogenic white rot *Phanerochaete carnosa*, the brown rot *Serpula lacrymans* and the ectomycorrhizal fungus *Paxillus involutus* are accepted for sequencing projects, in sequencing or in annotation processes (http://www.jgi.doe.gov/; see also Table 1.3). These selected fungi could not be more different in their lifestyle, thus analysis of the whole genome sequences could provide an interesting insight into the genomic basics of those different life styles.

For gene predictions of the whole genome sequences, it can be most crucial to provide data from a closely related model organism. The more sequences from higher basidiomycetes will be available, the better the annotations will become, as more and more sequences to compare will be available in the future. Comprehensive analysis of genomes from fungi adapted to different environments and coping with different substrates has the potential to reveal knowledge not only about the differences of life style on the genomic level, but also how evolution was modeling the genetic basics for these different ways of life.

1.4.1 Techniques for Genome Sequencing

At the moment two large institutes are involved in the sequencing of genomes from Agaricomycotina, the Broad Institute (http://www.broad.mit.edu/) and the Joint Genome Institute (JGI) (http://www.jgi.doe.gov/ see also Table 1.3). The Broad Institute is a research collaboration of the MIT, Harvard, its associated hospitals and the Whitehead Institute, mainly focusing on the relevance of genomes for medical use. In 2000, they organized together with the fungal research community the project "fungal genome initiative" (FGI) and sequenced so far over 25 genomes from fungi important in medicine, agriculture and industry. Genomes for sequencing are chosen by a committee of fungal scientists. The fungal's relevance for basic research, medicine, agriculture, biotechnology and as well the relevance for comparative genomics and the taxonomic position of species and with it evolutionary concerns are the criteria for selection of a fungus for genome sequencing. Groups of organisms according to their value for comparative genomics, evolutionary studies, medical use, and eukaryotic biology were formed. Only one of the here reviewed genomes was sequenced by the Broad Institute, that is *C. cinerea*; fitting into a cluster with the basidiomycetous plant pathogen *U. maydis* and

Table 1.3: List of higher basidiomycetes (Agaricomycotina) which are in line for genome sequencing, are sequenced or already have whole genome sequences annotated and published.

Fungus	Family	Number of predicted genes	Genome size	Characteristics	Sequencing institute & reference
Agaricus bisporus var. *bisporus* H97 and JB137-s8	Agaricaceae	-	-	Secondary decomposer	JGI*
Coprinopsis cinerea strain Okayama 7 #130	Psathyrellaceae	13,544	37.5 Mbp	Saprotrophic dung fungus	Broad** (Stajich et al., 2010)
Heterobasidion annosum TC 32-1	Bondarzewiaceae	-	-	Pathogenic white rot, causing root and butt rot in conifers	JGI*
Laccaria bicolor strain S238N	Tricholomataceae	20,614	65 Mbp	Ectomycorrhizal fungus	JGI* Martin et al. (2008)
Paxillus involutus	Paxillaceae	-	-	Ectomycorrhizal fungus	JGI*
Phanerochaete carnosa	Corticiaceae	-	-	White rot tree pathogen	JGI*
Phanerochaete chrysosporium strain RP78	Corticiaceae	11,777	30 Mbp	White rot fungus weak tree pathogen	JGI* Martínez et al. (2004)
Pleurotus ostreatus var. *florida* PC9 and PC15	Pleurotaceae	-	35 Mbp	White rot fungus	JGI*
Postia placenta strain MAD 698	Coriolaceae	17,173	33 Mbp	Brown rot fungus	JGI* Martinez et al. (2009)
Schizophyllum commune strain H 4-8	Schizophyllaceae	-	-	White rot fungus	JGI*
Serpula lacrymans	Serpulaceae	-	-	Dry rot fungus (decay without water)	JGI*

* http://www.jgi.doe.gov/
** http://www.broad.mit.edu/annotation/fgi/

the human pathogen *Cryptococcus neoformans*, *C. cinerea* was sequenced as a model organism (http://www.broad.mit.edu/annotation/fgi/, see also Table 1.3). The other institute involved in genome sequencing of higher basidiomycetes is the Joint Genome Institute (JGI), an association of several DOE (U.S. Department of Energy) genome centers located at the University of California (http://www.jgi.doe.gov/). The JGI is interested in generation of clean energy, environmental clean-up and characterization of the environment. High-throughput sequencing and computational analysis of sequence data is utilized to face these challenges.

Both institutes used the whole genome shotgun (WGS) method for genome sequencing. For this method, genomic DNA is fragmented randomly, size selected, and cloned for the production of a random library in *Escherichia coli*. Clones are sequenced randomly from both ends and the single sequences are assembled by computational methods (Edwards et al., 1990; Roach et al., 1995). Due to size selections, the length of each fragment is known. This additional information is used by the assembly program for the construction of scaffolds. Overlapping sequences are combined to 'contigs', those are further linked to 'scaffolds'. Remaining sequence gaps after the first shot gun sequencing can be closed by sequencing from a primer into the clone containing the gap. Physical gaps, meaning sequences which do not appear within any of the clones, are closed by selective primer choice and PCR using genomic DNA as a template (Hutchison, 2007). However, this strategy finds its restriction in fungal genomic DNA as repetitive sequences associated with rDNA, telomeres, and centromeres are common in fungi (Galagan et al., 2005b). Transposable elements are widely studied in ascomycetes (Cambareri et al., 1998; Hua-Van et al., 2000; Thon et al., 2004) but as well for basidiomycetes efforts to identify transposable elements are undertaken, showing that basidiomyctes contain many more transposable elements than various of the so far characterized ascomycetes (Martin et al., 2008). In *P. chrysosporium*, the second revised release of genome data was used to describe the structure, organization and function of repetitive elements (Larrondo et al., 2007). Those repetitive sequences, if present in the clone libraries, are often difficult to handle during the assembly of the sequence data. Though optimization and follow up analysis of sequenced genomes is ongoing, repetitive sequences still keep challenging for genome sequencing (Farman & Leong, 1995; Li et al., 2005). Having the whole DNA sequence established, the work is not yet done. Also automated annotation of genes is a challenging approach for bioinformatics. Coding densities of the fungal genomes are relatively dense (37 - 61%)

compared to other eukaryotes (Galagan et al., 2005b). The typical gene structure of higher basidiomycetes is embossed by high densities of relatively short introns (4 - 6 or more per gene) (Loftus et al., 2005; Martínez et al., 2004), probably inherited from an intron-rich common ancestor (Stajich et al., 2007). Alternative splicing is as well a great challenge for gene prediction methods. Fungi seem not to make use of alternative splicing as often as other eukaryotes (Modrek & Lee, 2002) but still alternative splicing is a regularly occurring event. Most extensively studied so far was the alternative splicing process in *C. neoformans* (Loftus et al., 2005).

Though knowing the principle characteristics of fungal gene structures, the gene finding process was still challenging for the first genome released from the phylum Basidiomycota, which was the one from *P. chrysosporium* (Martínez et al., 2004). Especially less conserved genes were hard to identify within the genome due to the fact that comparative genomes from closely related species are missing. To optimize the gene finding process in the case of *P. chrysosporium* genome, comparative methods and *ab initio* gene finding were compared (Martínez et al., 2004). For the *P. chrysosporium* genome already a second, optimized version was published (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Phchr1&advanced=1). For *C. cinerea* additional to the gene prediction provided by the Broad Institute, other alternative genome annotations obtained by different annotation algorithms are available (http://genome.semo.edu/cgi-bin/gb/gbrowse/cc/). *L. bicolor* gene predictions were done by combing several gene prediction algorithms taking advantage of the forerunners *P. chrysosporium*, *C. cinerea*, *C. neoformans* and *U. maydis* [(Martin et al., 2008); see also Table 1.3]. Most of the proteins showing sequence similarities to already known genes (70% of total) had a high similarity with genes from those four fungi (Martin et al., 2008). Still, the gene prediction keeps challenging as long as only few genomes from higher basidiomycetes are available.

1.4.2 Already Sequenced Genomes of Agaricomycotina

The first genome published from the phylum Basidiomycota was the one from *P. chrysosporium* strain RP78 in 2004 by the US DoE Joint Genome Institute (Martínez et al., 2004). *P. chrysosporium* is a white rot fungus, as it is degrading lignin from the wooden substrates. A special feature of *P. chrysosporium* is that it leaves the cellulose of the wood almost untouched in contrast to other white rot fungi. For the degradation of

lignin, white rot fungi developed sets of extracellular oxidative enzymes, such as multicopper oxidases, peroxidases, copper radical oxidases, and FAD-dependent oxidases, which act non-specifically by the formation of oxygen radicals (Kersten & Cullen, 2007). In addition to their ecological role, these enzymes have a high biotechnological potential (Cohen et al., 2002). Therefore, *P. chrysosporium* is an extensively studied fungus concerning extracellular oxidative enzymes and their application in biodegradation of organic pollutants (Hammel & Cullen, 2008; Rubilar et al., 2008). The genome of *P. chrysosporium* was extensively analyzed by *in silico* approaches as well as experimental approaches (Vanden Wymelenberg et al., 2006). Transcriptome analysis under various different growth conditions were performed in order to study the reaction of the fungus to natural as well as artificial media (Jiang et al., 2009; Sato et al., 2009; Vanden Wymelenberg et al., 2009; Zhang et al., 2009). In contrast to the strictly lignin degrading *P. chrysosporium* stands the mainly cellulose-degrading brown rot fungus *P. placenta*. The genome sequence of *P. placenta* has been released in 2007 and the whole genome has been published early in 2009 (Martinez et al., 2009). As a brown rot fungus, it mainly degrades the cellulose network of the plant cell walls and leaves a modified lignin structure. Though certain enzymes for active lignin decay as found in white rot fungi are missing, lignin still undergoes a chemical modification (Goodell, 2003). First comparative genetics of those two fungi revealed that brown rots, such as *P. placenta*, lost multiple gene families which are thought to be important for white rot fungi, such as *P. chrysosporium*, namely cellulases, lignin peroxidases, manganese peroxidases, copper radical oxidases, cellobiose dehydrogenase and pyranose-2-oxidase, indicating that brown rot fungi developed novel mechanisms for cellulose degradation (Goodell, 2003).

L. bicolor is the first ectomycorrhizal fungus sequenced and the genome sequence was released in 2007 (Martin et al., 2008). Symbiotic fungi, such as *L. bicolor* are studied mainly with the focus on the symbiosis with the tree species and the morphology of the fungi (Martin et al., 2001). Still, the sequenced genome of an ectomycorrhizal fungus can provide deep insight into symbiotic mechanisms especially in combination with the genome sequence of a host tree such as available for *Populus trichocarpa* (Tuskan et al., 2006). Also, the development of a range of molecular methods for *L. bicolor* is already in progress, making molecular studies of the ectomycorrhizal lifestyle possible. Only recently, RNA silencing by *Agrobacterium*-mediated gene transfer was for example used to knock down the gene for *L. bicolor* nitrate reductase. This resulted in transgenic

strains, which are strongly affected in growth on medium supplemented with nitrate as N source (Kemppainen et al., 2009).

The ink cap mushroom *C. cinerea* is a model organism for higher basidiomyctes since it is able under laboratory conditions to pass through a whole life cycle within two weeks. Molecular genetics, sexual development, and meiosis of this fungus are extensively studied [reviewed by (Kamada, 2002; Kües, 2000)]. For *C. cinerea*, many molecular tools for genetic studies are already well established. DNA-mediated transformation was already developed in the 1980's (Binninger et al., 1987). This gave the basis of the published studies for example on a homologous *C. cinerea* laccase gene expressed under different basidiomycetous promoters (Kilaru et al., 2006b). Gene silencing provides a powerful tool for the generation of knock-down strains, extremely useful for the study of single genes and gene products. Mutants with silenced genes have been successfully produced by double-stranded-RNA-mediated gene silencing (Namekawa et al., 2005). Also other methods for post-transcriptional gene silencing were successfully tested (Heneghan et al., 2007).

1.4.3 Genomes in Sequencing and Annotation Progress

S. commune is used as a model system for white rot fungi, sexual reproduction and fruiting body development (Palmer & Horton, 2006). Moreover, *S. commune* is a pathogen attacking wounded trees of all kind (Peddireddi, 2008). Genetical tools such as a transformation method of *S. commune* are long established (Muñoz Rivas et al., 1986) and optimized (Specht et al., 1991) and gene-knock-outs are possible in this fungus (Muñoz Rivas et al., 1986; Specht et al., 1991). The genome release of this species could give rise to large scale genetic and proteomic analysis of this model organism.

P. ostreatus is an edible white rot fungus and like *P. chrysosporium* already well studied for the use in bioremediation and concerning extracellular oxidative enzymes (Cohen et al., 2002). But also on the genetic level, *P. ostreatus* is not totally unknown. The genome size of the dikaryotic strain N001 was determined by pulsed field electrophoresis and a genetic linkage map was created (Larraya et al., 1999; Park et al., 2006; Santoyo et al., 2008). Transformation protocols were developed for *P. ostreatus* using polyethylene glycol and protoplasts (Li et al., 2006) and this was already used for the expression of green fluorescence protein (*GFp*) in *P. ostreatus* (Lin et al., 2008). For *P. ostreatus*, two monokaryotic strains are in sequencing and annotation progress, PC9 and PC15. They were produced from the commercial dikaryotic strain N001 by de-dikaryotization

(Park et al., 2006). The availability of two monokaryotic strains developed from one dikaryon reveals great possibilities for comparative genomic studies.

P. carnosa and *H. annosum* are severe pathogenic white rot fungi causing high financial losses to forest owners (http://chem-eng.utoronto.ca/˜bioproducts/research.htm). *H. annosum* causes root and butt rot of conifers in the Northern temperate regions of the world but especially in Europe. The preferred host of *H. annosum* is pine. The disease itself has been intensively studied already since decades (Asiegbu et al., 2005; Hodges, 1969) but genetics, biochemistry, and molecular aspects of the pathogenicity are less well understood (Asiegbu et al., 2005). However, recently, advances in molecular characterization of pathogenicity factors and application of *Agrobacterium*-mediated DNA transformation system have been made (Karlsson et al., 2003; Samils et al., 2006). The genome sequences of *H. annosum* (North American P-type) and *P. carnosa* can be important tools for an insight into pathogenicity of those fungi and may give rise to an effective combat of the diseases.

Also the brown rot fungus *S. lacrymans* causes enormous damage by the attack of mainly coniferous wood, indoor and outdoor, every year (Schmidt, 2007). *S. lacrymans* is a true dry rot fungus, meaning that the wood decay occurs even under dry conditions. A remarkable fact is that *S. lacrymans* is divided into two main species, one nonaggressive occurring naturally in North America and Asia (var. *shastensis*), and another aggressive lineage spread over all continents, in natural environments as well as in buildings (var. *lacrymans*) (Kauserud et al., 2007). This feature and the great damage the fungi are causing especially in the Northern hemisphere, where coniferous wood is mainly used as construction material, makes obvious why these fungi are chosen for sequencing. Techniques of molecular biology are little applied up to date to of *S. lacrymans* but sequencing of the genome and availability of molecular methods for other basidiomycetes could give rise to extensive studies of *S. lacrymans* on the genomic level. The genome sequence could reveal possibilities for the effective combat of this fungus.

P. involutus is the second ectomycorrhizal fungus in pipeline for sequencing. In symbiosis with birch (*Betula pendula*), transcriptional studies of expressed genes were already performed as well as microarray studies (Johansson et al., 2004). Several genes regulated by symbiosis and genes relevant for mycorrhizal development were identified within these studies [reviewed by (Breakspear & Momany, 2007)]. The genome was already characterized concerning complexity and size indicating a genome size of 23 Mbp

(Le Quéré et al., 2002) which is far smaller than that of *L. bicolor*. The relatively small genome size could be due to the fact that only low proportions of non-coding sequences and possibly only low fractions of repetitive DNA are present in the genome (Le Quéré et al., 2002). *P. involutus* provides a perfect organism for genome comparison with the ectomycorrhizal fungus *L. bicolor*.

Last but not least the commercially relevant, edible fungus *A. bisporus* is in sequencing progress. *A. bisporus* is commonly known as button mushroom and is the most widely cultivated mushroom all over the world and therefore already relatively well studied (Stoop & Mooibroek, 1999). As a saprotrophic fungus growing on compost (Stoop & Mooibroek, 1999), *A. bisporus* has a different substrate spectrum than the wood-degrading species. Already a large number of genes potentially relevant for fruiting body development and substrate usage, as well as genes encoding proteins involved in basic biochemical pathways were cloned and characterized in expression patterns (De Groot et al., 1998b; Stoop & Mooibroek, 1999). Breeding of *A. bisporus* is challenging because of its unusual secondary homothallic life-cycle (Raper et al., 1972). Instead of forming upon meiosis basidia with four basidiospores with one type of haploid nuclei resulting upon spore germination in a sterile monokaryotic mycelium, most basidia produce only two spores containing two nuclei of opposite mating type. Thus, after germination of such basidiospores a fertile dikaryotic mycelium, able to produce mushrooms with the meiotic basidiospores, is directly formed. Such dikaryotic offspring is not directly usable for crossing experiments. Still, approximately 2% of the produced basidia from *A. bisporus* carry three or four spores of which those with only one type of haploid nuclei are able to form monokaryotic mycelia upon germination which can be further used for mating with other strains (Summerbell et al., 1989). This is the reason for the small genetic diversity of available *A. bisporus* strains. Most of the commercially available strains are derived from only two strains HorstU1 and HorstU3 (Fritsche, 1983). Lack of genetic diversity makes the commercially cultivated strains of the fungus *A. bisporus* an easy target for fungal and bacterial infections as well as for spontaneous degeneration leading to crop losses and malformed mushrooms (Fritsche, 1983). Within the last decade, effort was made to overcome these problems, which makes the fungus also interesting for the scientific community. Transformation of *A. bisporus* using *A. tumefaciens* was developed (De Groot et al., 1998a) and optimized (Burns et al., 2006; Chen et al., 2000). This rose considerable interest for the application of *A. bisporus* for bio-manufacturing and molecular pharming (Kothe, 2001). A sequenced genome can play a crucial role in this process.

1.4.4 Application of Whole Genome Sequences

An essential part of the interpretation of the genomes from higher basidiomycetes has to be the comparison of the genomes available up to date. Comparison of three *Aspergillus* genomes from the ascomycetes showed the great value of this approach and gave insight into genome evolution and gene regulation (Galagan et al., 2005a). This could be a crucial and interesting step also in the analysis of the Agaricomycotina genomes available currently and the ones soon becoming available. Further, genome comparison could reveal insight into the regulation of gene expression. However, the community of researchers working on Agaricomycotina is comparably small and progress in genome analysis and post genomic research is therefore slow. A large scale comparison of some currently or soon available genomes could be the start for the postgenomic area of higher basidiomycetes. A remarkably interesting comparison would be the one of the genomes from two strains of the same species as will be available soon for *P. ostreatus* and *A. bisporus*. As the two strains in sequencing progress from *P. ostreatus* are obtained from the same dikaryotic strain, a comparative analysis in combination with specific experiments could give insight for example into degradation processes of the fungus.

Comparing the size of the already sequenced genomes and the number of predicted genes, *L. bicolor* has with 65 Mbp by far the largest genome (Martin et al., 2008). The size of the other three completed genomes of *C. cinerea*, *P. placenta* and *P. chrysosporium* with 37.5 Mbp, 33 Mbp, and 30 Mbp, respectively, is only about half the size (Martin et al., 2008). High variations are known to occur in the genome size of basidiomycetes. However, the number of predicted genes is by far not double for *L. bicolor* compared to the other fungi (Table 1.3). Transposable elements present in a higher portion than in the other sequenced genomes account for some part for the relatively large genome of *L. bicolor* (Martin et al., 2008). As the analysis of the *L. bicolor* genome was only recently published (Martin et al., 2008), only minor comparisons with the forerunner genomes of *P. chrysosporium* and *C. cinerea* were done concerning the ectomycorrhizal life style of *L. bicolor*.

Interestingly, the total number of predicted genes for transporters, especially ammonia transporters is larger in the genome of *L. bicolor* compared to the genomes of *P. chrysosporium* and *C. cinerea*, indicating an increased genetic potential for the uptake of nitrogen (Martin et al., 2008). This is also shown by the fact that the number of protease encoding genes is larger in *L. bicolor* (Martin et al., 2008).

The genome sequences from *P. chrysosporium* and *L. bicolor* were already extensively analyzed on the genomic level (Martin et al., 2008; Martínez et al., 2004). A large scale proteomic study was done with secreted proteins of *P. chrysosporium* growing on different substrates (Abbas et al., 2005; Vanden Wymelenberg et al., 2006). Recently, a proteomic and metabolomic analysis of *P. chrysosporium* exposed to benzoic acid has been published (Matsuzaki et al., 2008). These studies showed that the proteomic level can reveal a view on the organism from a totally different perspective. An extensive analysis on the genomic level is still missing for *C. cinerea*, though some effort was done to analyze some individual protein groups on the genetic level with *in silico* approaches. A high number of genes for peptidases, especially metalloprotases, were found to be present in the genome of *C. cinerea* (Lilly et al., 2008). Other analyzed gene families encode e.g. the already mentioned laccases (Kilaru et al., 2006a) or hydrophobins (Velagapudi, 2006). For the brown rot *P. placenta*, the extensive analysis of the genome was accompanied by systematic examination of the transcriptome and secretome (Martinez et al., 2009). This comprehensive study revealed genes for unique extracellular enzyme systems, including an unusual repertoire of extracellular glycoside hydrolases, while genes for exocellobio-hydrolases and proteins with cellulose-binding domains are absent in the genome of this fungus (Martinez et al., 2009).

Sequencing whole genomes can provide a deep insight not only into the biology of higher basidiomyctes but into eukaryotic biology in total. Still, genome sequencing is only the tip of the iceberg. The whole biology and way of life can only be completely understood in combination with the so called -omics approaches. Proteomics is a research field already established and applied for many fungi (Kim et al., 2007) and is already ongoing also for higher basidiomyctes (Abbas et al., 2005; Vanden Wymelenberg et al., 2006; Zorn et al., 2005b) providing insight into subproteomes and protein modification (e.g. PTM). Also a metabolomics approach was already started with *P. chrysosporium* (Matsuzaki et al., 2008). Large scale approaches in proteomics are hard to perform without the background of a completely sequenced and annotated genome, especially for a distinct phylogenetic group such as Agaricomycotina. Certain sequence similarities have to be present to perform a successful protein identification with peptide sequences revealed by mass spectrometry. Also genome-wide functional studies, in particular microarrays, are already available for various fungi. The *L. bicolor* genome for example was analyzed using a whole-genome oligoarray analysis and the study showed that almost 80% of the predicted genes are expressed in free living mycelium, ectomycorrhizal

root tips or fruiting bodies. Performance of similar studies on the other sequenced Agaricomycotina are only a question of time.

1.5 Objective of the Thesis

As outlined in the general introduction, the secretomes from higher basidiomycetous fungi such as *C. cinerea* are up to now not studied in much detail and only a few of the freely secreted proteins have been biochemically analyzed. The cell wall structure as the main contact zone to a fungus' environment is generally supposed to bear unknown enzymes involved in physiological processes such as substrate degradation, interaction with other organisms and pathogenicity (Bowman & Free, 2006). The saprotrophic model basidiomycete *C. cinerea* was chosen for this work, since it is a genetically and physiologically well studied fungus and the annotated genome sequence has been recently published thereby facilitating proteomic studies (*Coprinus cinereus* Sequencing Project, Broad Institute, http://www.broad.mit.edu). The aim of this thesis was to analyze and identify secreted proteins from *C. cinerea* with focus on the following objectives:

1. Optimization of proteomic techniques for the secretomes of higher basidiomycetes

The up to now applied methods for fungal protein analysis by 2-dimensional gel electrophoresis revealed an unsatisfactory protein separation, hindering analysis of protein secretion. An optimized protocol for the isolation of secreted fungal proteins for the application in 2-DE is presented (Chapter 2).

2. Proteomic analysis and identification of the freely secreted and cell wall associated proteins from *C. cinerea*

The cell wall proteome can be expected to be greatly different from the freely secreted proteome. The aim of this study was to isolate and to identify secreted proteins and their compartmentation within the cell wall structures, using modern methods of protein analysis. Proteins bound to the cell wall were separated concerning their linkage to the fungal cell wall polysaccharides and compared with the freely secreted proteome (Chapter 3).

3. Analysis of possible phosphorylation as post-translational modifications (PTM) on proteins in the cell wall of *C. cinerea*

Fungal cell wall proteins are known to be highly glycosylated and other PTMs were

also suggested to occur on cell wall proteins (Freeze, 1985; Ivatt et al., 1984; Leisola et al., 1987). The possibility of protein phosphorylation in the secretome of *C. cinerea* was examined (Chapter 4).

4. Analysis of growth-dependent changes in the fungal secretome

Fungi likely adapt their secretomes to the prevalent environmental conditions and substrate availability. The complete secretome including the freely secreted and the cell wall proteins of *C. cinerea* was studied in different stages of growth (Chapter 5).

Finally, the results are globally discussed concerning their biological relevance for the fungus *C. cinerea* (Chapter 6).

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2 Optimized protocol for the 2-DE of extracellular proteins from higher basidiomycetes inhabiting lignocellulose

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Research Article

Optimized protocol for the 2-DE of extracellular proteins from higher basidiomycetes inhabiting lignocellulose

Basidiomycetes inhabiting lignocellulose comprise an important class of filamentous fungi with ecological and biotechnological relevance. Extracellular enzymes of wood-degrading fungi such as laccases, manganese-dependent (or independent) peroxidases, cellulases and xylanases are of considerable interest for biotechnological applications. Still, proteomic studies of fungal secretomes based on 2-DE can be very challenging due to low protein concentrations and variable amounts of fungal metabolites. Comparison of different standard methods for protein precipitation has demonstrated their limited applicability to fungal secretomes. The extracellular metabolites impair standard methods for protein quantification and can result in a strong overestimation of total protein. We have developed an optimized protocol for the precipitation of extracellular proteins from liquid cultures of *Coprinopsis cinerea* growing in an exponential phase on a complex media. We found that a considerable amount of gelatinous material can be removed from the liquid culture supernatants by high-speed centrifugation. Fungal proteins can be effectively enriched by TCA precipitation and coprecipitated metabolites hampering 2-DE can be removed through the application of Tris/acetone. Following our protocol makes it possible to concentrate proteins from culture supernatants and to simultaneously remove most of the impeding compounds from a given sample. We have applied this procedure in the 2-DE of secretomes from the model organism *C. cinerea* as well as other basidiomycetes such as *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Polyporus brumalis* and *Schizophyllum commune*.

Keywords:
2-DE / Basidiomycetes / Extracellular proteins / Fungal secretome / Protein precipitation
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1 Introduction

Higher basidiomycetes comprise an important class of filamentous fungi with ecological and commercial relevance. Many species (*e.g. Agaricus*, *Lentinus* and *Pleurotus*) are cultivated on an industrial scale for fruiting body production or as a source of enzymes, chemicals and therapeutics [1–3]. Numerous basidiomycetous fungi are unique in their degradation of complex lignocellulosic substrates and are responsible for the mineralization of wood biomass in nature. Enzymes of wood-degrading fungi, particularly those secreted during lignocellulose degradation, such as laccases, manganese-dependent (or independent) peroxidases, cellulases and xylanases, are of considerable interest for biotechnological applications [4–9]. Up to now all purified enzymes have been glycoproteins, many of which are secreted in multiple isoforms (*e.g.* [10]). Interest in fungal proteins has been reflected in the past few years by the increasing number of fungal genome-sequencing projects (http://www.jgi.doe.gov/; http://www.broad.mit.edu/). Actually, only a few genome annotations have been released or are in a final stage for basidiomycetes, *e.g. Coprinopsis cinerea*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Schizophyllum commune* and *Heterobasidion annosum*. Proteomic studies using high-resolution techniques for protein separation and mass spectrometric identification of single proteins are of great value for investigating fungal degradation processes and biotechnological protein production [11, 12]. The degradation of complex lignocellulosic substrates is, however, not

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Abbreviations: BCA, bicinchoninic acid assay; **BSM,** basal medium; **EP,** extracellular polysaccharide; **LMWM,** low-molecular-weight metabolites; **MW,** molecular weight; **MWCO,** molecular weight cut-off; **PhMe,** phenol/methanol; **RuBP,** ruthenium(II) Tris(bathophenantroline disulfate)

only related to extracellular enzymes but is also enhanced and regulated by low-molecular-weight metabolites (LMWM) secreted by the fungus [10]. In addition, numerous metabolites are secreted to fend off competitive organisms, for signaling, as virulence factors in pathogenic processes and to influence and regulate the fungal environment [13, 14]. The extracellular metabolites cover a wide range of chemical compound classes such as low-molecular organic acids [15], fatty acids [16], phenols, quinones and other aromatic compounds [13, 17, 18]. As a result of degradation processes, the complexity of extracellular compounds with low molecular weight (MW) actually increases and many of the LMWM undergo chemical modifications during fungal growth [19, 20].

In addition to LMWM, fungi produce miscellaneous extracellular polysaccharides (EPs) with a broad MW range extending from approximately 10 to 500 kDa including soluble glycans and cross-linked EPs associated to the mycelia [21–23]. This mucilagous material is mainly distributed around the fungal hyphae [24] forming a so-called hyphal sheath. The role of these EPs has been described as a means of immobilizing extracellular enzymes, adhering the fungus to its solid substrates, providing protection from toxic compounds, preventing dehydration of the mycelium and enhancing carbon storage (*e.g.* [25–27]). The storage of carbon is especially evident in growing cultures where the content of soluble EPs is particularly high during the early exponential phase [28]. Amounts of soluble polysaccharides depend on the fungal strain and culture conditions [29] and their production appears to be typical for lignocellulose-degrading fungi [30]. The glycan structure is usually branched and is composed of glucose, galactose, mannose, xylose and glucosamine as an aminosugar [31, 32]. In addition, polysaccharides can be modified by methylation and esterification or linked to lipids and small peptides [21, 33–35].

In order to analyze fungal secretomes with 2-DE, very diluted protein solutions must be concentrated and separated from large amounts of other metabolites. It is very difficult to actually obtain 2-D gels from secretomes of higher basidiomycetes with a resolution sufficient for spot analysis. The aforementioned compounds can precipitate together with proteins and totally impede 2-DE. In studies on extracellular proteins of basidiomycetes attempts have been made to overcome these problems by ultrafiltration or dialyses prior to sample concentration [36–39]; in spite of such attempts the hitherto existing sample preparation methods are still not applicable for a wide range of fungal secretomes.

In this study we have developed an optimized protocol for the precipitation of extracellular proteins from liquid cultures of *C. cinerea*. Our protocol makes it possible to concentrate proteins from the culture supernatant and simultaneously remove the compounds impeding the process from the sample. We have also applied this protocol successfully to other higher basidiomycetes and obtained satisfactory 2-D gels of fungal secretomes in a relatively expedient way.

2 Materials and Methods

2.1 Chemicals

Yeast extract was obtained from Serva Electrophoresis (Heidelberg, Germany) and agar was supplied by Oxoid (Hampshire, UK). All other chemicals were of p.A. quality unless otherwise noted.

2.2 Fungal cultures

P. ostreatus var. *florida* PC9 (obtained from Antonio G. Pisabarro, Universidad Pública de Navarra, Pamplona, Spain), *Ph. chrysosporium* strain RP78 (obtained from Dan Cullen, USDA Forest Products Lab, Madison, USA) and *Polyporus brumalis* CBS 470.72 were grown on BSM (basal medium, [40]). Fungi were preserved on BSM with 1.5% agar at 25°C and 50 mL liquid BSM precultures were inoculated with three 10 mm diameter agar pieces and cultivated stationary for 6 days. Liquid cultures (50 mL) for use in experiments were inoculated with 5 mL of the homogenized liquid precultures (1 min at 8000/min, Ultraturrax T25, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany). *P. ostreatus*, *Ph. chrysosporium* and *Po. brumalis* were grown in stationary cultures at 25°C. Cultures of *P. ostreatus* were harvested after 6 days of growth whereas *Ph. chrysosporium* and *Po. brumalis* were cultivated for 15 days. *S. commune* 4–39 (CBS 341.81) was grown in stationary liquid cultures at 25°C in minimal medium for 15 days [41]. *C. cinerea* Okayama7 ♯130 (*Coprinus* research community, FGSC ♯9003) precultures were grown on a medium according to Kjalke *et al.* [42] with modifications: glucose 20 g, yeast extract 10 g, $CaCl_2 \times 2H_2O$ 0.5 g, KH_2PO_4 2 g, $MgSO_4 \times 7H_2O$ 0.05 g and agar 15 g/L. Three pieces of the 6-day-old agar culture with diameters of 10 mm each were used for inoculation of 100 mL liquid precultures (the same medium without agar). After 3 days of growth, precultures were homogenized and 5 mL aliquots were used as an inoculum for the experimental cultures (100 mL). All liquid cultures of *C. cinerea* were grown with continual shaking (120 rpm/min) at 37°C for 3 days.

2.3 Protein precipitation

The culture supernatants were collected by vacuum filtration with Whatman filter paper No 1 (⌀ 90 mm, Schleicher & Schuell, München, Germany) and stored on ice or at −20°C until used. Supernatant proteins were precipitated by applying several different methods.

2.3.1 TCA/acetone precipitation

TCA/acetone precipitation was performed according to Damerval *et al.* [43] with some modifications. An aliquot of the culture supernatant was mixed with five volumes of ice-cold TCA/acetone (20%/80% w/v) and incubated overnight

at −20°C. Subsequently, precipitated proteins were separated by centrifugation at 4000 × *g* for 20 min at 4°C. The pellet was washed with ice-cold acetone until the washing solution displayed an almost neutral pH (usually three to four times), then air dried and finally stored at −20°C.

2.3.2 Phenol/methanol precipitation

Phenol/methanol (PhMe) precipitation was performed by the modified method of Hurkman and Tanaka [44]. Tris-saturated phenol was prepared through the extraction of 90% liquid phenol (Fluka, Munich, Germany) with an equal volume of 0.5 M Tris-buffer, pH 8.0. The phenol phase was separated and extracted once again with 0.05 M Tris-buffer, pH 8.0. The final pH value of saturated phenol was approximately 8.0. Saturated phenol was stored at 4°C overlaid with 50 mM Tris buffer, pH 8.0. For sample preparation, 3.3 g sucrose, 100 mg DTT and 1 mL extraction buffer (0.5 M Tris buffer, pH 8.5, 50 mM EDTA and 1 M KCl) were added to 10 mL of the culture supernatant and mixed until dissolved. Supernatant proteins were extracted by adding 10 mL saturated phenol for 20 min at room temperature while shaking. Phenol phase separated by centrifugation for 5 min at 1700 × *g* and 4°C was mixed with five volumes of ice-cold methanol/ammonium acetate (100 mM). Proteins were precipitated overnight at −20°C and collected by centrifugation at 1700 × *g* at 4°C for 20 min. The protein precipitate was resuspended in ice-cold acetone containing 0.2% DTT, incubated for 1 h at −20°C and collected again at 1700 × *g* for 5 min (4°C). The washing step was repeated until the pH of the washing solution become neutral. The pellet was air dried and stored at −20°C.

2.3.3 Optimized precipitation protocol

The culture supernatant was frozen and stored at −20°C for at least a few hours. After thawing, supernatant samples were centrifuged at 48 400 × *g* and 4°C for 30 min to separate precipitated polysaccharides. Supernatants with low protein content were concentrated 10- to 20-fold by freeze-drying. Protein precipitation was performed by adding 10% v/v TCA solution from a 100% TCA stock solution (100 g TCA in 45.4 mL water) [45]. After mixing, samples were incubated on ice overnight and subsequently centrifuged at 1700 × *g* and 4°C for 15 min. Excess TCA was removed from protein precipitate through subsequent washings with a few milliliter of ice-cold 20% Tris-buffer (50 mM, pH 7.5) in acetone v/v. In most instances, three washing steps were required to remove TCA from the protein pellets. Finally, protein samples were washed with pure, ice-cold acetone, air-dried and then stored at −20°C for further processing.

2.3.4 Other precipitation methods

Precipitation with solid TCA was performed by adding 10% TCA w/v to the culture supernatants. After overnight incubation samples were centrifuged and washed as described above for TCA/acetone precipitation. Chloroform/methanol precipitation was performed according to Wessel and Flügge [46] and ethanol precipitation according to Zellner *et al.* [47]. For protein precipitation with acetone or ACN, ice-cold solvents and culture supernatant were mixed 4:1 v/v and centrifuged after overnight incubation at −20°C. The pellet was washed once with ice-cold acetone and air-dried. Protein concentration (20-fold) by ultrafiltration was performed with a 10 kDa MW cut-off (MWCO) membrane (Vivaspin 2, 10 000 MWCO PES, Sartorius, Göttingen, Germany) following which samples were washed twice with ten volumes of water. For protein concentration with the 2-D-Clean-Up Kit (Amersham Biosciences) culture supernatants were first concentrated 10-fold by ultrafiltration without washing steps.

2.4 Protein separation by 2-DE

The protein samples were dissolved in the IEF sample buffer: urea 8 M (PlusOne, Amersham Biosciences, Freiburg, Germany), CHAPS 4% w/v, DTT 50 mM, Triton X100 5% v/v, IPG buffer (pH 3–10) 0.5% v/v (Amersham Biosciences), and traces of Bromphenol Blue, according to the Amersham Biosciences protocol. In experiments in which the total protein amount loaded *per* gel had to be determined, samples were dissolved in the IEF sample buffer without DTT, IPG buffer and Bromphenol Blue [48]. A small sample aliquot was diluted for protein measurement and the remaining sample was spiked with DTT, IPG buffer and Bromphenol Blue to the final concentration mentioned above. For maximal protein recovery, the samples were stirred with a magnetic stirrer for up to 2 h and sonicated [49] at a temperature <25°C. If necessary, the pH of the dissolved samples was adjusted using 1.5 M Tris-HCl, pH 8.8 and after centrifugation the samples were loaded on IPG strips (100 μg total protein *per* 18 cm Immobiline DryStrip, pH 3–10) in ceramic strip holders (Amersham Biosciences). IPG strips were rehydrated for 12 h at 20 V and 20°C (Ettan IPGphor, Amersham Biosciences). IEF was performed under the following conditions: 150 V for 1 h, 1 h at 200 V, 1 h at 500 V, 1000 Vh at 1000 V, followed by 2.5 h gradient from 1000 to 8000 V and finally focused for 28 000 Vh at 8000 V. The maximal current *per* strip was set at 50 μA. The focused samples were stored at −20°C. Prior to separation in the second dimension, IEF strips were incubated twice for 10 min in 6 mL of the equilibration buffer (urea 6 M, SDS 1% w/v, glycerol 30% v/v, Tris Base 40 mM and traces of Bromphenol Blue) containing 10 mM DTT in the first step and 100 mM iodoacetamide in the second step, respectively. The separation in the second dimension was performed with 12% SDS-PAGE (25.5, 20.5 and 0.10 cm), at 3000 V (400 mA, 13 W) for 30 min followed by 3000 V (400 mA, 100 W) for 4–5 h using an Ettan DALTsix electrophoresis unit (Amersham Biosciences). The MW marker for the second dimension was obtained from Fermentas (♯SM0431,

St. Leon-Rot, Germany). The gels were stained with a RuBP (ruthenium(II) Tris(bathophenantroline disulfate)) [50] according to the protocol described by Lamanda *et al.* [51]. Finally, gels were scanned using an FLA-5100 fluorescence reader (Fujifilm, Düsseldorf, Germany) with 50 μm resolution. Sixteen-bit reader files were converted to TIF-format using an AIDA image analyzer (v4.10.020, Raytest, Straubenhardt, Germany).

2.5 Determination of protein concentration

2.5.1 Standard assays

The total protein concentration was measured by three different methods: the Bradford assay using a protein determination reagent from Pierce (Coomassie Plus, Thermo Scientific, Bonn, Germany), Lowry assay according to Peterson [52] and a bicinchoninic acid assay (BCA) obtained from Pierce (BCA-Protein-assay, Thermo Scientific, Bonn, Germany). Ultrapure BSA (GERBU Biochemicals, Gaiberg, Germany) was used as a calibration standard for all methods. Fresh samples from fungal cultures supernatants were centrifuged for 10 min at 13 000 × *g* before processing. Concentrated protein samples dissolved in electrophoresis buffers were diluted with pure water to fit the measurement range and to reduce the concentrations of buffer components below the interfering limits for protein assays.

2.5.2 SDS-PAGE assay

Dilutions of the protein sample in a sample buffer (60 mM Tris Base, pH 6.8, 2% SDS, 10% glycerol and traces of Bromphenol Blue) were separated by 12% SDS-PAGE (Multilong, Biometra, Göttingen, Germany) at 20 mA for approximately 3 h. The calibration curve for the determination of the protein amount was obtained by applying BSA standards (2, 1, 0.4 and 0.2 μg of protein *per* lane) separated simultaneously with the samples on the same gel. An AIDA image analyzer was used for gel processing and calculating total protein. The following parameters were used: baseline determination at search level 1, smooth parameter 10 pixel and search level 0 for peak determination (not smooth). Integration parameters for BSA bands were set manually to prevent any distortion of the protein measurements by BSA contaminations. The resulting calibration curve ($R^2 = 0.9851$) was used for the calculation of total protein in the fungal samples.

3 Results

3.1 Determination of the protein concentration in culture liquid

All fungal strains tested were grown in media commonly used for physiological experiments in either stationary or shaking liquid cultures and harvested during the exponential growth phase. *C. cinerea*, as well as other basidiomycetes cultivated under laboratory conditions secretes proteins in low concentrations into the culture media: typically 5–50 μg/mL. Performing the 2-DE of fungal secretomes we recognized that loading the gels with protein amounts assayed by commonly used methods very often resulted in an unexpectedly low abundance of protein spots; this effect was never observed when analyzing fungal intracellular proteins. Measurement of the total protein amount in native culture supernatant of *C. cinerea* varied dramatically depending on the method used: protein amount assayed using the Bradford method was 42.9 ± 2.37 μg/mL and varied from the protein amounts measured by the Lowry and BCA assays which detected 738.9 ± 51.3 and 1701.7 ± 253.3 μg/mL, respectively. No total protein was measured in control samples of the culture media without fungus. To provide an alternative measurement, we used the SDS-PAGE separation of proteins with RuBP staining and a densitometric quantification of proteins as a more significant method for 2-DE approaches. Culture supernatants were concentrated 10-fold by ultrafiltration using a 3 kDa MWCO membrane and analyzed simultaneously with BSA standards on SDS-PAGE. Densitometric quantification revealed a protein concentration in the native supernatants of 36.9 ± 2.48 μg/mL and indicated that, in all likelihood, only the Bradford assay which does not lead to a profound overestimation of the protein amount. The values measured by the SDS-PAGE approach were lower than protein amounts assayed by other methods, nevertheless, they could also be too high due to a background caused by interfering fungal metabolites.

Determination of total protein amount in samples precipitated with the optimized protocol performed by Lowry, BCA and Bradford assays revealed 47.4 ± 0.3, 45.4 ± 6.6 and 91.2 ± 62.5 μg/mL, respectively. The values obtained from Lowry and BCA assays were more than two times greater compared with total protein determination by SDS-PAGE (19.9 ± 1.1 μg/mL); the Bradford assay did not provide reproducible results for these samples even after several repetitions. For better compatibility we used the SDS-PAGE values for calculating the protein amount used in the 2-DE experiments. We observed comparable problems in protein determination in native culture supernatants and samples prepared for 2-DE from culture supernatants of other basidiomycetous fungi. No protein was assayed in control samples prepared from the culture media not inoculated with fungi.

3.2 Sample concentration

Preliminary experiments indicated that standard methods originally developed for concentration of intracellular proteins cannot be easily transferred to secretomes of basidiomycetous fungi. We examined those protocols for samples from the genetic model organism *C. cinerea* and

found that severe limitations began to manifest themselves even during the dissolution of protein precipitates. Solubilization of pellets was time-consuming and failed to recover proteins completely in both SDS-PAGE and IEF sample buffers. Proteins precipitated in the presence of TCA were particularly difficult to dissolve. The addition of NaOH, as suggested by Nandakumar *et al.* [53], or the sonication of the sample [49] and subsequent stirring over a period of few hours did not result in complete dissolution of the pellets. Thorough washing of protein pellets with acetone did not completely remove TCA, Tris-buffer was still needed to adjust the pH of the samples dissolved in electrophoresis buffers. Concentration of proteins by ultrafiltration with a 10 kDa MWCO membrane was difficult for the culture supernatant tested when performed on large volumes of liquid. The ultrafiltration membrane was rapidly blocked during sample concentration probably by soluble EPs, making sample concentration very time-consuming.

Comparison of preparation methods through application of SDS-PAGE using a secretome sample from *C. cinerea* revealed significant differences in protein patterns (Fig. 1). The commonly used methods with TCA/acetone and chloroform/methanol (Fig. 1: Lanes 4 and 9, respectively) revealed no smearing in SDS-PAGE separation at the low protein concentration used in this experiment. In the trials in which organic solvents were used to precipitate proteins, the performance of ethanol was the poorest; a loss of proteins was observed with all three methods (Fig. 1: Lanes 5–7). Sample concentration by ultrafiltration shows strong losses of proteins and even an absence of some protein bands (Fig. 1: Lane 10). The 2-D Clean-Up Kit (Fig. 1: Lane 11) performed better than TCA/acetone and chloroform/methanol did. Good protein concentration was also obtained with the PhMe precipitation method (Fig. 1: Lane 8). High-speed centrifugation of the culture supernatant at 48 000 × *g* and precipitation of proteins by TCA without acetone followed by washing of the protein precipitate with Tris/acetone (optimized protocol) resulted in smaller amounts of precipitate as compared with the TCA/acetone method and performed very well in a follow-up SDS-PAGE examination (Fig. 1: Lane 1). Removal of the mucilaginous material prior to sample precipitation with lower centrifugal force (1700 × *g*) revealed slight smearing (Fig. 1: Lane 2) as compared with high-speed centrifugation. A higher background was also observed when the proteins were precipitated with solid TCA (Fig. 1: Lane 3) instead of TCA stock solution. No significant differences in protein patterns were observed in these three experiments (Fig. 1: Lanes 1–3) when tested by densitometry with background subtraction.

Quantification of proteins in the 1-D gel by densitometry revealed that Lanes 1–3 and 8 (Fig. 1) showed comparable quality and data were assigned as 100%. Standard TCA/acetone and chloroform/methanol protocols only recovered 60 and 54% of the total protein, respectively. Precipitation of proteins using acetone, ACN and ethanol resulted in considerable protein losses: only 30, 60 and 8% proteins were recovered, respectively. Ultrafiltration using

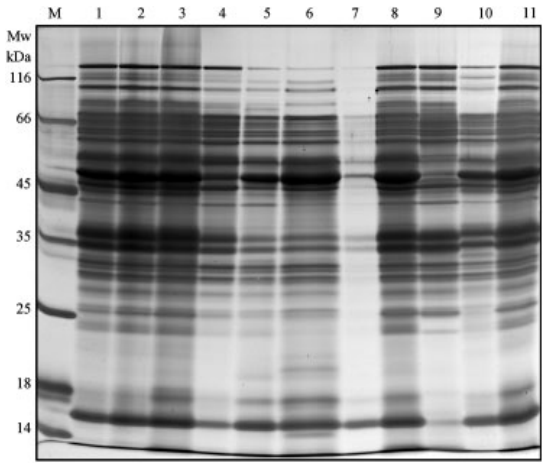

Figure 1. SDS-PAGE of supernatant proteins from 3 days cultures of *C. cinerea* concentrated by various methods. Lane 1 represents the sample obtained by the optimized precipitation protocol with supernatant centrifuged at 48 000 × *g* and precipitated with TCA stock solution; Lane 2 as sample 1 but the supernatant was centrifuged at 1700 × *g* prior to the precipitation; Lane 3 as sample 1 but supernatant proteins were precipitated with solid TCA. Further samples were precipitated with TCA/acetone (4), acetone (5), ACN (6), ethanol (7), PhMe (8) and chloroform/methanol (9); Lane 10 – proteins concentrated by ultrafiltration with 10 kDa MWCO membrane (Vivaspin 2, Sartorius) and Lane 11 – sample obtained by the 2-D Clean-Up Kit (Amersham Biosciences). All samples originated from 250 μL of culture supernatant which is corresponding to about 5 μg of total protein. Lane M corresponds to the protein marker (SM0431, Fermentas). Gel was stained with RuBP.

10 kDa MWCO membrane has so far been successfully applied to separate proteins by SDS-PAGE but only 35% of proteins were recovered from the culture supernatant of *C. cinerea* by this protocol. The Clean-Up Kit (Amersham Biosciences) for protein precipitation for 2-DE performed well in SDS-PAGE examination, although only 68% of the proteins were recovered.

3.3 Examination of the protocols by 2-DE

All of the above protocols were examined using 2-DE except for low-order ethanol precipitation and the Clean-Up Kit, which was not applicable for large sample volumes of fungal supernatants. Protein samples used in all comparisons came from the same sample volumes corresponding to 100 μg total protein obtained by the optimized protocol. IEF in the first dimension was performed with 18 cm Immobiline DryStrip-gels (pH 3–10) using the protocol recommended by the manufacturer. In samples prepared by standard TCA/acetone protocol, the presence of TCA-anions (not removable even by extensive acetone washings) resulted in an excess current during IEF, slowing down the focusing program. Upon completion of the focusing, the final current

reached approximately 40 μA and remained constant even when focusing time was prolonged. Surprisingly, protein samples prepared by methods without TCA caused problems by IEF as well and the final current of those samples was in the range 30–40 μA. Closer inspection of the IEF-gel strips revealed dramatic shrinkage of the gels within the final 3–4 cm of the acidic ends. It was not possible to overcome this problem through the use of additional filter paper pads soaked with IEF-buffer and placed between gel and electrodes or through application of samples using a cup-loading method (Ettan IPGphor cup loading strip holder, Amersham Biosciences).

Problems observed in the IEF finally manifested themselves in the 2-DE of samples prepared by the five standard methods tested. TCA/acetone precipitation has often been reported for samples with low protein concentrations. This is typical for most of the fungal culture supernatants. Unfortunately, samples prepared by TCA/acetone resulted in unfocused proteins with strong horizontal smearing (Fig. 2A). It was only possible to overcome this effect by reducing the sample amount far below 40 μg of protein loaded *per* gel at the expense of less-abundant proteins. We made various other attempts to eliminate inhibiting substances prior to sample precipitation by this very commonly used method. In total 10% of ethanol or acetic acid was added to the sample supernatants to remove any excess of fungal EPs. After overnight incubation at 0°C, precipitated substances were separated by centrifugation and supernatant proteins were precipitated by TCA/acetone as described above. Although both pre-treatments resulted in appreciable amounts of precipitates in the first centrifugation step, the final 2-D gels still proved to be very unsatisfactory (gels not shown).

Low recovery of proteins precipitated by acetone was also manifested in the 2-DE. This preparation method only recovered approximately 30% of the proteins (about 30 μg loaded *per* gel), as indicated by SDS-PAGE; therefore, the focusing of proteins was satisfactory (Fig. 2B). This separation was not achieved using higher sample amounts (data not shown). Proteins precipitated by ACN (about 60 μg protein loaded *per* gel) were less focused compared with the sample from acetone precipitation and, despite the low level of recovery, resulted in the best separation among the standard protocols tested (Fig. 2C). PhMe precipitation is certainly one of the most laborious methods and is, in addition, difficult to handle for large sample volumes. Concentration of proteins by freeze-drying and redissolving in water was still necessary prior to extraction; nevertheless, this proved to be the best of all standard methods tested with respect to protein recovery (Fig. 2D). *C. cinerea* secretome prepared by ultrafiltration with a 10 kDa MWCO membrane failed completely in attempts to separate proteins by 2-DE (Fig. 2E).

We found that precipitation of proteins with TCA and without acetone results in good protein recovery and in lower coprecipitation of non-proteinaceous metabolites visible as smaller volume of precipitate. For effective removal of TCA ions hampering IEF, we applied a washing solution of ice-cold 20% Tris-buffer (50 mM, pH 7.5) in acetone v/v. Still, protein pellets obtained from samples precipitated with solid TCA were difficult to dissolve and protein spots in the 2-DE were not sharp with horizontal smearing on 2-D gels and vertical streaks for abundant proteins were also observed (Fig. 3A). These problems were minimized by precipitation using TCA-stock solution (Fig. 3B). In contrast to solid TCA precipitation and other protocols tested, only a few seconds of sonication were sufficient to dissolve protein samples in the IEF sample buffer. The Tris/acetone washing step was not efficient if applied to samples precipitated by the TCA/acetone method.

One of the most important steps in the preparation of fungal secretome samples was removing as much of the gelatinous material as possible from the culture supernatant. In comparison to samples prepared at a lower level of centrifugal force of $1700 \times g$ – normal for table centrifuges – centrifugation of supernatant samples at about $48\,000 \times g$ for at least 30 min resulted in a much better separation of proteins in 2-DE without vertical tailing and with a clear background, retaining the good protein recovery compared with data shown in Fig. 3B and C. The aforementioned step was even more effective when samples were frozen and then thawed. The separation achieved was far greater than obtained by the standard protocols. A comparison of single spots in 2-DE obtained by all methods tested and optimized protocol using a 2-D-evaluation software was not possible due to the poor resolution of the gels and low protein yields in samples prepared by standard protocols.

Problems observed in examination of the methods were caused only by fungal metabolites and were not generated by the culture medium. The aforementioned problems in sample preparation were minimized in cultures growing in the late starvation stage. Control samples prepared from the culture medium alone indicated no problems in the IEF even if prepared by standard protocols, *e.g.* TCA/acetone precipitation. In addition, the medium control samples spiked with protein standards resulted in a very distinct separation of proteins in 2-DE. In no cases were protein spots originating from culture media components (*e.g.* yeast extract) observed in 2-D gels.

3.4 IEF

We also attempted to improve protein separation from samples prepared using the optimized precipitation protocol by optimization of the focusing step. Further dramatic improvements were not achieved even through an extensive modification of the IEF program. The optimal IEF-program – slightly different from the standard program recommended for Immobiline DryStrips – is described in Section 2. Contradictory to our experience with fungal intracellular proteins, IEF of secretome of *C. cinerea* (and also other fungi) failed to remove any excess of mobile ions in the low-voltage focusing steps. Regardless of the focusing

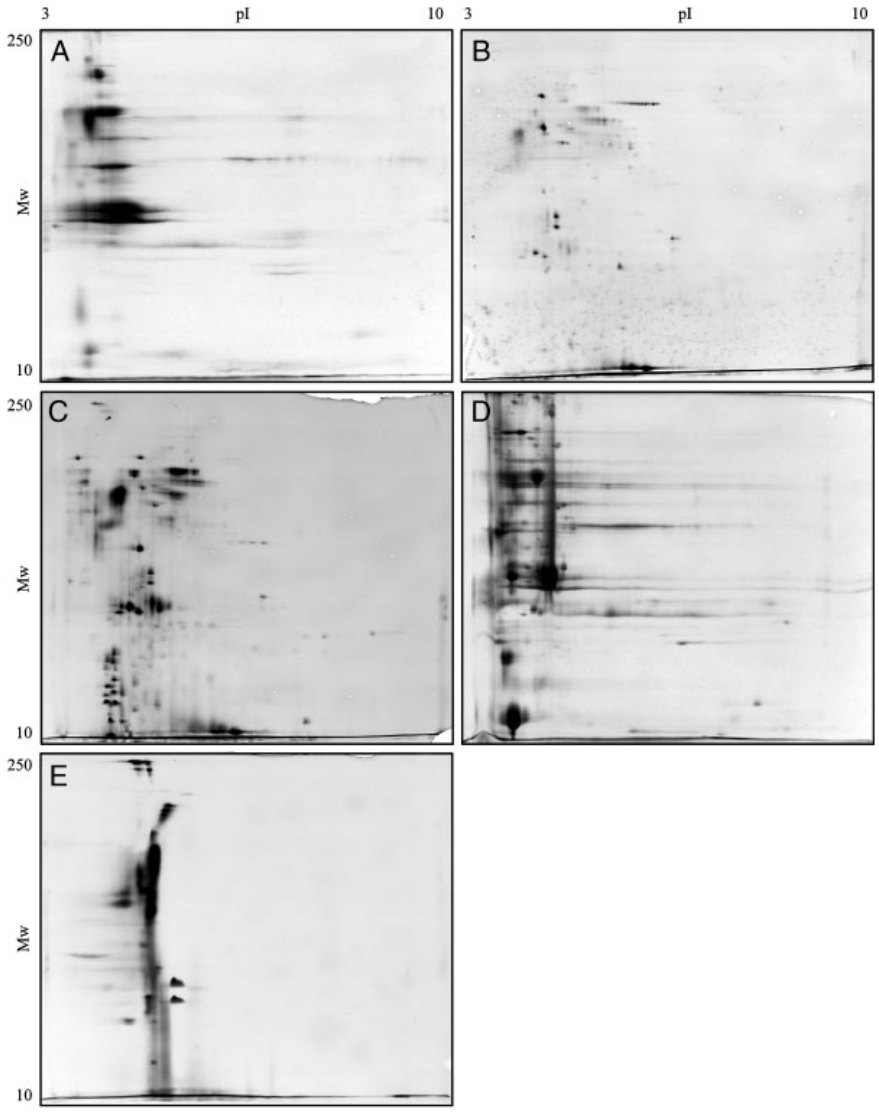

Figure 2. 2-DE of supernatant proteins from 3 days shaking cultures of *C. cinerea* concentrated using various standard precipitation methods: A – TCA/acetone, B – acetone, C – ACN, D – PhMe and E – 10 kDa MWCO membrane. Proteins were separated on 18 cm Immobiline DryStrip pH 3–10 (Amersham Biosciences) in the first dimension and on a 12% SDS-PAGE in the second dimension; Gels were stained with RuBP. Samples of proteins in each gel were obtained from equal volumes of the culture supernatant.

program, the final current at 8000 V used in the final focusing step remained constant at approximately 30 μA *per* strip. Therefore, it was of utmost importance to keep the focusing time as short as possible but long enough for sufficient protein focusing; extending the total focusing time resulted in lower resolution of acidic proteins. This effect was caused by gel shrinking in the acidic part of the IEF strips following prolonged focusing time. The gel shrinking could not be prevented by addition of ethylene glycol to the IEF buffer. Ultrafiltration of the samples dissolved in the IEF buffer using a 3 kDa MWCO membrane followed by resuspending the concentrated sample (10 ×) in a fresh volume of the IEF buffer did not remove the ions responsible for the high current. Passive or onside rehydration and cup-loading of samples failed to further improve the resolution as well. Also ineffective was the attempted reduction of protein with 2-mercaptoethanol or (Tris(2-carboxyethyl)-phosphine hydrochloride) and alkylation with 2-vinylpyridine [54], application of the DeStreak kit (Amersham Biosciences) as well as changing concentration of detergents in the IEF-buffer. Application of thiourea, which is useful for dissolving hydrophobic and high MW proteins, was disadvantageous since it resulted in a streaking phenomenon in the acidic region of the gels, thus confirming previous observations [55, 56].

Extended examination of IEF parameters, including the use of the aforementioned approaches in combination, did not result in a significant improvement, indicating that the sample preparation prior to the IEF step plays a crucial role by in the 2-DE of *C. cinerea* secretome. The optimal amount

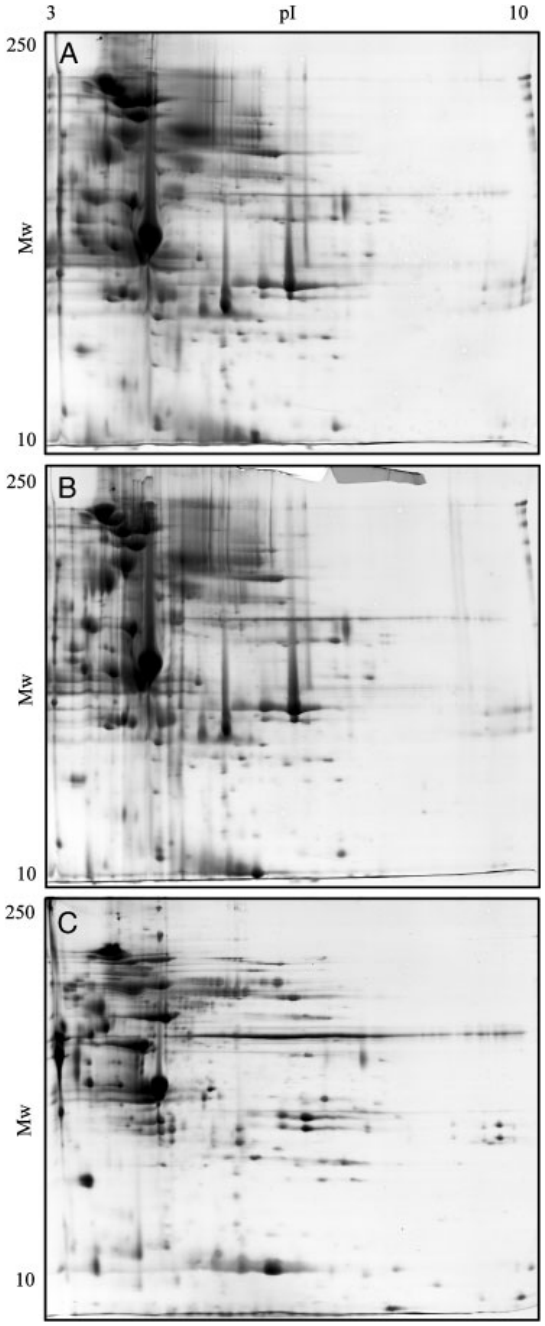

Figure 3. 2-DE of supernatant proteins from 3 days shaking cultures of *C. cinerea* concentrated using modified TCA-based precipitation methods; A – supernatant was centrifuged at 1700 × *g* and proteins precipitated with solid TCA; B – supernatant was centrifuged at 1700 × *g* and proteins precipitated with TCA stock solution and C – the optimized protocol (Supernatant was centrifuged at 48 000 × *g* prior to precipitation and proteins precipitated with TCA stock solution.); for separation conditions see Fig. 2. All gels were loaded with proteins obtained from equal volumes of the culture supernatant as in Fig. 2 and corresponded to 100 μg protein *per* each gel.

of proteins analyzed by 2-DE without strong discrimination of the low-abundant proteins or reducing the separation quality was 50–100 μg *per* 11 or 18 cm Immobiline DryStrip and 1 mm SDS-PAGE-gel.

3.5 Application of the optimized protocol to secretomes of other basidiomycetes

Among the higher basidiomycetes that we studied, the *C. cinerea* caused the most severe problems in 2-DE. It was, however, also difficult to obtain well-resolved protein spots in 2-DE using the standard protocols for secretomes from other fungi. We were able to successfully apply the optimized protocol developed for *C. cinerea* for 2-D-separation of secretomes from *Po. brumalis*, *S. commune*, *Ph. chrysosporium* and *P. ostreatus* (Fig. 4A, B, C and D, respectively) growing in liquid cultures and different culture media.

4 Discussion

Higher basidiomycetes inhabiting wood secrete numerous enzymes essential for the degradation of complex polymeric substrates. The composition of extracellular enzymes depends strongly on the substrate and on the stage of growth. It is most complex during the exponential growth phase. Proteomic studies of extracellular fungal enzymes can, however, be very challenging. The first problem is the initial quantification of the total amount of protein as commonly performed in experiments with extracellular proteins. Conventional methods such as Bradford, Lowry and BCA assays have been unreliable for fresh supernatants of *C. cinerea*, even after protein precipitation for 2-DE. An excessive overestimation of protein amounts has been observed, particularly for the BCA and Lowry assays. Both methods are based on the principle of Cu^{2+} reduction and formation of color complexes. There is, however, a problem involved since fungal metabolites that are formed extracellularly or secreted by fungi [31, 32] possibly reduce Cu^{2+} and thus give a false-positive response in these assays. Polyphenolic compounds and phenolic acids have been shown to interfere with the Bradford assay [57, 58]. The overestimation of protein content from the precipitated samples could, nevertheless, be caused by concentrated fungal phenolic compounds [59] co-precipitated with the extracellular proteins. In addition, the Bradford assay was barely reproducible for these samples. This effect could have resulted from irreproducible microprecipitation of the CBB dye during measurement and was typical for *C. cinerea* samples; this effect has not been observed for samples from other fungi tested. Colorimetric assays for protein determination also depend on protein glycosylation and hydrophobicity and are strongly influenced by IEF-buffer components [60–62]. Due to the lack of proper standards they do not assay the absolute protein amount. Similar

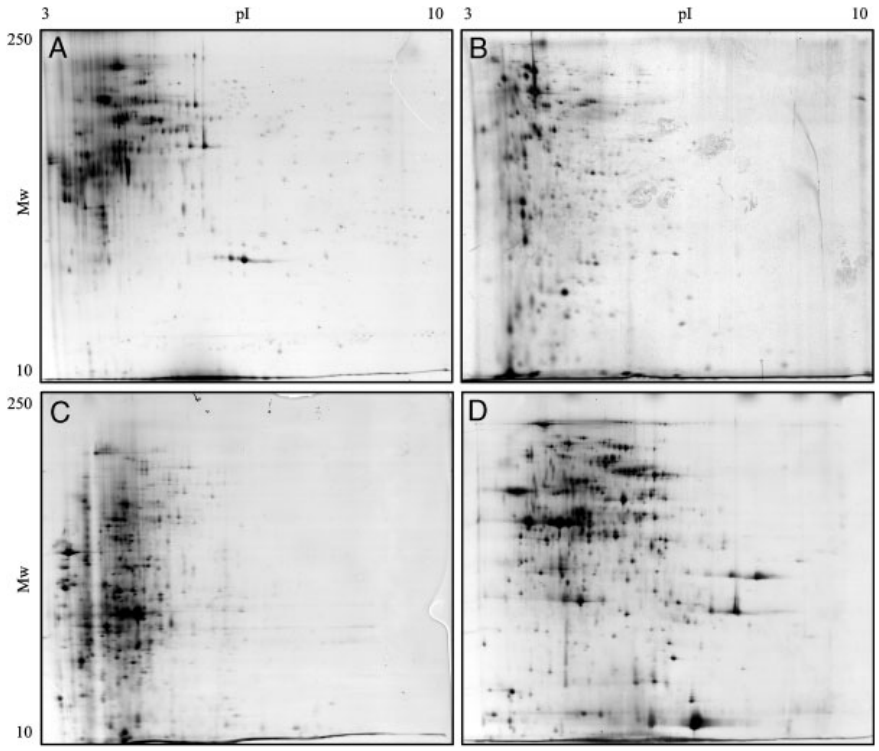

Figure 4. 2-DE of supernatant proteins from 15 days stationary cultures of *Po. brumalis* (A), and *S. commune* (B), 6 days stationary cultures of *Ph. chrysosporium* (C) and *P. ostreatus* (D) obtained with the optimized protocol; for separation conditions see Fig. 2. About 100 μg of total protein (determined by SDS-PAGE after protein precipitation) were loaded on each gel.

effects have been reported for fungal culture liquids in which protein measurements varied by twofold [63] and even as much as fivefold for purified extracellular enzymes [64]. Quantification of proteins in fungal supernatants free of interference can be achieved through total amino acid analysis [65]; however, peptides linked to soluble polysaccharides, which are present in large amounts and not available for analysis by 2-DE [21, 66, 67], are also detected by this method. Gel-based protein quantification, which we performed with RuBP protein staining, provides a wide dynamic range and a linear dependence for the determination of the protein amount [51]. Though more laborious, this method of measurement was more reliable for *C. cinerea* secretome samples and displayed better applicability for gel-based protein separations.

Altering amounts of extracellular metabolites hampers protein analysis; moreover, the amounts of total proteins secreted by different fungi can vary on the order of two magnitudes for our samples. Single proteins are also secreted in great excess, thus hindering detection of less abundant proteins. In liquid cultures of wood-degrading basidiomycete *Ph. chrysosporium*, only 16–40 unique extracellular proteins have been identified [38, 36] and the corresponding 2-D gels show – as in our experiments – rather complex protein patterns. Clear spot separation commonly achievable for intracellular proteins from basidiomycetes or secretomes of ascomycetes [68–71] is difficult to realize because most extracellular fungal proteins are heavily glycosylated or modified and typically appear as fuzzy spot chains on 2-D gels. In addition, most protein spots focus during IEF within the acidic range on 2-D gels and the fungal secretome can become even more complex due to a turnover of the secreted proteins and a possible proteolytic degradation. Problems in IEF focusing of secretomes from higher basidiomycetes can impede the protein separation and the strong background in 2-D-gels appearing as vertical streaks can hamper the recognition of single spots. This effect manifests itself particularly in media containing readily available carbon sources such as glucose or cellulose and can be caused by the fungal conversion of simple sugars to carbon-storage polysaccharides [72]. Most of the methods tested resulted in large amounts of gelatinous – in some cases colored – pellets difficult to dissolve. This was a common difficulty in all samples examined making protein dissolution in electrophoretic buffers very laborious and incomplete. Differences in the yield of the protein recovery of the various protocols were quite apparent in the SDS-PAGE and 2-DE comparisons. Previous studies of secretomes from *P. sapidus* [37] and *Ph. chrysosporium* [38, 36] applied ultrafiltration using a 10 kD MWCO membrane followed by an extensive dialysis of samples. This approach has thus far been successful and has allowed protein separation by 2-DE; however, in our study it proved to be useless for samples from *C. cinerea*. For our samples ultrafiltration did not solve the problems in 2-DE and only 35% of proteins were

recovered from the culture supernatant. A preliminary analysis of the published genome of *C. cinerea* revealed a large number of low MW proteins with secretion signals (data not shown). The inefficiency of ultrafiltration was either caused by losses of small proteins or by adsorption of proteins to the concentrated gelatinous material. The Clean-Up Kit (Amersham Biosciences) for protein precipitation for 2-DE performed well for intracellular proteins from *Aspergillus fumigatus* [68], but it was less successful for samples of extracellular proteins used in this study. In the latter cases it recovered only 68% of the proteins in comparison to other protocols and, since this procedure was developed for small sample volumes, it is very cost-intensive when used for several milliliters of samples with low protein concentration. Among the standard methods tested, PhMe, which was developed for protein precipitation from plant material with high contents of polysaccharides and polyphenols [44], performed best and was useful for secretomes from cultures in stationary or starvation stages.

In the cultures tested, extracellular fungal metabolites seem to focus within the acidic range of the 2-D gel, as does the majority of extracellular proteins. It was not possible to solve this problem by cup-loading of samples within the alkaline gel range, which points to a mobility of these impeding substances in the electric field applied during IEF. We assume that acidic metabolites with high MW (possibly polysaccharides) in the sample may clog the IEF-gel or increase sample viscosity, thus interfering with the protein mobility and hampering efficient focusing. In addition, these compounds could cause an intensive background, which interfered with silver and CBB protein staining and hindered spot analysis. This effect was less pronounced with the RuBP staining method.

In studying secretomes of lignocellulose inhabiting fungi we were searching for a 2-DE method applicable to our samples. Unfortunately, no published protocol was successful. We tried combining a great variety of different approaches and tested their applicability for a wide range of fungal samples. Since all precipitation methods tested – except the optimized precipitation protocol – failed to separate proteins in the following 2-DE, it was not possible to perform either a quantitative or a qualitative comparison of single protein spots. The resulting protocol was not only the best but the only one applicable for *C. cinerea*. It was also successful for other fungal secretomes. As far as we can ascertain from our experience, the optimized protocol described in this study performed best at higher protein concentration and was not impeded by components of culture media such as yeast extract, malt extract, glucose or mineral salts. For this reason and to facilitate the handling of samples, fungal supernatants containing low amount of proteins can be concentrated up to 20-fold by freeze-drying before the precipitation step. The method presented performs well in routine use and is reproducible for a large number of samples. We believe that it can be applied in comparative proteomic studies of various physiological conditions of many higher basidiomycetes.

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The authors have declared no conflict of interest.

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3 The Free and the Cell Wall Secretomes in *Coprinopsis cinerea* Differ in Composition

3.1 Abstract

The fungal cell is surrounded by a complex glycan network forming the fungal cell wall. As an extracellular organelle, it provides contact to the close environment of the fungus and it interacts with the fungal substrate. Thus, the fungal secretome, including the freely secreted proteins, the hyphal sheath and the cell wall proteins (CWPs), can be expected to contain a huge number of proteins involved in cell wall formation and substrate degradation. In this study, a proteomic approach was performed on the secreted proteins of the model basidiomycete *Coprinopsis cinerea*, fractionated into the freely secreted proteins, proteins of the hyphal sheath, NaCl-extractable CWPs, SDS-extractable CWPs, NaOH-extractable CWPs and non-extractable CWPs. Separation of the fractionated proteins by two-dimensional gel-electrophoresis (2-DE) and further identification of single highly abundant spots revealed a significant difference between the proteins secreted into the culture supernatant and the proteins being attached to the cell wall.

To identify as many proteins as possible from the fractionated secretome we used a 1-DE shotgun approach. From the five fractions analyzed in total, we identified 162 proteins with overlappings between different fractions: 41 proteins were freely secreted, 61 hyphal sheath proteins and 59, 50 and 6 NaCl-, SDS- and NaOH-extractable cell wall proteins, respectively. The free secretome and the hyphal sheath shared the highest number of proteins; in contrast, there was little overlapping with proteins from the different cell wall fractions. Identified proteins from the free secretome included

mainly glycoside hydrolases and peptidases as well as several oxidases such as various FAD/FMN-containing oxidoreductases, reductases and a copper radical oxidase. Within the cell wall fractions, proteins homologues to cell wall constructing enzymes of the yeasts *Saccharomyces cerevisiae* and *Candida albicans* were detected. Experimental and computational approaches on the fungal genome identified no typical Pir proteins, as found in *S. cerevisiae*, in *C. cinerea*. The experimental approach for detection of GPI-anchored proteins revealed three different proteins only one of them with a putative GPI-anchor site, while computational analysis of the genome from *C. cinerea* detected 652 proteins with putative GPI-anchor sites and five beneath the 162 proteins identified in this experiment.

3.2 Introduction

The fungal cell wall is a complex and dynamic network consisting mainly of glycans and glycoproteins (Bowman & Free, 2006). Providing not only the shape of the cell but also protecting the cell from osmotic and physical stresses, the fungal cell wall presents a protection shield against environmental threats (Klis et al., 2002). The dense network of the cell wall compounds constitutes a barrier against molecules harmful to the fungal cell (Klis et al., 2006). Moreover, the cell wall acts as a tool for penetrating substrates and as a scaffold required entering the hosts intracellular space.

The main component of the fungal cell wall is β-1,3-glucan, making up almost 50% of the wall dry weight (Klis et al., 2006). The non-reducing end of β-1,3-glucan is linked to β-1,6-glucans (Kollár et al., 1997) that comprise about 5% of the cell wall dry weight in *Saccharomyces cerevisiae* (Kapteyn et al., 1999b). Another minor component of the cell wall polysaccharides is chitin making up 1-2% of the dry weight (Kapteyn et al., 1999b). Polysaccharides - as the main structural components of the cell envelope - enclose various glycoproteins (De Nobel et al., 1990; Zlotnik et al., 1984). Studies of the fungal cell wall structure were mainly done for model organisms such as the ascomycetous yeasts *S. cerevisiae* and *Candida albicans* (Chaffin et al., 1998; Lesage & Bussey, 2006). The cell walls of basidiomycetes are so far less studied, though morphological studies were done already several decades ago (Bottom & Siehr, 1979, 1980; Schaefer, 1977; Wessels & Sietsma, 1979).

Fungi produce also extracellular glycans released into its enviroment or aggregated

with the cell wall to a so called hyphal sheath (Bes et al., 1987). The composition of this extracellular mucilagous layer depends on the fungal species but the main component is β-1,3-glucan branched with β-1,6-glucans (Gutiérrez et al., 1995) as it is found in the cell wall itself. The hyphal sheath is distributed around the fungal hyphae (Gutiérrez et al., 1995) and covalently linked to the cell wall glucans and chitin (Sietsma & Wessels, 1981). Though the role of the hyphal sheath is not completely understood, various functions have been proposed in previous studies. The hyphal sheath can act as an additional protective layer against environmental threats such as toxic molecules or radicals (Vesentini et al., 2007). Storing of nutrients in the form of extracellular glucans, used by the fungus under starvation conditions, was shown by Stahmann et al. (1992). As the hyphal sheath forms the contact between the fungus and its substrate, it could also act as a reaction space for extracellular degradation enzymes (Gutiérrez et al., 1995).

Proteins, such as hydrophobins and agglutinins, are well known to be located in the fungal cell wall (Bowman & Free, 2006), but also numerous enzymes are directly attached to the fungal cell wall. Oxidoreductases, phosphatases, glucosidases, chitinases, and chitin synthases were already detected in many fungal species by means such as specific enzyme tests and electron microscopy [for an extensive review see (Rast et al., 2003)]. Attachment of the proteins to the cell wall polysaccharides can occur by non-covalent or covalent linkages (Klis et al., 2001). Cell wall proteins (CWP) extractable by sodium-dodecyl sulfate (SDS) or salts are associated with the cell wall components by hydrogen bonds, van der Waals or electrostatic forces, or by reducible disulphide bridges (Pitarch et al., 2002). Other covalently bound CWPs are linked either by a glycophosphatidylinositol (GPI) anchor at the C-terminal end of the protein (Kapteyn et al., 1996) or through alkali sensitive linkages which was described for so called Pir-proteins (proteins with internal repeats) (Kapteyn et al., 1999a; Mrsa et al., 1997). Several of those covalently linked CWPs were particularly well studied in yeasts, such as *S. cerevisiae* and *C. albicans* (De Groot et al., 2005), but also in other ascomycetes, in particular, *Trichoderma reesei* (Lim et al., 2001), *Yarrowia lipolytica* (Jaafar et al., 2003), and *Schizosaccharomyces pombe* (De Groot et al., 2007) covalently linked cell wall proteins were identified. Only few studies of the fungal cell wall included loosely bound CWPs (Bhat, 2000; Delgado et al., 2001; Pitarch et al., 2002). Pitarch et al. (2002) performed a proteomic analysis on the fractionated cell wall proteins of *C. albicans* and showed a highly complex cell wall proteome, differing significantly from

fraction to fraction. The majority of the envelope proteins could be extracted with hot SDS and β-mercaptoethanol and therefore these are most likely loosely attached to the cell wall.

Different strategies for the isolation and identification of CWPs from either intact cells (Casanova et al., 1992; López-Ribot et al., 1996) or from isolated cell walls (Ebanks et al., 2006; Pitarch et al., 2002) have been described. Alternatively, an approach for the study of CWPs is the analysis of proteins secreted by regenerating protoplasts directly into the medium (Pardo et al., 1999; Pitarch et al., 1999).

Hardly anything is known about the CWPs of higher basidiomycetes (Agaricomycotina). Many fungi from this subphylum are involved in degradation of complex substrates such as wood and other lignocellulosic substrates and are the main organisms responsible for the mineralization of wooden biomass and other recalcitrant plant materials and oxidative and hydrolytic decomposing of organic materials. For this purpose, most basidiomycetes produce numerous enzymes responsible for degradation including laccases, manganese-dependent (or -independent) peroxidases, cellulases, and xylanases. Single enzymes have already been detected within the fungal cell wall or the associated hyphal sheath (Barrasa et al., 1998; Ruel & Joseleau, 1991).

In this study, we chose *Coprinopsis cinerea* (also known as *Coprinus cinereus*) being a model organism for higher basidiomycetes (Agaricomycotina). With an available annotated genome (Stajich et al., 2010), this fungus is a perfect target for proteomic studies (*Coprinus cinereus* Sequencing Project, Broad Institute, http://www.broad.mit.edu). Freely secreted proteins (culture supernatant), proteins of the hyphal sheath, NaCl-extractable CWPs, SDS-extractable CWPs, and NaOH-extractable CWPs were studied by 2-dimensional gel electrophoresis (2-DE) and SDS-PAGE (SDS-Polyacrylamide gel electrophoresis) for a shotgun protein identification by LC-MS^2 (Liquid chromatography coupled mass spectrometry).

3.3 Material and Methods

3.3.1 Chemicals

Yeast extract and malt extract were obtained from Oxoid (Hampshire, UK) and agar was supplied by Serva Electrophoresis (Heidelberg, Germany). All other chemicals were of p.A. quality unless otherwise noted.

3.3.2 Fungal Cultures

Coprinopsis cinerea Okayama7 (#130), (*Coprinus* research community, FGSC #9003) was precultivated on YMG (yeast, malt, glucose) medium (Rao & Niederpruem, 1969) containing 4 g glucose, 4 g yeast extract and 10 g malt extract, and for solidification 1.5% agar per liter. Three 10 mm diameter pieces of 6-days-old agar cultures were used for inoculation of 100 ml YMG liquid precultures. After three days of growth, precultures were homogenized (1 min at 8000 min^{-1}, Ultraturrax T25, Janke & Kunkel, IKA Labortechnik, Staufen, Germany) and 5 ml aliquots were used as inoculum for 100 ml YMG experimental cultures. All liquid cultures were incubated under shaking conditions (120 rpm/min). Experimental cultures were grown to the early exponential phase (3 days of growth) at 37 °C. Biomass of the fungal cultures was determined by ergosterol measurements and determination of the dry weight (DW) (for detailed information see Material and Methods Chapter 5).

3.3.3 Protein Fractionation

All buffers used for cell wall and protein extraction contained 1 mM phenylmethylsulfonyl fluoride (PMSF) as protease activity inhibitor. Fungal mycelium was separated from the culture supernatant by vacuum filtration (Whatman filterpaper No 1, ∅ 90 mm, Schleicher & Schuell, München, Germany). The supernatant containing soluble proteins was frozen at −20 °C and later thawed for protein precipitation. Hyphal sheath proteins were extracted from the collected mycelium of 100 ml cultures using 25 ml Tris-buffer (0.5 M, pH 7.5) containing 2% (v/v) Tween 80 at room temperature for 30 min under gentle shaking. The procedure was repeated and solutions containing extracted proteins from the hyphal sheath, were pooled and frozen at −20 °C until further proceeding.

The remaining mycelium was lyophilized and ground with a ball mill (Retsch MM2000, Haan, Germany) 3 times for 2 min (amplitude of 80) under liquid nitrogen cooling. The cell wall was isolated as described by Lim et al. (2001). Briefly, the broken mycelial fragments were washed twice with cell breaking buffer (CBB; Tris-base pH 7.5 20 mM, NaCl 200 mM, EDTA (Ethylene diamine tetraacetic acid) 2 mM, Tween 80 0.01%, PMSF 1 mM) and collected by centrifugation (650g for 10 min). The mycelial fragments were suspended in 10 ml CBB, layered on top of 30 ml of 30% sorbitol solution

and centrifuged at 650g for 20 min. The pellet of the cell wall fraction was washed with CBB once and subsequently once with water. The collected cell walls were lyophilized and stored at −20 °C. All centrifugation steps were performed at 4 °C. Cell wall purity was controlled by enzyme tests using 3 different marker enzymes: malate dehydrogenase (Sassoon & Mooibroek, 2001), isocitrate dehydrogenase (Soundar et al., 2006), and glucose-6-phosphate dehydrogenase (Ibraheem et al., 2005). The enzyme activity of those three enzymes was measured from aliquots of the supernatants obtained from each cell wall purification step and finally on the pure cell wall fraction.

Ionically bound proteins were extracted from the pure cell wall fraction with Tris-buffer (20 mM, pH 7.5) containing 1 M NaCl by incubation for 1 h at room temperature under gentle shaking. The NaCl-extractable fraction was collected by centrifugation and the extraction was repeated two more times. Subsequently, non-covalently bound cell wall proteins were extracted four times by boiling with SDS-extraction buffer, 1 ml/10 mg cell wall [Tris-base 60 mM, pH 7.5, EDTA 50 mM, SDS 2%, β-mercaptoethanol 0.5%, PMSF 1 mM (Masuoka et al., 2002)] and all extracts were combined. Extracted cell walls were further incubated with 30 mM NaOH (1 ml/10 mg cell wall) at 4 °C overnight as described previously for *C. albicans* (Pitarch et al., 2002). The so obtained NaOH-extractable protein fraction from the cell wall obtained from one cell wall preparation was pooled. Non-extractable CWPs were broken down directly within the previously extracted cell wall by CNBr (Fluka, Buchs, Switzerland) and subsequently treated with trypsin (Sequencing Grade Modified Trypsin, Promega, Mannheim, Germany) as previously described by Ebanks et al. (2006).

3.3.4 Protein Precipitation

Protein samples from the culture supernatant or the hyphal sheath fraction were centrifuged at 48400g at 4 °C for 30 min to precipitate polysaccharides. Protein precipitation was performed by addition of 10% (v/v) of the sample volume from a 100% TCA (Trichloroacetic acid) stock solution [100 g TCA in 45.4 ml water (Sivaraman et al., 1997)]. After mixing, samples were incubated overnight on ice and centrifuged at 1700g and 4 °C for 15 min. Excess of TCA was removed from protein precipitates by subsequent washings with 2 ml of ice-cold 20% Tris-buffer (50 mM, pH 7.5) in acetone (v/v). In most cases, three washing steps were required to remove TCA from the pellet (Fragner et al., 2009). Finally, protein samples were washed with pure, ice-cold

acetone, air dried, and stored at −20 °C until further use. NaCl, SDS, and NaOH extractable proteins were concentrated 10 to 20 times by freeze drying and precipitated with chloroform/methanol (Wessel & Flügge, 1984) and stored at −20 °C.

3.3.5 Protein Quantification

Dilutions of protein samples in sample buffer (60 mM Tris-base pH 6.8, 2% SDS, 10% glycerol, and traces of Bromphenol blue) were separated by 12% SDS-PAGE (Multilong, Biometra, Göttingen, Germany) at 20 mA for about 3 h. A calibration curve for the determination of the protein amount was obtained with bovine serum albumin (BSA) standards (2 µg, 1 µg, 0.4 µg, and 0.2 µg of protein per lane) separated simultaneously with the samples on the same gel. An AIDA image analyzer (v4.10.020, Raytest, Straubenhardt, Germany) was used for gel processing and calculations of the total protein amount (Fragner et al., 2009). The following parameters were used: baseline determination with search level 1, smooth parameter 10 pixel, and search level 0 for peak determination (not smoothed). Integration parameters for BSA bands were set manually to prevent distortion of the protein amount measurements by buffer contaminations. Obtained calibration curve ($R^2 = 0.9851$) was used for calculation of total protein amount in fungal samples.

3.3.6 Protein Separation by 2-DE

The protein samples were dissolved in the IEF (Isoelectric focusing) sample buffer: urea 8 M (PlusOne, Amersham Biosciences, Freiburg, Germany), CHAPS (3-[(3-cholamidopropyl) -dimethylammonio]-1-propanesulfonate) 4% (w/v), DTT (Dithiothreitol) 50 mM, Triton X100 5% (v/v), IPG (Immobilized pH gradient) buffer (pH 3-10) 0.5% (v/v) (Amersham Biosciences), and traces of Bromphenol blue, according to the Amersham Biosciences protocol for isoelectric focusing . For maximal protein recovery the samples were sonicated (Manadas et al., 2006) at a temperature <25 °C. After centrifugation the samples were loaded on IPG strips (100 µg total protein per 18 cm Immobiline DryStrip, pH 3-10) in ceramic strip holders (Amersham Biosciences). IPG-strips were rehydrated for 12 h at 20 V and 24 °C (Ettan IPGphor, Amersham Biosciences). IEF was performed under the following conditions: 150 V for 1 h, 1 h at 200 V, 1 h at 500 V, 1000 Vh at 1000 V, followed by 2.5 h of a gradient from 1000 V to 8000 V,

and finally focused for 28000 Vh at 8000 V. The maximal current per strip was set at 50 µA. The focused samples were stored at −20 °C. Prior to separation in the second dimension, IEF-strips were incubated twice for 10 min in 6 ml of equilibration buffer (urea 6 M, SDS 1% (w/v), glycerol 30% (v/v), Tris-base 40 mM, and traces of Bromphenol blue) containing 10 mM DTT in the first step and 100 mM iodoacetamide in the second step, respectively. The separation in the second dimension was performed with 12% SDS-PAGE (25.5, 20.5, 0.10 cm), at 3000 V (400 mA, 13 W) for 30 min followed by 3000 V (400 mA, 100 W) for 4-5 h using an Ettan DALTsix electrophoresis unit (Amersham Biosciences). The molecular weight marker for the second dimension was obtained from Fermentas (#SM0431, St. Leon-Rot, Germany). The gels were stained with RuBP [ruthenium(II) tris(bathophenanthroline disulfate)] (Rabilloud et al., 2001) according to the protocol described by Lamanda et al. (2004). Finally, gels were scanned using an FLA-5100 fluorescence reader (Fujifilm, Düsseldorf, Germany) with 50 µm resolution. 16 bit reader files were converted to TIF-format using an AIDA image analyzer (v4.10.020, Raytest, Straubenhardt, Germany).

Spot detection and gel matching was done with Proteomweaver (Version 3.1.0.7; Definiens AG München, Germany). The parameters for spot detection were set as follows: radius limit: 25.04; intensity limit: 312120; contrast limit: 94.2857; border contrast: 0.2; normalization point: 95. The minimal significant factor for the spot detection was calculated as 1.595. Two technical replications were used for the statistical analysis of the 2-DE-gels.

3.3.7 Mass Spectrometry and Identification of 2-DE Spots

In-gel digestion of protein spots was done according to Havlis et al. (2003) with some modifications. Briefly, gel pieces were washed twice with water for 15 min and subsequently dehydrated with 100% acetonitril (ACN) for 20 min and dried in a vacuum centrifuge (Concentrator 5301, Eppendorf, Wesseling-Berzdorf, Germany) for 10 min. Subsequently, the gel pieces were soaked in 2 µM trypsin solution (Sequencing Grade Modified Trypsin, Promega, Mannheim, Germany) in 50 mM ammoniumbicarbonate buffer (pH 8) at 4 °C for 1 h and digested for 30 min at 58 °C. Peptides were extracted from the gel pieces on a shaker first with 150 µl 5% formic acid (v/v) for one hour, followed by extraction with 150 µl 5% formic acid, 50% acetonitril (v/v), twice. Extracts were combined, dried in a vacuum centrifuge and stored at −20 °C until proceeding. For

further processing, peptides were dissolved in 5 µl of 5% formic acid. 4 µl of the peptide solution was analyzed by LC (HP 1100, Agilent, Böblingen, Germany) equipped with an active flow splitter (AcuRate, LC Packings, Dionex, Amsterdam, Netherlands) reducing the flow rate from 120 µl/min to 2 µl/min. Peptides were loaded onto a 180 µm id capillary column packed with 3 µm Reprosil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany), prepared from a 14 cm silica capillary (Polymicro, Phoenix, USA) with a Reprosil-Pur 120Si 3 µm frit (Zhang & Huang, 2001). The programmed LC injector was left for 20 min in the injection position and samples were eluted into the column by an isocratic flow of 98% of solvent A (0.1% formic acid in water) and 2% of solvent B (0.1% formic acid in 90% ACN). After switching the injector to a bypass position, peptides were eluted by a four step gradient of B: 5 min to 15%, 90 min to 40%, 5 min to 50%, followed by 5 min to 90% B. After additional elution for 5 min in 90% B, the column was conditioned by a reverse gradient to the starting conditions (5 min) and isocratic flow for further 15 min. The ESI (Electron spray ionization) nebulizer was set to 20 psi pressure of N_2, 5.0 l/min dry gas, 300 °C dry temperature, and to 4500 V capillary voltage. Ion trap parameters were optimized to tryptic digests and averages of four precursor ions scans and of four secondary mass scans were used. Single charged precursor ions were not allowed and fragmented peptides were excluded from sequencing for 1 min after two spectra have been attained. Analysis of the raw data was performed with Daltonic DataAnalysis version 3.0 (Bruker Daltonic GmbH, Bremen, Germany) and proteins were identified by searching the dataset against a database of the annotated genome of *C. cinerea* using local Mascot software (local server V. 2.0.04, Matrix Science). The following settings were used for the database search: fixed modification: carbamidomethylation on cysteine; variable modification: oxidation on methionine; peptide mass tolerance: 1.4 Da; peptide charge: 1+,2+,and 3+; MS/MS tolerance: 0.4 Da; missed cleavages allowed: 1. For final identification and predicted function protein BLAST (Basic Local Alignment Search Tool) against NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/guide/), PFAM (Protein families; http://pfam.sanger.ac.uk/), and InterPro (http://www.ebi.ac.uk/interpro/) were used.

3.3.8 Protein Separation and LC-MS^2 for the Shotgun Analysis

A 3 mg protein sample from each isolated fraction was dissolved in 200 µl SDS-PAGE sample buffer, reduced with 20 mM DTT for 20 min at 95 °C and subsequently alkylated with 200 mM iodoacetamide for 20 min at room temperature in the dark (Washburn et al., 2001). Stacking gel (4%) was prepared with a purpose-built comb creating a 35 x 12 mm well sufficient to accommodate the protein sample. Proteins were separated on 12% resolving gel by SDS-PAGE (Multilong from Biometra) at 15 mA for 2-3 h. Thereafter, the gel was rinsed with water and stained for few minutes with CBB G250 (Serva, Heidelberg, Germany). The gel area with visualized protein patterns was cut into five equal lanes and each lane was further divided with a razor blade into equal 10-13 strips, depending on the protein sample. Gel strips were finally cut in approximately 1 mm^3 pieces and frozen for few hours in LoBind protein tubes (Eppendorf) prior to in-gel digestion. In-gel digestion of proteins was performed according to Havlis et al. (2003) as described above. For further processing, peptides were dissolved in 15 µl of 5% formic acid.

4 µl of the peptide solution were analyzed by LC (HP 1100, Agilent, Böblingen, Germany) as described above. Each sample was analyzed in three subsequent identical LC separations with three different mass ranges for precursor ion selection by ESI-MS^2 (Esquire 3000, Bruker Daltonik, Bremen, Germany) as described by Rappsilber et al. (2002): m/z = 300-500, m/z = 500-750, or m/z = 750-1500, respectively. These were matched to SPS settings of the Esquire 5.0 software (Bruker Daltonic) enhancing target masses of m/z = 450, 600, or 800, respectively. In all three analysis, ESI nebulizer was set to 20 psi pressure of N_2, 5.0 l/min dry gas, 300 °C dry temperature, and to 4500 V capillary voltage. Ion trap parameters were optimized to tryptic digests and averages of four precursor ions scans and of four secondary mass scans were used. Single charged precursor ions were not allowed and fragmented peptides were excluded from sequencing for 10 min after two spectra have been attained.

3.3.9 Shotgun Data Analysis and Protein Identification

Lists of compounds mass spectra were generated from raw MS/MS data with Data Analysis 3.0 (Bruker Daltonic) and compound lists from the three LC-MS/MS analysis of one sample were combined into a single dataset (in mgf-format). Mascot (local server V. 2.0.04, Matrix Science) was used to search these data against a database

constructed by Dr. Majcherczyk from six annotations of *C. cinerea* genome: Augustus, m300_Glean, GLEAN (retrained), Twinscan, SNAP (retrained) (*Coprinus* research community, http://genome.semo.edu), Broad annotated genome (www.broad.mit.edu, available also at NCBI) and SwissProt database (about 302000 database entries). The protein database used was transformed also to a reverse sequence database and used for false/positive searches (http://www.psidev.info/). Carbamidomethylation on cysteine as fixed modification and oxidation of methionine as variable modification of peptides were used for searches of peptides with charges 1+, 2+, and 3+ and monoisotopic masses. Peptide mass tolerance was 1.4 Da and peptide MS/MS tolerance 0.4 Da; one missed cleavage was allowed.

Peptide data were extracted from Mascot generated files (in dat-format) using a Visual Basic (VB)-script and transferred via Microsoft Access 2007 to a structured query language (SQL) database operated by Microsoft SQL Server 2005. A unique database was created for each protein fraction combining all data obtained from a single 1-DE gel. SQL queries were used to extract proteins with at least two peptides with scores higher than the corresponding identity score and only these proteins were reported. Analogous data processing was performed on false/positive results from Mascot searches. Further SQL queries were used to calculate the protein sequence coverage and reporting confidently identified proteins. Further, average peptide scoring (APS) was applied to the obtained LC-MS^2 data (Chepanoske et al., 2005; Shadforth et al., 2005). For the identified proteins unique ID-numbers were assigned, which are listed together with the protein properties and obtained LC-MS^2 data in the supplements (Tables A.1 and A.2). Sequences of identified proteins were used for prediction of signal peptides by the SignalP 3.0 Server (www.cbs.dtu.dk/services/SignalP) and homology searches were performed by BLASTp against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein families, domains, repeats and sites were detected by searching the PFAM (http://pfam.sanger.ac.uk), InterPro (http://www.ebi.ac.uk/interpro), and CDD (Conserved Domains Database; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) databases. No attempts were made to manually modify or alter protein predictions by the available computer annotations. Only in exceptional cases (as it the case for the proteins in the Tables 3.11 and 3.12) predicted N-termini were controlled.

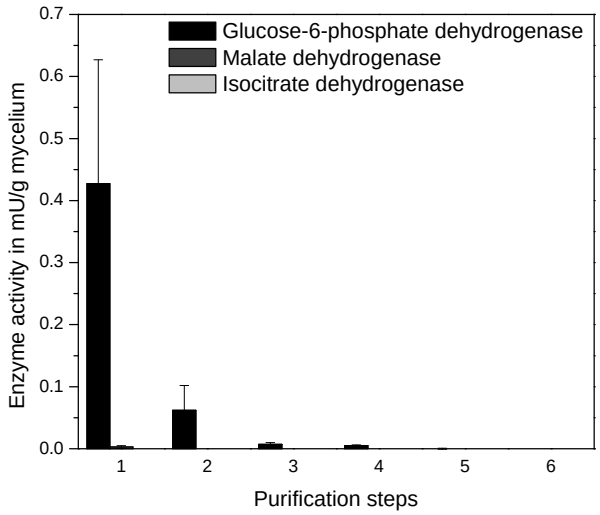


Figure 3.1: Enzyme activities of typical housekeeping enzymes measured during the isolation of the cell wall from *Coprinopsis cinerea* cultures (for 3 days grown at 37 °C on YMG medium) in order to control the contaminations of the cell wall fraction with intracellular proteins. From each step during the isolation procedure (see Material and Methods this chapter) aliquots were taken for the enzyme measurement, enzyme activity tests were performed as described elsewhere (Ibraheem et al., 2005; Sassoon & Mooibroek, 2001; Soundar et al., 2006).

3.4 Results

The here presented work gives an overview of the proteins from *C. cinerea* occurring in different fractions of the secretome when growing the fungus in a liquid complete medium at 37 °C. The freely secreted proteins of the culture supernatant, the proteins of the hyphal sheath and the differently bound cell wall proteins of exponentially growing *C. cinerea* cultures were isolated as described in Material and Methods. Intracellular protein contaminations of the cell wall fractions were tested by enzyme tests of housekeeping enzymes such as malate dehydrogenase, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase [(Ibraheem et al., 2005; Sassoon & Mooibroek, 2001; Soundar et al., 2006) Figure 3.1].

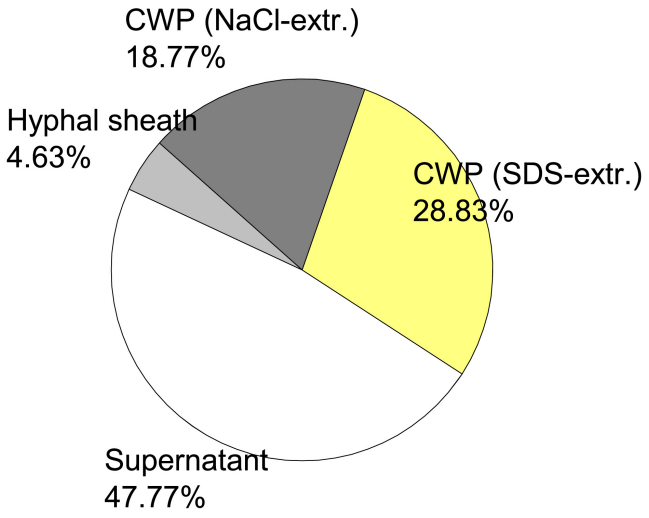


Figure 3.2: Relative distribution of the total protein amount between different fractions of the secretome of *C. cinerea* when grown at 37 °C for 3 days in YMG medium (100% corresponds to 3.7 mg protein per g DW); the protein concentration was measured by an in-gel assay (Fragner et al., 2009).

3.4.1 2-DE Analysis of the Secreted Protein Fractions

Measurement of the total protein amount in the different fractions of the secretome showed an unequal distribution (Figure 3.2). In total, 3.7 mg protein per gram DW biomass was detected. Almost half (1.7 mg/g dry weight or 47.7%) of the proteins was directed to the culture supernatant. Only 170 µg/g (4.6%) was found in the hyphal sheath and the cell wall fractions comprised 692 µg/g (18.7%) and 1.1 mg/g (28.8%) in the NaCl- and SDS-extractable fraction, respectively. NaOH-extractable proteins made up only 7.4 µg/g (0.2%) of the totally secreted protein, which was a too low concentration in order to perform 2-DE gel analysis for this fraction (other results from this fraction are described in Section 3.4.3). However, 2-DE was performed for all other fractions and the obtained gels were analyzed with Proteomweaver software, determining the number of spots and matching the gels.

Large differences in spot numbers and positions on the 2-DE gels became obvious for the different fractions (Figure 3.3). The number of spots in the cell wall fractions was almost double than in the freely secreted protein fraction (Figure 3.3 A) and in the hyphal sheath fraction (Figure 3.3 B). Also the distribution of spots differed significantly between the freely secreted fractions and the cell wall fractions (Figure 3.3 C and D).

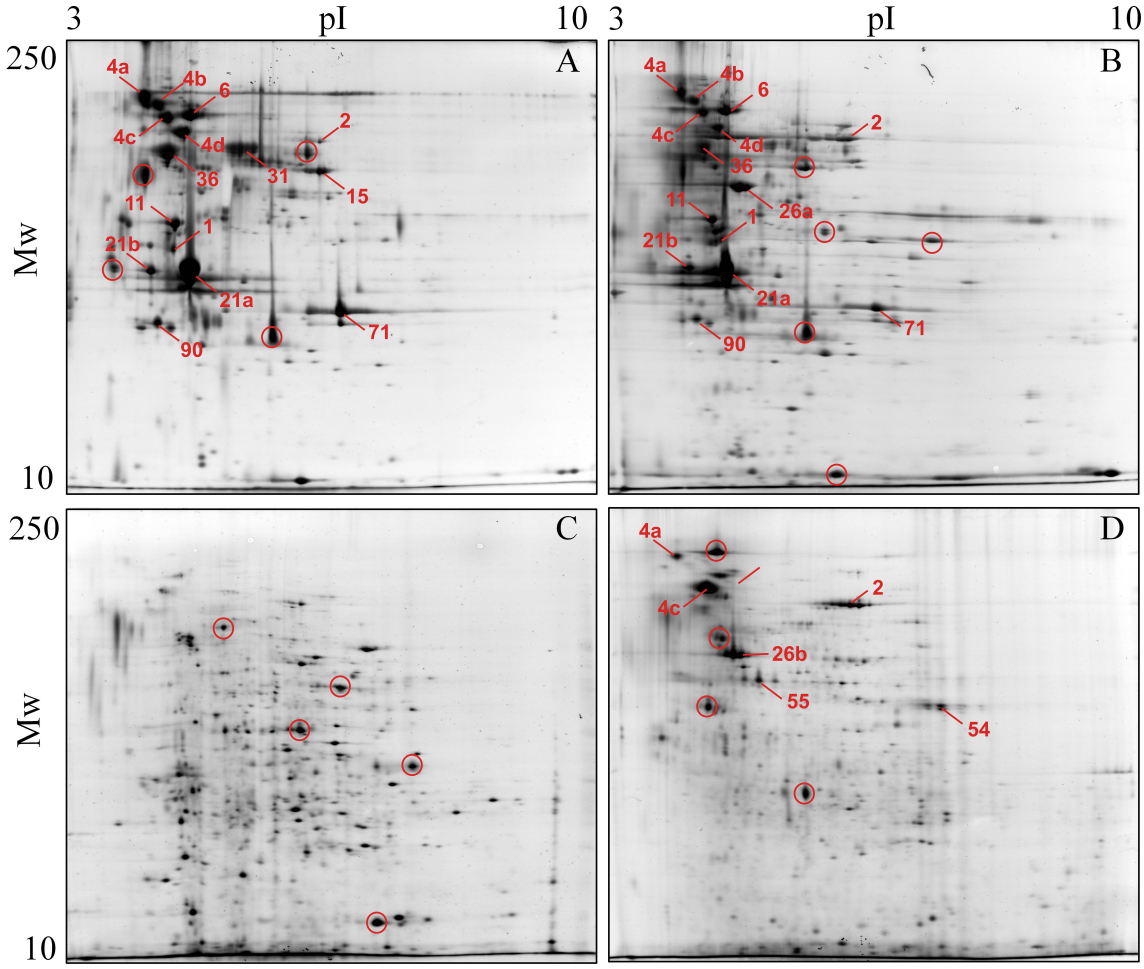


Figure 3.3: 2-DE-gels of the fractionated secretome from 3-days-old YMG cultures of *C. cinerea* grown at 37 °C. All gels were loaded with 100 µg of protein. A: freely secreted proteins; B: hyphal sheath proteins; C: cell wall proteins (CWP) extracted with NaCl; D: CWPs extracted with hot SDS under reducing conditions. Proteins were separated on 18 cm Immobiline DryStrips pH 3-10 (Amersham Biosciences) in the first dimension and on a 12% SDS-PAGE in the second dimension. Gels were stained with RuBP. The spots labeled with numbers could be detected by LC-MS^2 and identified by searching the LC-MS^2 data against the annotated genome of *C. cinerea*, whilst the encircled spots gave no positive results in the LC-MS^2 analysis. Protein function was predicted by BLAST, InterPro and Pfam searches.

Table 3.1: A matrix of the number of spots matching between the gels of the different protein fractions (analyzed with Proteomweaver (Version 3.1.0.7 Definiens AG München, Germany)) is shown in this table. Two technical replicates of 2-DE analysis were used for each fraction.

Fractions	Supernatant	Hyphal sheath	NaCl-extractable	SDS-extractable
Supernatant	**96**			
Hyphal sheath	33 ± 6	**88**		
NaCl-extractable	23 ± 2	26 ± 4	**196**	
SDS-extractable	28 ± 4	27 ± 5	57 ± 4	**161**

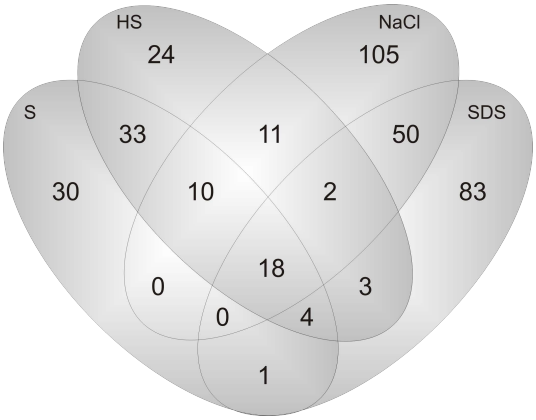


Figure 3.4: Venn diagram showing the spots overlapping between the different fractions of the secretome from *C. cinerea* grown at 37 °C in shaking cultures in liquid YMG medium (see also Table 3.1).

Whilst the freely secreted proteins and the proteins of the hyphal sheath were mainly clustered in the high molecular weight range from about 220 to 100 kDa and at low pI values from 3 to 6, the proteins of the cell wall fractions were distributed over the whole pI range and various high molecular as well as numerous low molecular weight proteins were present (Figure 3.3). In the supernatant as well as the hyphal sheath fraction, approximately 10 to 15 high abundant spots, representing a single protein, were visible mainly in the acidic pI range. This was not the case for the NaCl-extractable fraction (Figure 3.3).

96 and 88 spots were in total detected on the 2-DE gels of the supernatant and the hyphal sheath fraction, respectively. Further, 196 and 161 spots were detected on the gels from the NaCl-extractable and the SDS-extractable fraction, respectively. The pattern of the 2-DE gels showed a high degree of similarity between the freely secreted proteins and the hyphal sheath proteins. This observation was confirmed by the number

of spots matching between those two fractions (Table 3.1 and Figure 3.4). 33 ± 6 spots were at corresponding positions in those two fractions, this corresponds to 34% and 38% of the spots in the freely secreted protein fraction and the hyphal sheath fraction, respectively. Matching between the latter fractions and the two fractions extracted from the cell wall revealed very distinct distributions of proteins, as in total only 18 spots are overlapping between all fractions (Figure 3.4). The NaCl-extractable and SDS-extractable fraction shared only 11.7% and 17.4% of spots with the supernatant fraction and 13.3% and 16.7% of spots with the hyphal sheath fraction, respectively. The NaCl-extractable fraction and the SDS-extractable fraction had in total 52 ± 5 matching spots (Table 3.1). This corresponds to 29.1% and 35.4% of the the total number of spots from the NaCl- and the SDS-extractable protein fraction, respectively.

High abundant spots of the 2-DE gels were picked for the identification by LC-MS^2. In total, 19 of 38 picked and analyzed spots were positively identified by LC-MS^2 (Table 3.2). These 19 spots represented 14 different proteins coming either from the freely secreted proteins, the hyphal sheath proteins and the SDS-extractable fraction (compare Figure 3.3 A to D, respectively). Those spots in gels being too weak for protein identification but corresponding to positively identified spots in other gels were included in the intensity analysis performed by Proteomweaver software. Spot intensities in the single fractions were calculated in relation to the total amounts of protein being present in the different fractions. The highest amount of most of the proteins was obviously present in the hyphal sheath fraction (see Figure 3.5 a to c). Only two proteins (ID-numbers 54 and 55) were most abundant in the SDS-extractable fraction.

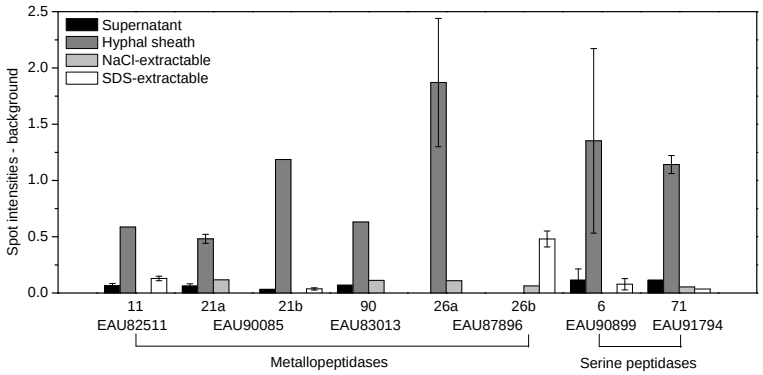
For the identification of the detected proteins, the obtained LC-MS^2 data sets were searched against the Mascot database containing the whole annotated genome sequence of *C. cinerea*. For the prediction of the protein function, the obtained protein sequences were blasted (pBLAST) against the whole NCBI database and the InterPro database, containing Pfam, CDD, Panther, Tigr, Superfamily, SMART (Simple Modular Architecture Research Tool), Uniprot databases for conserved domains. In all cases, a potential function could be assigned to the proteins (Table 3.2).

Many of the identified spots (6 of 19) appear to represent peptidases, as predicted from the conserved domains and the BLAST searches. Four different metallopeptidases from three distinct clans (M28, M36 and M43) and two serine peptidases (clan S8) were present (compare Table 3.2). Three of the metallopeptidases (ID-numbers 11, 21, 90) were found within the freely secreted proteins as well as the hyphal sheath proteins.

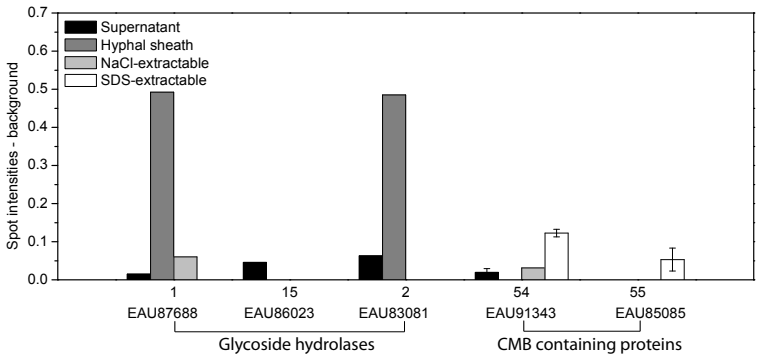
Table 3.2: Spots identified from the 2-DE-gels by LC-MS^2.

ID*	Protein accession**	Proposed protein function	Signal peptide	Theoretical MW (kDa)	Theoretical pI value	Fraction***
4	EAU83456	Copper radical oxidase	+	105	4.2	1, 2, 4
36	EAU82165	Oxidoreductase (3)	-	53	4.4	1, 2
31	EAU80813	Oxidoreductase (2)	+	61	5.3	1
2	EAU83081	Alkaline phosphatase	+	72	6.31	1, 2, 4
1	EAU87688	Glycoside hydrolase fam 3 (1)	+	97	4.5	1, 2
15	EAU86023	Glycoside hydrolase fam 15	+	61	6.4	1
6	EAU90899	Serine peptidase SB/S8 (1)	+	95	4.7	1, 2
71	EAU91794	Serine peptidase SB/S8 (2)	+	36	7.8	1, 2
11	EAU82511	Metallopeptidase MA/M36 (1)	+	63	4.5	1, 2
21	EAU90085	Metallopeptidase MA/M43 (1)	+	29	4.5	1, 2
90	EAU83013	Metallopeptidase MA/M43 (3)	+	30	4.4	1, 2
26	EAU87896	Metallopeptidase MH/M28 (2)	+	52	4.9	2, 4
54	EAU91343	CBM-containing protein (1)	-	34	8.6	4
55	EAU85085	CBM-containing protein (2)	+	43	6.8	4

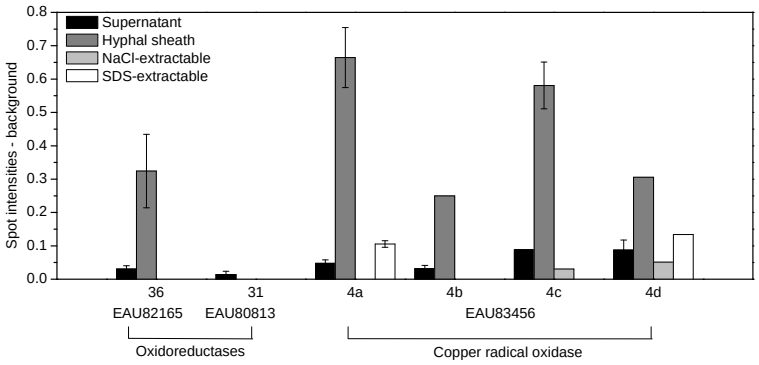
* The ID numbers in this table correspond to the spot numbers in Figure 3.3;
for peptide sequences and scores see Table A.1 in the Appendix
** NCBI protein accession http://www.ncbi.nlm.nih.gov/Genbank/
Molecular weight and pI value are obtained from EMBOSS (http://emboss.sourceforge.net/
*** 1: Freely secreted protein fraction; 2: Hyphal sheath fraction;
3: NaCl-extractable fraction; 4: SDS-extractable fraction



(a) Peptidases



(b) Glycoside hydrolases and carbohydrate binding proteins



(c) Oxidoreductases and copper radical oxidase

Figure 3.5: Relative intensities of identified spots in the 2-DE gels of the different fractions of the secretome of 3-days-old *C. cinerea* cultures grown at 37 °C (see Figure 3.3). Numbers beneath columns refer to specific proteins, numbers with a, b, etc. to spots representing the same protein. For identification of the proteins NCBI accession numbers are given. Spot intensities measured by Proteomeweaver were multiplied by a factor reflecting the differences in the protein amount of the different protein fractions.

The fourth of the metallopeptidases from clan M28 (ID-number 26) was identified from the hyphal sheath fraction and the SDS-extractable cell wall fraction only. Remarkably, the peptidases represent the most abundant spots in all gels regardless of the respective fraction (compare Figure 3.3 a).

Two glycoside hydrolases from the families 3 and 15 (ID-number 1 and 15) were identified from spots of the freely secreted protein fraction. The glycoside hydrolase from family 3 was found in the supernatant and the hyphal sheath fraction, while the glycoside hydrolase from family 15 was only detected in the supernatant. Alkaline phosphatase (ID-number 2) was identified from a spot in the supernatant and in the hyphal sheath fraction. Also this protein showed the highest spot intensity in the hyphal sheath fraction (compare Figure 3.5 b). Further, two different FAD/FMN-containing oxidoreductases were found in the supernatant (ID-number 31 and 36). Four different high abundant spots (ID-number 4) in the supernatant and the hyphal sheath fraction were identified as glyoxal oxidases similar to a copper radical oxidase from *P. chrysosporium* (Whittaker et al., 1996) . Two of them were as well present in the SDS-extractable cell wall fraction (Figure 3.3).

3.4.2 1-DE Shotgun Proteomics of the Secretome

2-DE analysis of the different fractions of the fungal secretome showed that the fractions differ significantly from each other in their protein composition. However, the identification of proteins by LC-MS^2 proofed to be limited in this approach. To identify more proteins of the secretome from *C. cinerea*, a 1-DE shotgun approach of the secreted protein fractions was performed (Dowell et al., 2008; Merrihew et al., 2008; Wolters et al., 2001). The proteins isolated from the four different fractions were separated by 1-DE, all lanes were cut into 13 equal slices containing proteins and analyzed by LC-MS^2. Five identical lanes were analyzed as replicates from each fraction (Figure 3.6).

Two approaches have been performed for confident protein identification. In the first, only proteins with at least two peptides having a Mascot score higher than the identity score were accepted. These very stringent rules for protein identification revealed in total 116 proteins. In the second, average peptide score (APS) in combination with minimum threshold levels was used for the identification of proteins from the LC-MS^2 data. This analysis was performed by Dr. A. Majcherczyk (Chepanoske et al., 2005; Shadforth et al., 2005). This approach detected 46 additional proteins, summing up to

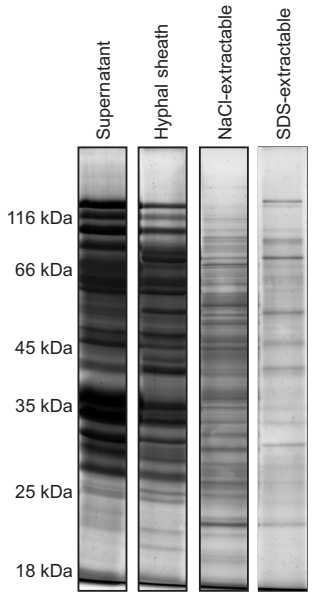


Figure 3.6: 1-DE separation of the proteins from the supernatant, the hyphal sheath, the NaCl- and SDS-extractable protein fractions from 3-days-old *C. cinerea* cultures grown at 37 °C. For shotgun masspectrometry, from each fraction five identical lanes were cut into 13 slices and each slice was individually analyzed by LC-MS^2.

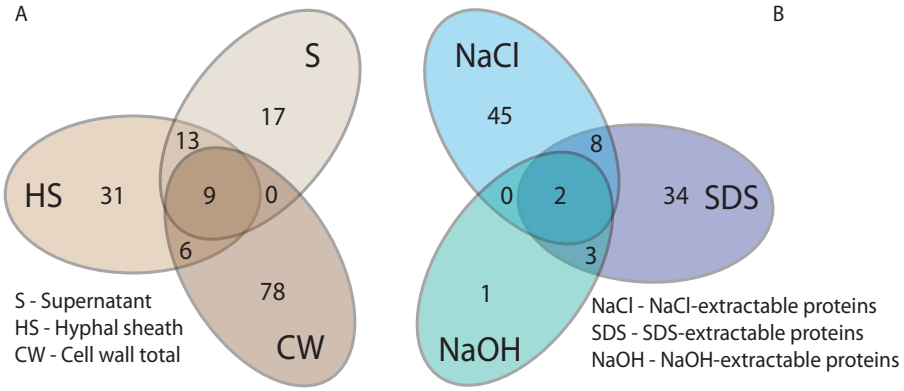


Figure 3.7: Overlap of the totally identified proteins of *Coprinopsis cinerea* from the supernatant fraction (S), the hyphal sheath fraction (HS) and the totally extractable cell wall proteins (CW) (Figure A) and overlap of proteins within the different protein fractions of the cell wall, NaCl-, SDS-, and NaOH-extractable proteins (Figure B).

a total of 162 unique proteins (Tables 3.3 to 3.11).

Proteins identified from all fractions in total counted for 162 unique proteins (Tables 3.3 to 3.11). The highest number of proteins was identified in the hyphal sheath fraction, with 61 (28%) proteins, while only 41 (19%) freely secreted proteins were identified. 59 (27%) NaCl-extractable proteins and 50 (23%) SDS-extractable proteins were identified, respectively. NaOH treatment of the cell wall resulted in identification of only six (3%) proteins; however, these proteins appeared already in previous fractions analyzed. All proteins identified previously in the 2-DE approach were as well found in the 1-DE shotgun approach.

Low overlapping of identified proteins between fractions was observed (Figure 3.7). 13 proteins of the hyphal sheath were also detected in the freely secreted protein fraction; this presents the highest overlap occurring between fractions. However, 31 and 17 unique proteins, respectively, were unique to these fractions. Only 9 proteins shared between the freely secreted, the hyphal sheath and the cell wall secretome, when combining the data from all cell wall fractions. Amongst the three analyzed fractions of the cell wall secretome, only two proteins were in common. These were the alkaline phosphatase (ID-number 2) and an elongation factor (ID-number 113).

Table 3.3: Putative oxidoreductases found within different fractions of the secretome from *C. cinerea* grown for 3 days on liquid YMG at 37 °C. Classification was done after BRENDA enzyme database (http://www.brenda-enzymes.info/index.php4) and properties were determined by NCBI protein BLAST (www.ncbi.nlm.nih.gov/).

ID*	Protein accession**	Predicted function	Fractions*** 1	2	3	4	5	Signal peptide	Expected properties
4	EAU83456	Copper radical oxidase	1	2		4		+	Extracellular; glyoxal oxidase; H_2O_2 providing enzyme Vanden Wymelenberg et al. (2006) Whittaker et al. (1996)
14	EAU90814	Oxido-	1	2				+	FAD/FMN- and C-terminal
31	EAU80813	reductase	1	2				+	berberine domain containing
36	EAU82165		1	2		4		+	proteins
85	EAU82898			2				+	(Kutchan & Dittrich, 1995)
119	TS_1_199_191			2				+	
APS1	EAU80782		1					+	
APS36	EAU85448		1					+	
32	EAU87895	Aryl-alcohol oxidase		2				+	Choline dehydrogenase related flavoproteins pfam.sanger.ac.uk/
61	EAU86801	Catalase			3			-	Extracellular; dismutation of H_2O_2; Peraza & Hansberg (2002)
50	EAU89294	Superoxide			3	4		-	Extracellular location;
131	EAU86088	dismutase		2				+	antioxidant defense Chaturvedi et al. (2001)
81	EAU81725	Aldo/Keto			3			-	Possibly intracellular
APS19	EAU88751	reductase			3			-	location
95	EAU81656	Redoxin-containing protein		2	3			-	Similar to thioredoxin; peroxiredoxin; reduction of H_2O_2 Thön et al. (2007)
APS6	retrain_ccin Contig112-snap.73	Glutathione reductase		2				-	Reduction of glutathione to glutathione disulfide Noguera-Mazon et al. (2006)
118	EAU90873	Peroxidase			3			-	Plant peroxidase superfamily; contains fungal cytochrome C oxidases
APS28	EAU83399	Short-chain dehydrogenase			3			+	Unknown
APS22	EAU89899	Ferric reductase				4		+	Transmembrane component; iron assimilatory system; Haas (2003)

* Protein identification number generally used within this work

** NCBI protein accession http://www.ncbi.nlm.nih.gov/Genbank/

*** 1: Freely secreted proteins, 2: hyphal sheath proteins, 3: NaCl-extractable proteins, 4: SDS-extractable proteins, 5: NaOH-extractable proteins

Table 3.4: Putative oxidoreductases with predicted intracellular localization found within different fractions of the secretome from *C. cinerea* grown for 3 days on liquid YMG at 37 °C. Classification was done after BRENDA enzyme database (http://www.brenda-enzymes.info/index.php4) and properties were determined by NCBI protein BLAST (www.ncbi.nlm.nih.gov/).

ID*	Protein accession**	Predicted function	Fractions*** 1	2	3	4	5	Signal peptide	Expected properties
42	EAU87137	Aldehyde dehydrogenase			3			-	Intracellular, possibly part of the thioredoxin system Thön et al. (2007)
67	EAU91058	Formate dehydrogenase			3			-	Intracellular Watanabe et al. (2008)
57	EAU87654	Malate			3			-	Intracellular; mitochondrial
86	EAU82346	dehydrogenase			3			-	membrane protein Maloney et al. (2004)
74	EAU92918	Phosphogluconate dehydrogenase			3			-	Decarboxylase; requires NADH; pentose phosphate pathway He et al. (2007)
87	EAU81873	Succinate dehydrogenase				4		-	Mitochondrial membrane protein Silkin et al. (2007)
106	EAU89492	Alcohol dehydrogenase				4		-	Intracellular Suvarna et al. (2000)
68	EAU86652	Dihydrolipoyl dehydrogenase			3			-	FAD/NAD dependent; pyruvate dehydrogenase complex (BRENDA)

* Protein identification number generally used within this work
** NCBI protein accession http://www.ncbi.nlm.nih.gov/Genbank/
*** 1: Freely secreted proteins, 2: hyphal sheath proteins, 3: NaCl-extractable proteins, 4: SDS-extractable proteins, 5: NaOH-extractable proteins

Table 3.5: Putative glycoside hydrolase families (GH) and other carbohydrate processing enzymes as a subgroup of hydrolases found in different fractions of the secretome from *C. cinerea* grown for 3 days on liquid YMG at 37 °C. Classification was done after the CAZy-database (http://www.cazy.org/fam/acc_fam.html).

ID*	Protein accession**	Family	Fractions***					Signal peptide	Representative enzyme
			1	2	3	4	5		
1	EAU87688	GH 3	1	2		4	5	+	Family of β-
27	EAU81960			2				+	glucosidases
APS45	EAU87685					4		+	
17	EAU92553	GH 5	1	2		4		+	Cellulases, β-mannosidase
132	EAU80670	GH 6	1					+	Cellobiohydrolase
15	EAU86023	GH 15	1	2				+	Glucoamylase,
APS14	EAU86122		1					+	α-trehalase
79	EAU80873	GH 24		2				+	Lysozyme
APS2	EAU80866			2				+	
5	EAU80752	GH 31	1	2				+	α-
18	EAU80766		1	2				+	glucosidases
25	EAU81201	GH 47				4		+	α-mannosidase
12	EAU87930	GH 62		2				+	α-L-arabino-
102	EAU87931		1					+	furanosidase
43	EAU90116	GH 72			3	4		+	β-1,3-glucanosyl-
58	EAU92645			2	3	4		-	transglycosylase Gas1p
20	EAU86655	GH unknown	1	2				+	1,3-β-glucosidase
24	EAU91840	Ceramidase		2				+	N-acylsphingosine amidohydrolase

* Protein identification number generally used within this work
** NCBI protein accession http://www.ncbi.nlm.nih.gov/Genbank/
*** 1: Freely secreted proteins, 2: hyphal sheath proteins, 3: NaCl-extractable proteins, 4: SDS-extractable proteins, 5: NaOH-extractable proteins

Table 3.6: Putative peptidase families as a subgroup of hydrolases found in different fractions of the secretome from *C. cinerea* grown for 3 days on liquid YMG at 37 °C, defined after http://expasy.org/cgi-bin/lists?peptidas.txt

ID*	Protein accession**	Predicted function/properties	Clan	Sub-family	Fractions** 1	2	3	4	5	Signal peptide
		Metallopeptidases								
11	EAU82511		MA	M36	1	2				+
19	EAU88550	Require a divalent	MA	M49		2	3			-
21	EAU90085	metal ion for	MA	M43	1	2				+
26	EAU87896	their activity	MH	M28		2				+
38	EAU91256	Rao et al. (1998)	MA	M43	1	2				+
69	EAU87908		ME	M16				4		-
90	EAU83013		MA	M43	1					+
104	EAU90001		MH	M28	1					+
124	EAU92210		MF	M17			3			-
APS8	EAU90172		MH	M20	1					-
APS20	EAU87254		MA	M43		2				+
APS24	EAU86243		MA	M3			3			-
APS29	EAU91824		MH	M28				4		-
		Serine peptidase								
6	EAU90899		SB	S8	1	2	3	4		+
71	EAU91794	Group of serine	SB	S8	1	2				+
88	EAU82352	residues at the	SC	S33		2		4		+
92	EAU82190	active site	SB	S8		2				+
135	EAU91702	Rao et al. (1998)	SB	S8		2				+
		Aspartic type peptidase								
23	EAU84813	Catalytic activity dependent on aspartic acid residues Krysan et al. (2005)	AA	A1	1	2		4		+

* Protein identification number generally used within this work
** NCBI protein accession http://www.ncbi.nlm.nih.gov/Genbank/
*** 1: Freely secreted proteins, 2: hyphal sheath proteins, 3: NaCl-extractable proteins, 4: SDS-extractable proteins, 5: NaOH-extractable proteins

Table 3.7: Putative esterases and other hydrolases classified after BRENDA enzyme database as subgroup of hydrolases found in different fractions of the secretome from *C. cinerea* grown for 3 days on liquid YMG at 37 °C (http://www.brenda-enzymes.info/index.php4).

ID*	Protein accession**	Predicted function	Fractions*** 1	2	3	4	5	Signal peptide	Expected properties
3	EAU81840	Carotinoid ester lipase	1	2		4		+	Extracellular Zorn et al. (2005)
8	EAU93605	Metallophospho-		2				+	Serine/threonine specific
73	EAU81511	esterase		2				+	protein phosphatase
105	EAU93615			2				+	(normally intracellular) (BRENDA)
33	EAU86468	Dienelacton hydrolase	1	2				+	Chlorocatechol degradation pfam.sanger.ac.uk/
37	EAU93188	Glycerophos-phoryldiester phospho-diesterase		2				+	Broad specificity for glycero-phosphodiesters (in plants); cell wall construction in plants Hayashi et al. (2008)
APS44	EAU86921	1-Phosphatidyl-inositol phospho-diesterase	1					+	Phosphatidyl-inositol-specific phospholipase C Ryan et al. (2001)
84	EAU91144	Lipase		2				+	Extracellular; degradation of the plant cell wall McAuley et al. (2004)
112	EAU83363	Polysaccharide	1					+	Extracellular or cell wall
APS41	EAU85387	deacetylase			3			+	Jorge et al. (1999)
122	EAU91099	Acetyl-xylan esterase		2				+	Releases acetate from acetylated structures Ding et al. (2007)
2	EAU83081	Alkaline phosphatase		2	3	4	5	+	Extracellular providing P as nutrient Guimaraes et al. (2003)
64	EAU86905	Acid phosphatase	1					+	Extracellular Oshima (1997)
103	EAU88102	Phosphatic acid phosphatase				4		-	Extracellular Oshima (1997)
APS54	TS.ccin_ 1.174.103	Extracellular phosphatase		2				+	
APS57	EAU92592	Endoribo-nuclease	1					-	RNA processing Drider & Condon (2004)

* Protein identification number generally used within this work
** NCBI protein accession http://www.ncbi.nlm.nih.gov/Genbank/
*** 1: Freely secreted proteins, 2: hyphal sheath proteins, 3: NaCl-extractable proteins, 4: SDS-extractable proteins, 5: NaOH-extractable proteins

Table 3.8: Putative lyases, isomerases, transferases and synthases found in different fractions of the secretome from *C. cinerea* grown for 3 days on liquid YMG at 37 °C, classified after BRENDA enzyme database (http://www.brenda-enzymes.info/index.php4).

ID*	Protein accession**	Predicted function	Fractions*** 1	2	3	4	5	Signal peptide	Properties and references
29	EAU85209	Oxalate decarboxylase				4		+	Extracellular in fungi; formate producing; Mäkelä et al. (2002)
56	EAU92397	Enolase				4		-	Lyase; glycolytic enzyme Edwards et al. (1999)
63	EAU82343	Fructose bisphosphate aldolase			3			-	Lyase; glycolytic enzyme Elshafei et al. (2005)
APS9	EAU90126	Pyruvate carboxylase			3			-	Lyase; citrate cycle (BRENDA)
APS43	EAU86914	Aconitase			3			-	Citrate cycle (BRENDA)
28	EAU84825	Disulphide isomerase			3			+	Isomerase Wilkinson et al. (2005)
83	EAU86629	Triosephosphat isomerase			3			-	Isomerase Logsdon et al. (1995)
100	EAU84882	S-methyltransferase			3			-	Transfer of of methyl groups (BRENDA)
133	EAU93086	Acetyltransferase			3			-	Transfer of acetyl groups (BRENDA)
52	EAU82113	Peptidyl-prolyl		2				-	Isomerase
108	ccin_1.51-g27.1	cis-trans		2				+	Wang et al. (2001)
139	EAU93364	isomerase		2				-	
APS25	EAU90964			2				+	
APS27	EAU91055			2				+	
APS21	EAU84924	Para-hydroxy-benzonate-polyprenyl-transferase			3			-	Prenyltransferase superfamily (BRENDA)
APS40	EAU83254	Glutathione-S-transferase	1					-	Glutathione metabolism (BRENDA)
APS3	EAU82052	Citrate			3			-	Enzyme of the
APS37	EAU80161	synthase			3			-	citrate cycle
APS50	EAU83052				3			-	(BRENDA)

* Protein identification number generally used within this work
** NCBI protein accession http://www.ncbi.nlm.nih.gov/Genbank/
*** 1: Freely secreted proteins, 2: hyphal sheath proteins, 3: NaCl-extractable proteins, 4: SDS-extractable proteins, 5: NaOH-extractable proteins

Table 3.9: Putative proteins without predicted enzymatic activity found in different fractions of the secretome from *C. cinerea* grown for 3 days on liquid YMG at 37 °C. Classified after the NCBI protein BLAST (www.ncbi.nlm.nih.gov/).

ID*	Protein accession**	Predicted function	Fractions*** 1	2	3	4	5	Signal peptide	Properties and references
16	EAU86031	Porin				4	5	-	Mitochondrial membrane protein
30	EAU92338	Calreticulin			3	4		+	Calnexin-like; chaperon of the ER Feitosa et al. (2007)
45	EAU81622	Septin			3			-	Surface growth
115	EAU92914				3			+	Momany et al. (2001)
47	EAU91906	Heat shock			3			-	Eroles et al. (1997)
116	EAU90954	protein 20			3			-	López-Ribot et al. (1996)
49	EAU88955	Ricin-B-like				4		-	Lectin like protein with carbohydrate binding motive Candy et al. (2001)
APS12	EAU81467	protein				4		-	
APS32	EAU89191					4		+	
54	EAU91343	CBM-containing			3	4		-	
55	EAU85085	protein				4		+	
60	EAU84741	Heat shock 31-like	1	2				-	López-Ribot et al. (1996)
96	EAU88450	Heat shock			3			-	Eroles et al. (1997)
97	EAU87549	protein 70			3			-	López-Ribot et al. (1996)
137	EAU81969	Ceratoplatanin-like		2				+	Phytotoxin Pazzagli et al. (1999)
138	EAU92199	WSC-domain protein		2				+	Lodder et al. (1999)
APS13	EAU86184	Actinin-like protein		2				-	Important for cytokinesis Wang et al. (2009)
APS15	EAU90475	MFS monosaccharide transporter				4		-	Transport of small molecules pfam.sanger.ac.uk/
APS26	EAU90205	Cytochrome C1				4		+	pfam.sanger.ac.uk/
APS30	EAU92402	Proteasome subunit			3			-	Protein degradation pfam.sanger.ac.uk/
APS49	EAU88891	Activator of 90 kDa heat shock protein			3			-	ATPase homolog 1 pfam.sanger.ac.uk/
APS51	TS.ccin_1.112.139	Heat shock protein 90			3			-	pfam.sanger.ac.uk/

* Protein identification number generally used within this work

** NCBI protein accession http://www.ncbi.nlm.nih.gov/Genbank/

*** 1: Freely secreted proteins, 2: hyphal sheath proteins, 3: NaCl-extractable proteins, 4: SDS-extractable proteins, 5: NaOH-extractable proteins

Table 3.10: Putative intracellular contaminations found in different fractions of the secretome from *C. cinerea* grown for 3 days on liquid YMG at 37 °C. Classified after BRENDA enzyme database (http://www.brenda-enzymes.info/index.php4) and NCBI protein BLAST (www.ncbi.nlm.nih.gov/).

ID*	Protein accession**	Predicted protein function	Fractions*** 1	2	3	4	5	Signal peptide	Properties and references
9	EAU87957	Elongation factor		2	3			-	Protein synthesis
41	EAU92297				3			-	Sheu & Traugh (1992)
113	EAU93024				3	4	5	-	
53	EAU90579	Mitochondrial carrier enzyme				4		-	Energy transfer pfam.sanger.ac.uk/
59	EAU84527	Ubiquitin activating enzyme			3			-	Ubiquitin attached pfam.sanger.ac.uk/
66	EAU88183	Nucleoside diphosphate kinase	1	2	3			-	Signal transduction Hasunuma et al. (2003)
75	EAU93181	ADP/ATP carrier protein				4		-	ADP/ATP transporter pfam.sanger.ac.uk/
91	EAU84422	14-3-3-like protein			3			-	Intracellular signalling pfam.sanger.ac.uk/
101	EAU84514	Ran-binding protein			3			-	Nuclear import of proteins He et al. (1998)
110	EAU91660	Ribosomal protein				4	5	-	
114	EAU92441						5	-	
125	EAU84827				3			-	
APS11	EAU80499					4		-	
APS17	EAU84719					4		-	
APS31	EAU83020					4		-	
APS33	EAU93015					4		-	
117	EAU87151	Nucleosome assembly protein			3			-	Formation of nucleosome structures Ishimi & Kikuchi (1991)
123	EAU88709	ATPase			3			-	Plasma membrane localized; respiration Ohta et al. (1997)
126	EAU89150				3			-	
130	EAU88638	Ubiquitin ligase protein			3			-	Catalysation of the nucleotidyl transfer Shuman & Lima (2004)

* Protein identification number generally used within this work

** NCBI protein accession http://www.ncbi.nlm.nih.gov/Genbank/

*** 1: Freely secreted proteins, 2: hyphal sheath proteins, 3: NaCl-extractable proteins, 4: SDS-extractable proteins, 5: NaOH-extractable proteins

Table 3.11: Putative proteins with unknown function; no significant BLASTp hits or conserved domains were detected (www.ncbi.nlm.nih.gov/).

ID*	Protein accession**	Predicted function	Fractions*** 1	2	3	4	5	Signal peptide
34	EAU91944	Unknown protein 1				4		-
35	EAU86523	Unknown protein 2	1	2				+
46	EAU83394	Unknown protein 3	1	2	3	4		+
65	EAU850095	Unknown protein 4	1	2				+
70	EAU84200	Unknown protein 5			3			-
72	EAU82201	Unknown protein 6				4		+
78	EAU87630	Unknown protein 7				4		+
89	EAU88538	Unknown protein 8	1					+
93	EAU92705	Unknown protein 9	1					-
94	EAU83673	Unknown protein 10		2				-
98	EAU87109	Unknown protein 11				4		+
111	EAU85945	Unknown protein 13	1					+
129	EAU81962	Unknown protein 14				4		+
140	EAU81416	Unknown protein 16	1					+
APS48	EAU88534	Unknown protein 18		2				+
APS39	EAU82636	Unknown protein 19				4		-
APS35	EAU93299	Unknown protein 20				4		+
APS34	EAU93037	Unknown protein 21			3			-
APS16	EAU80978	Unknown protein 22			3	4		+
APS7	GLEAN_08660	Unknown protein 23				4		+
APS52	retrain_ccin_ Contig167-snap.9	Unknown protein 24		2				-

* Protein identification number generally used within this work
** NCBI protein accession http://www.ncbi.nlm.nih.gov/Genbank/
*** 1: Freely secreted proteins, 2: hyphal sheath proteins, 3: NaCl-extractable proteins, 4: SDS-extractable proteins, 5: NaOH-extractable proteins

3.4.2.1 Protein Identification

The enzymes were ordered according to the international enzyme classification (IUPAC). Most importantly, all proteins that were previously identified by the 2-DE analysis were also found in the 1-DE shotgun approach.

18% of the identified proteins belong to the class of oxidoreductases (Tables 3.3 and 3.4); amongst them was a copper radical oxidase, eight different FAD/FMN-containing oxidoreductases, two superoxide dismutases, two undefined oxidoreductases and two reductases, one of them unknown and one xylose-reductase (Table 3.3). Most of these oxidoreductases were identified in the freely secreted protein fraction and the hyphal sheath fraction. However, some of the identified oxidases are expected by function and lack of secretion signal to be intracellular enzymes (Table 3.4) such as malate dehydrogenase (used as marker enzyme for intracellular contaminations; Figure 3.1), succinate dehydrogenase, aldehyde dehydrogenase and formate dehydrogenase. These putative intracellular contaminations were mainly found in the NaCl-extractable fraction.

With 33% of the identified proteins, the hydrolases make up the largest group of enzymes identified. The hydrolases were subdivided into the following groups: carbohydrate processing enzymes (Table 3.5), peptidases (Table 3.6), esterases and other hydrolases (Table 3.7). 14 different glycoside hydrolases of nine distinct families (Pfam families 3, 5, 6, 15, 24, 31, 47, 62, and 72) and one of an unknown family were identified. 12 of 18 glycoside hydrolases were identified in the hyphal sheath fraction, while only 6 glycoside hydrolases were detected in the cell wall fractions.

19 different peptidases were identified (Table 3.6) in the secretome of *C. cinerea*, most of them were predicted to be metallopeptidases (13), but as well five serine peptidases and one aspartyl peptidase were detected. Moreover in the group of hydrolases, 8 putative esterases (lipases) were detected, amongst them an alkaline phosphatase and two acidic phosphatases.

Four proteins of the class of lyases, making up about three percent of the totally identified proteins, were exclusively detected in the cell wall fractions. Further, seven isomerases were detected in the hyphal sheath and in the NaCl extractable protein fraction and five transferases mainly detected in the NaCl-extractable fraction (summarized in Table 3.8).

Proteins with functions other then enzymatic were listed together in one table (Table 3.9). Amongst them were 6 heat shock proteins, detected in the cell wall fractions,

the freely secreted and the hyphal sheath fraction. Identified proteins without any or unreliable BLAST hits and without any predicted domains or functions were constituted as unknowns (Table 3.11). Although previous enzymatic tests [malate dehydrogenase, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase (Ibraheem et al., 2005; Sassoon & Mooibroek, 2001; Soundar et al., 2006)] indicated that the pure cell wall is free of intracellular contaminations, typical intracellular proteins such as ribosomal proteins, chaperons and some metabolic enzymes were detected in addition to the intracellular enzymes reported above to be found in the NaCl-extractable fraction (Table 3.10).

3.4.3 Search for Potential Pir and GPI Proteins of *C. cinerea*

3.4.3.1 Pir Proteins

The treatment of the pure cell wall fractions with 30 mM NaOH was previously used in other fungi for the extraction of alkali-sensitive linked cell wall proteins, so called Pir proteins (proteins with internal repeats) (Mrsa & Tanner, 1999). In the case of *C. cinerea*, such alkaline treatment revealed 6 different proteins, namely a glycoside hydrolase from family 3 (ID-number 1), an alkaline phosphatase (ID-number 2), a porin (ID-number 16), an elongation factor (ID-number 113) and two ribosomal proteins (ID-numbers 110 and 114). However, these were not only detected in the NaOH-extractable fraction but were as well present in one of the other fractions.

Typical proteins with internal repeats (Pir) contain a signal peptide, a Kex2 cleavage site, a glutamine-rich domain with a variable number of internal repeats and a C-terminal domain with four cysteine residues following the motive C-65/66-C16-C-12-C (Ruiz-Herrera et al., 2008) (Figure 3.8). None of the six proteins identified in this study from the NaOH-fraction had the typical cysteine motive, internal glutamine rich repeats or the Kex2 cleavage site. Signal peptides were however only predicted for the potential glycoside hydrolase (family 3)(ID-number 1) and the alkaline phosphatase

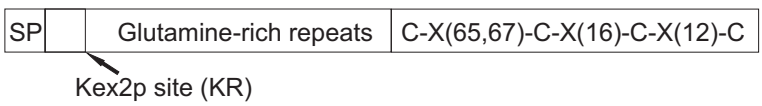


Figure 3.8: Schematic structure of Pir proteins (proteins with internal repeats; SP: Signal peptide). The C-terminal motive is in Prosite formate, as used for the FUZZPRO analysis to identify Pir proteins from the genome of *C. cinerea*; after Ruiz-Herrera et al. (2008)

Table 3.12: Proteins resulting from an *in silico* search for proteins with internal repeats (Pir) in the genome of *C. cinerea*.

Protein accession	Number of amino acids	C-motive	Signal peptide	Kex2 site	Repeats	Predicted function/ domain
EAU87325	384	215 - 313	-	+	-	MYND zinc finger; transcription factor
EAU82768	1365	340 - 438	+	+	-	Furin-like cysteine rich repeats; surface receptor
EAU91114	317	36 - 133	+	+	-	GCC2 and GCC3 family protein; unknown function
EAU81977	1083	531 - 628	+	+	-	TNFR/NGFR cysteine-rich region surface receptor

(ID-number 2). An *in silico* approach to identify proteins with internal repeats in the genome of *C. cinerea* was performed using the Pir-motive C-X(65,67)-C-X(16)-C-X(12)-C (Figure 3.8) in a FUZZPRO analysis of the whole genome (http://www.hgmp.mrc.ac.uk/Software/EMBOSS/) according to Weig et al. (2004). This approach revealed four putative proteins which contain a Kex2 cleavage site, in three of the four cases also a signal peptide and the typical cysteine motive but the latter is in none of the three cases at the C-terminus. Further, none of the four proteins shows the Pir-specific sequence repeats. Accordingly, none of the four proteins can be considered to be typical Pir proteins (Table 3.12).

3.4.3.2 GPI-Anchored Proteins

Further, we tried to experimentally identify putative GPI proteins as previously described by Ebanks et al. (2006) by digestion of the cell wall remaining after extraction of the other protein fractions with CNBr followed by a trypsin digest. For *C. cinerea*, this method revealed three different proteins (Table 3.13); an alkaline phosphatase (ID-number 2), previously detected in the hyphal sheath and the other cell wall fractions, a superoxide dismutase (ID-number 50) and newly one putative GPI-anchored protein without predicted function. However, only the newly detected protein (ID-number 149) has a putative GPI-anchor site predicted by the BIG-Π-predictor from Eisenhaber et al. (2004) and by the NCBI BLASTp search (for a precise description of the GPI anchor

Table 3.13: Proteins identified from the CNBr-trypsin digestion of the cell wall remaining after the extraction of the prior fractions.

ID-number	Protein code	Proposed protein function
50	EAU89294	Superoxide dismutase 2
2	EAU83081	Alkaline phosphatase
149	EAU90038	Putative GPI protein

and the GPI-attachment site see Figure 1.1). An *in silico* approach for the identification of putative GPI-anchored proteins in the genome of *C. cinerea* performed with the BIG-Π-predictor from Eisenhaber et al. (2004) revealed in contrast 652 putative GPI proteins. However, amongst the 162 identified proteins five showed a putative GPI-anchor site when analyzed with the BIG-Π-predictor Eisenhaber et al. (2004). These were two glycoside hydrolases (ID-numbers 43, 58), a polysaccharide deacetylase (ID-number APS41) and two unknown proteins (ID-numbers 46, 111).

3.5 Discussion

A proteomic analysis of the fractionated secretome from *C. cinerea* was performed in this study. Freely secreted and hyphal sheath proteins were extracted from the culture supernatant and the whole mycelium, respectively. From the subsequently isolated cell wall, NaCl-, SDS- and NaOH-extractable proteins were extracted and finally a CNBr-trypsin digestion was performed in order to detect possible GPI-anchored proteins. Protein profiles and compositions revealed a significant difference between the freely secreted and the cell wall proteome with only low overlapping of proteins. To control the purity of the isolated cell wall from intracellular contaminations, three representative intracellular enzyme activities (malate dehydrogenase, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase) were spectrometrically measured, showing no detectable activity in the purified cell wall fraction (Figure 3.1). However, protein identification revealed several typically intracellular proteins, mostly of proteins known to appear in high abundance (Table 3.10). In the enzyme tests, about 100 µg of protein was used whereas 3 mg, 30 times as much, was loaded on the 1-DE gels for the LC-MS^2 analysis. In the 2-DE analysis, only spots of about 50 to 100 ng were concentrated enough for a positive identification. Transferred to the enzyme test, this would be an equivalent of maximum 1.7 to 3.3 ng protein. This value certainly would be too low to detect any of the control enzymes.

Two strategies were used to analyze the fractionated secretome from *C. cinerea*: a 2-DE approach and a 1-DE shotgun approach. Both applied strategies showed their advantages. With the 1-DE shotgun approach it was possible to identify totally 162 different proteins from the fractionated secretome. In contrast, 2-DE electrophoresis of the protein fractions gave information about the general characteristics in protein composition of the fractions, such as molecular weight, pI-value or posttranslational modifications but it was unfortunately limited to the identification of high abundant protein spots.

3.5.1 2-DE Analysis of the Fractionated Secretome from *Coprinopsis cinerea*

C. cinerea was grown in liquid shaking cultures in YMG medium, containing yeast-extract, malt and glucose. The single protein fractions were harvested from these liquid cultures and 2-DE was performed as described in Material and Methods. From the 2-DE profiles of the secreted proteins from *C. cinerea*, it was obvious that the cell wall proteins differ strongly from the proteins attached to the hyphal sheath and the freely secreted proteins (Figure 3.3). The number of spots detected on the gels of the different fractions gives an indication of a highly complex cell wall proteome with numerous different proteins.

Comparing the number of spots with the total amount of protein in the single fractions makes obvious that the freely secreted protein fraction consists under the tested growth conditions (YMG complete medium) of a comparable small protein diversity but with a number of high abundant proteins (Figure 3.3). Most of these are in the high molecular weight range and have low pI values (between 3.5 and 5). The 2-DE profile of the free secretome and the hyphal sheath proteins showed a similar profile (Figures 3.3 A and B), although the individual protein amount in this fraction is much lower (Figure 3.2). In contrast, the cell wall fractions seem to bear many different low abundant proteins (Figures 3.3 C and D). The free secretion of only a few but highly abundant proteins likely indicates an adaption of the fungus to the given culture medium (Ravalason et al., 2008; Sato et al., 2007), while the cell wall proteome is expected to be more independent from the culture conditions. In this study, the fungus was grown in YMG medium containing e.g. proteins from yeast extracts and the manifold occurrence of peptidases in the free secretome (see also below) is consistent with an adaption of the freely secreted enzymes to the available nutrients of the medium offered.

Most of the identified protein spots show a shift of molecular weight and pI-value compared to the theoretic values into the higher range, respectively for the pI either into the lower or the higher value range. This is commonly caused by posttranslational modifications (PTM) of extracellular proteins (compare the positions of e.g. spots with the ID-numbers 2, 11, 15, 54, 55, 71 shown in Figure 3.3 and listed in Table 3.2 with their theoretical pI and MW-values and spot positions on the 2-DE gels). For instance, glycosylations cause an increase in molecular weight and are well known modifications for extracellular fungal proteins (Peberdy, 1994). Glycosylations are thought to protect proteins from protease digestion (Marinaro et al., 2000), which seems to be important considering for example the large number of peptidases identified from the secretome of *C. cinerea* in this study (Table 3.2).

Another indication for extensive posttranslational modification of the *C. cinerea* secretome is the formation of spot trains in the 2-DE gels (chains of densely arranged spots of proteins with the same MW) (Pietrogrande et al., 2006). This phenomenon was for example observed for the alkaline phosphatase (ID-number 2) detected in all except of the freely secreted fraction (Figure 3.3). Various different types of PTMs, changing the charge of the amino acids, can be responsible for the formation of spot trains (Pietrogrande et al., 2006).

However, not only different PTMs result in the distribution of one protein over several spots. This can also be caused by the formation of isoforms (different protein forms derived from one gene, resulting e.g. from alternate splicing or different N- or C-terminal processing of the protein product) as it was observed in this study to occur for the copper radical oxidase (ID-number 4; Figure 3.3 A and B). The later was distributed over totally 4 distinct spots with different molecular weight and pI value.

While the 2-DE gels give exiting information on the complexity of the different secretome fractions and the extensive variations between them (Figure 3.3), identification of specific proteins by LC-MS^2 is unfortunately only limited due to the often too low protein amount in the spots. In consequence, information on individual proteins is restricted to the high abundant proteins.

3.5.2 1-DE Shotgun Approach

Since the analysis of 2-DE gels had its limitations in protein identification, a 1-DE shotgun approach of the different secreted protein fractions was performed. The advantage of a 1-DE shotgun approach over the analysis of single spots from 2-DE gels is that it is less laborious and, most importantly, a 1-DE shotgun approach makes it possible to identify also low abundant proteins. This is due to the fact that the total amount of protein loaded on 1-DE gels can be much higher than for 2-DE gels. The 1-DE approach resulted in the identification by LC-MS^2 of totally 162 proteins from all analyzed secretome fractions which were grouped into the IUPAC enzyme classes (International Union of Pure and Applied Chemistry), proteins without enzymatic function, unknown proteins and putative intracellular contaminations (see Tables 3.3 to 3.11).

3.5.2.1 Extracellular Oxidoreductases

A larger part of identified enzymes belongs to the class of oxidoreductases (Tables 3.3 and 3.4) distributed over all examined fractions, except the NaOH-extractable protein fraction. Oxidoreductases with a classical secretion signal were predominantly identified in the freely secreted fraction and the hyphal sheath (Table 3.3), while oxidoreductases lacking a classical secretion signal were mainly detected in one of the cell wall fractions. Amongst these were also typically intracellular enzymes of, for example, the citric acid cycle (Table 3.4). The function of the latter is discussed more extensively in Section 3.5.2.5.

However, many of the oxidoreductases are known or expected to be involved in substrate degradation and to be secreted and thus to come in close contact to the substrate, meaning here the culture supernatant and the hyphal sheath as the outer interface of the fungal hyphae.

Many of the predicted oxidoreductases are putative H_2O_2 providing enzymes, such as the eight different FAD-domain (Flavin Adenine Dinucleotide) containing oxidoreductases (ID-numbers 14, 31, 32, 36, 85, 119, APS1 and APS36) identified in this study in the 1-DE approach. Two of these putative oxidoreductases were already identified from the 2-DE gels as high abundant spots (ID-numbers 31 and 36). Often involved in the oxidation of sugars, FAD-containing oxidoreductase might be an important source for H_2O_2 (Kutchan & Dittrich, 1995). H_2O_2 might also be provided by the identified putative copper radical oxidase (CRO; ID-number 4) similar as postulated for glyoxal

oxidases in the white rot fungus *P. chrysosporium* (Vanden Wymelenberg et al., 2006). The 2-DE analysis of the *C. cinerea* secretome showed that this copper radical oxidase is distributed over four high abundant spots with different molecular weight and pI value in the freely secreted and the hyphal sheath fraction, indicating the formation of isoforms. This putative copper radical oxidase from *C. cinerea* contains 4 copies of a WSC domain, defined as a carbohydrate binding domain (www.ebi.ac.uk/interpro/). These might be responsible for the binding of the carbohydrate substrate to the enzyme.

In total, eight different enzymes for the supply of H_2O_2 were identified; in contrast to this only one putative peroxidases (ID-number 118) as potential consumer of H_2O_2 was detected. However, the well known *C. cinerea* oxidase (Cip) was not detected in this study (Baunsgaard et al., 1993). The putative peroxidase identified in this study is a new enzyme for *C. cinerea* and is related to the plant peroxidase superfamily class I, containing peroxidases dependent on a steady-state concentration of H_2O_2 and further fungal cytochrome C oxidases located in the mitochondria, but however no secreted peroxidases (http://pfam.sanger.ac.uk/). Thus, this specific peroxidase might be an intracellular contamination.

Although *C. cinerea* is a fungus which is normally not involved in the degradation of wooden substrates, but rather a dung inhabiting fungus (Kües, 2000), there is nevertheless evidence that the fungus shows some lignocellulose degrading abilities (e.g. on straw and wood chips) (Navarro-González, 2008). The detection of putative lignocellulose degrading enzymes, e.g. the putative copper radical oxidase confirm, these observations. *C. cinerea* seems to possess an enzymatic system for the degradation of lignocellulose which is possibly an evolutionary artefact and might be induced under stress, such as provided by an artificial liquid medium. On natural organic substrates, the fungus grows on the surface with air contact (Kües, 2000).

An alternative use for H_2O_2 besides the consumption by peroxidases is possibly the defence against competitors as known for plants (Salzer et al., 1999) and as suggested for e.g. ectomycorrhizal fungi (Gafur et al., 2004). Besides the H_2O_2 producing enzymes, one catalase (ID-number 61) was detected in *C. cinerea* within the NaCl-extractable cell wall proteins, possibly responsible for the detoxification of H_2O_2 and other reactive oxygen intermediates, known as a self-protection mechanism (Abbott et al., 2009). Also the two putative superoxide dismutases (ID-numbers 50 and 131) reported in other species to be located extracellularly, soluble in the growth medium and cell surface attached (Chaturvedi et al., 2001), may play a crucial role in the antioxidant defence by

removing mainly O_2-radicals (Chaturvedi et al., 2001). The combination of superoxide dismutases and catalase could be a very effective defense system against O_2-radicals, H_2O_2 and reactive oxygen intermediates and could provide the fungus with a defence system against the attack of competitive microorganisms.

In contrast to the rather unexpected variety of H_2O_2 producing enzymes, the finding of a ferric reductase (ID-number APS22) is not as much surprising as iron is an essential element for almost all organisms, used in a variety of cofactors. For this reason, iron uptake is a crucial part of the nutrient supply. In this experiment, one ferric reductase (ID-number APS22), shown to be attached to the cell surface in other fungi (Haas, 2003), was identified from the NaCl-extractable fraction of the *C. cinerea* cell walls. This protein was shown in other fungi to be essential for the iron uptake into the cell being part of the reductive iron assimilatory system (Haas, 2003).

3.5.2.2 Glycoside Hydrolases

Most of the hydrolases in this study were identified to be glycoside hydrolases, mainly detected in the freely secreted and the hyphal sheath fraction. Though the glycoside hydrolases present in the free secretome are most likely involved in the nutrient supply, many of them are as well present in the hyphal sheath. Thus, it can not be excluded that several of these glycoside hydrolases have also a function in the formation of the hyphal sheath. The few glycoside hydrolases found within the cell wall fractions are most probably involved in the formation and rebuilding of the cell wall structure (Bernard & Latgé, 2001).

Among the glycoside hydrolases detected in this study, two homologues of the Gas1p protein (β-1,3- glucanosyltransglycolases) from glycoside hydrolase family 72 of *S. cerevisiae* were found in the cell wall fractions of *C. cinerea*. The Gas-proteins play an important role in the biosynthesis of the cell wall (Mouyna et al., 2000a,b) and catalyze the reaction of splitting β-1,3-glucan molecules and transferring the generated reducing end to a non-reducing end of an other β-1,3-glucan molecule (Mouyna et al., 2000a). Usually in yeasts, β-1,3- glucanosyltransglycolases are bound to the cell wall by GPI anchors but they were as well found to be freely secreted (Mouyna et al., 2000a). In *C. cinerea*, the two identified Gas1p-homologues seem not to be GPI-anchored because they were detected in the hyphal sheath, the NaCl- and the SDS-extractable protein fractions. Inspection of the protein sequence, however, revealed for both proteins the presence of a putative GPI anchoring site (see Figure 1.1 in Chapter 1). It remains

a question whether the computational prediction of GPI anchor attachment site as applied for ascomycetes (Eisenhaber et al., 2004) is also applicable for basidiomycetes.

For this study, *C. cinerea* was grown on a glucose-based complete medium to the early exponential growth phase. Growth curves, created by the measurement of ergosterol and the dry weight of fungal cultures (see Figure 5.1 in Chapter 5) show that at the time point of harvest (day 3) the dry weight of the fungal cultures was highest, while the ergosterol values were still low and the glucose was totally consumed. This indicated a high accumulation of biomass different than living fungal mycelium. A hypothesis therefore might be that the glucose was used up by the fungus to form the hyphal sheath, possibly as a way of nutrient storage. This possibly can explain the high number of different glycoside hydrolases in the freely secreted and the hyphal sheath fraction probably with the purpose to store nutrient (for further discussion see Chapter 5).

3.5.2.3 Peptidases

In the class of hydrolases, also many different peptidases were identified (Table 3.6), both in the 1-DE shotgun approach as well as is in the 2-DE gels. Since peptidases occur in a huge diversity in nature, they were generally arranged into clans and families according to their evolutionary ancestors and their catalytic activity (http://merops.sanger.ac.uk/cgi-bin/make_clan_index?type=peptidase). In this study, peptidases were predominantly detected in the freely secreted and the hyphal sheath fraction and most of them bear a classical secretion signal.

Extracellular proteases from basidiomycetes were not intensively studied until now. Some work on white rot fungi indicate that specific extracellular proteases are used for the regulation of enzyme activity. In *P. ostreatus*, Palmieri et al. (2001) showed a regulation of laccase activity by a specific processing protease purified from the culture supernatant. In *P. chrysosporium* grown in nitrogen limited submerged cultures, protease activities were observed to increase during carbon starvation (Dosoretz et al., 1990). However, it remains unclear which individual purpose the many different peptidases may have in the cultures of *C. cinerea*.

Besides the possible role of enzyme processing by specific proteases, as proposed by Palmieri et al. (2001) for the activation of laccase, secreted peptidases in general contribute to provide nitrogen and essential amino acids for the fungus. The artificial medium used in this study most probably contains a higher concentration of organic

nitrogen than the natural substrate of *C. cinerea* (horse dung) expected to be poor of organic nitrogen. Such difference in organic nitrogen in form of proteins and peptides might be an explanation for the occurrence of these various different peptidases. Unnaturally high organic nitrogen concentration in the medium might be responsible for the expression of various peptidases.

In *C. cinerea*, a superfamily of fungalysin extracellular metalloproteases associated to the metalloprotease family M36 was predicted from the annotated genome (Lilly et al., 2008). Our proteomic analysis of the *C. cinerea* secretome revealed one of the 8 predicted fungalysin metalloproteases (EAU82511). Fungalysin family peptidases in ascomycetous fungi are secreted when the fungi are grown in protein rich media (Jousson et al., 2004).

3.5.2.4 Other Hydrolases

The third group of identified hydrolases were esterases and lipases, listed in Table 3.7. Esterases catalyze the reversible splitting of esters into an acid and an alcohol (http://www.brenda-enzymes.org/). A related enzyme to the glycerophosphoryl diester phosphodiesterase (ID-number 37) is known to act in the periplasma of bacteria, possibly linking the cell wall polysaccharides to the cell membrane (Tommassen et al., 1991). A homologue to an extracellular carboxyesterase (ID-number 3) characterized from *Pleurotus sapidus*, hydrolyzing xanthophyll esters as commonly being present in plant cell walls (Zorn et al., 2005), was detected in the *C. cinerea* secretome, and might also be involved in substrate degradation of compounds of present in malt or yeast extract. Phosphatidylinositol phosphodiesterases (ID-number APS44) are ubiquitous enzymes important in intracellular signal transduction pathways of eukaryotic cells (Ryan et al., 2001). Possibly also in basidiomycetous fungi it plays a role in signal transduction and was found to be cell-wall-bound in ectomycorrhizal fungi (Gibbson & Mitchell, 2005).

Polysaccharide deacetylases are not well studied enzymes for fungi. Only few evidence exists that these enzymes are involved in the cell wall biogenesis as they are deacetylating for instance N-acetylglucosamine, a component of chitin, for the elongation of the chitin chains (Jorge et al., 1999).

Phosphatases are participating in numerous processes inside and outside of the cell; this includes the supply of phosphate for nutritional purpose (Guimaraes et al., 2003).

The phosphatases are most probably responsible for the supply of phosphate during cell growth. The YMG medium used for this study has a high phosphorus content (67 μg/ml; see Chapter 4)) and this possibly induced the production of high amounts of phosphatase. The finding of an acidic phosphatase in the free secretome is coherent concerning the fact that the culture liquid had an acidic pH at the time point of harvest. In contrast, an alkaline phosphatase was detected in the hyphal sheath and in all cell wall fractions. This indicates the formation of an enclosed milieu in the hyphal sheath and the cell wall deviating in pH from the large outer environment.

3.5.2.5 Possible Intracellular Contaminations and Moonlighting Proteins

In this study, we also found some typically intracellular proteins mostly within the cell wall fractions of *C. cinerea* but also in the freely secreted protein fraction; amongst these were heat shock proteins of the families 20, 31, 70 and 90 (ID-numbers 47, 60, 96, 97, 116 and APS51), enolase (ID-number 56), proteins of the citric acid cycle such as malate dehydrogenases (ID-numbers 57, 86), citrate synthases (ID-numbers APS3, APS37, APS50) and succinate dehydrogenase (ID-number 87). The highest number of these putative intracellular enzymes was found in the NaCl-extractable fraction, indicating in fact a contamination of intracellular proteins bound to the purified cell walls. Since the NaCl-extractable fraction is the first fraction extracted upon cell breakage from the isolated cell wall, possibly attached intracellular contaminations are most likely washed off in this extraction step. However, also the SDS-extractable fraction revealed putative intracellular contaminations such as succinate dehydrogenase. The latter is the only enzyme in the citric acid cycle which is bound to the mitochondrial membrane (Silkin et al., 2007). Technically, the cell wall can not be separated from the cell membrane by a density gradient as used in this study, and also the mitochondrial membranes are therefore isolated together with the fungal cell walls in the same density fraction.

Though the isolation of cell membrane proteins requires different isolation techniques, the treatment of cooking the cell wall with SDS and β-mercaptoethanol-containing buffer revealed few typical membrane proteins, amongst them were not only succinate dehydrogenase but as well the transmembrane proteins porin (ID-number 16) and septin (ID-number 45 and 115).

Generally, very high abundant intracellular proteins (chaperones, elongation factors and ribosomal proteins) are more likely to contaminate fractions of the isolated cell

wall proteins. In this study, such abundant intracellular proteins were mainly identified from the NaCl-extractable cell wall fraction (Table 3.10). The contaminating proteins detected in the secretome of *C. cinerea* were from various enzymatic groups and in the group of proteins without enzymatic function (Table 3.10). In *C. albicans* and *S. cerevisiae* glycolytic enzymes and chaperones were shown to be attached to the cell wall and it is believed that this is not only a methodical problem of cell wall isolation (Alloush et al., 1997; Eroles et al., 1997; Gil-Navarro et al., 1997; López-Ribot & Chaffin, 1996; López-Ribot et al., 1996). For example, 3-phosphoglycerate kinase (Alloush et al., 1997) or glyceraldehyde-3-phosphate dehydrogenase (Gil-Navarro et al., 1997), both glycolytic enzymes, are as well located in the cell wall of yeasts in addition to their expected intracellular localization. Also enolase, as well a glycolytic enzyme, and proteins of the heat shock protein family 70 (Hsp 70), a family of chaperones, were detected in the cell wall of *C. albicans* (Edwards et al., 1999). However, the biological function of such protein binding in the cell wall is not as clear. Saving of proteins after lysis of neighboring cells on the cell walls in order to avoid loss of valuable organic substances is one possibility discussed for this phenomenon.

From this experiment it can not be differentiated whether the putative intracellular proteins are secreted e.g. by a yet unknown mechanism and attached to the cell wall or whether they are intracellular contaminations within the isolated cell wall fractions. Regarding the observations in other fungi cited above, it can not be excluded that typically intracellular enzymes are located also in the cell wall of *C. cinerea*. However, until now a purpose for such proteins in the cell wall is not understood. In *C. albicans*, these unconventional cell wall proteins seem to play a role during the infection of the host by serving the fungus in the penetration of the host cell (Eroles et al., 1997). Nombela et al. (2006) suggest that those proteins are "moonlighting" proteins, performing multiple functions, depending on their location.

3.5.2.6 Unknown Proteins

In total, 24 proteins without any predicted function or domain were detected within totally 162 identified proteins corresponding to 14.8% of the identified proteins. This rather high number of totally unknown proteins within the secretome analyzed in this study might give an indication of the still uncharacterized proteins being encoded in the whole genome of *C. cinerea*. Also in other fungi with available genomes, a rather high

number of proteins without predicted function were detected in the genome sequence, for example in *L. bicolor* (Martin et al., 2008) or *U. maydis* (Kämper et al., 2006). The ongoing sequencing of other basidiomycetous genomes will indicate whether also other species contain as much genes for unknown proteins, being either specific to a species or occurring also in other basidiomycetes. Such comparative genomics might initiate the characterization of such proteins.

3.5.3 *In Silico* Prediction of Putative Pir and GPI Proteins

3.5.3.1 Pir-Proteins

In yeasts, the extraction of the cell wall under mild alkali conditions revealed proteins with internal repeats (Ecker et al., 2006), being functionally involved in the recovery from heat shocks (Tohe et al., 1993) and in the maintenance of the cell wall structure (Mrsa & Tanner, 1999). The extraction of the cell walls from *C. cinerea* with 30 mM NaOH in this work revealed no proteins with internal repeats. Only an alkaline phosphatase (ID-number 2), a glycoside hydrolase of family 3 (ID-number 1), a porin (ID-number 16), an elongation factor (ID-number 113) and two ribosomal proteins (ID-numbers 110 and 114) were identified from the NaOH-extract of the cell wall. The ribosomal proteins as well as the elongation factor can be considered as intracellular contaminations. The putative alkaline phosphatase and the glycoside hydrolase from family 3 were already identified in one or more of the fractions obtained prior to NaOH extraction. After this extraction an appreciable amount of protein is still present in the cell wall. Possibly, a complete extraction of all cell wall proteins can not be achieved most likely due to structural hindrances.

An *in silico* approach for the identification of putative Pir proteins showed that in the genome of *C. cinerea* genes for four proteins with the typical cysteine motives and the Kex2 cleavage site are present (Figure 3.8). The cysteine motive of characterized Pir proteins is typically located at the C-terminal end (Weig et al., 2004) but only in one of the four putative proteins with internal repeats found in *C. cinerea* the cysteine motive is in fact located at the C-terminus (EAU87325). However, this protein shows no signal peptide. The other three putative proteins have the cysteine motive in the middle of the amino acid sequence. However, a closer look on the four protein sequences showed that also the typical repeats in the internal of the sequence are low conserved to each other, unlike as described for ascomycetes (Weig et al., 2004). This might result from

the fact that the repeats are not as conserved as in yeasts and possibly vary in their sequence within one protein, making them hardly detectable. In conclusion, none of the four proteins is fulfilling the requirements for typical Pir proteins, only the typical Kex cleavage site was found for all putative proteins in this analysis.

Further, BLAST searches with known Pir proteins from *S. cerevisiae* or *C. albicans* detected no homologues to the genes of the yeast Pir proteins in the genome of *C. cinerea*. This leads to the conclusion that in *C. cinerea* likely no typical Pir proteins exist. In this content it is interesting to note that an *in silico* approach for the identification of Pir proteins in *U. maydis* (Ruiz-Herrera et al., 2008) as well revealed no proteins with typical Pir motives in this fungus. This suggest either that the existence of Pir proteins is restricted to ascomycetes or that Pir-like proteins derived from basidiomycetes bear completely different sequence motives.

3.5.3.2 GPI-Anchored Proteins

The experimental approach for the identification of GPI-anchored proteins revealed only three different proteins after the digestion of the remaining CWPs with CNBr and trypsin. This method was successfully used before for the identification of GPI-proteins in *C. albicans* (Ebanks et al., 2006). However, in *C. cinerea* only one of the so identified proteins has a putative GPI-anchor site, as predicted by the BIG-Π-predictor from Eisenhaber et al. (2004) and homologies to other GPI anchored proteins in the NCBI BLASTp search (Table 3.13; for a precise description of the GPI anchor and the GPI-attachment site see Figure 1.1). The other two proteins, alkaline phosphatase (ID-number 2) and superoxide dismutase (ID-number 50) were already detected in various of the previously extracted fractions and bear no putative GPI-anchor site. Possibly the combined digestion of the remaining cell wall proteins with CNBr and trypsin was not successful due to steric hindrances caused by cell wall polysaccharides. Basidiomycetes are known to produce many different metabolites and modified polysaccharides, as well as charged molecules (Carbonero et al., 2008; Ng, 1998; Rosado et al., 2002; Scherba & Babitskaya, 2008). Presence of such compounds might prevent the extraction of peptides from the cell wall network due to for example electrostatic forces. Although no experimental evidence was found, it seems unlikely that the cell wall of *C. cinerea* does not contain GPI-anchored proteins because the protein concentration in the cell wall is still remarkably after the extraction of all fractions.

A screen of the whole genome sequence for motives typical for GPI proteins in fungi

(De Groot et al., 2003) revealed 652 putative GPI proteins, which would correspond to a percentage of 4.8% of all proteins (13,544 predicted genes for *C. cinerea*). However within the 162 proteins identified in course of this work only one protein revealed to be a putative GPI protein (ID 149; EAU90038), as mentioned above. This protein was as well found within the 652 putative GPI proteins predicted with the BIG-Π-predictor Eisenhaber et al. (2004) to have a GPI anchor. In ascomycetes the percentage of putative GPI proteins lies around 1% as also predicted with the big-Π predictor program from Eisenhaber et al. (2004). As the sequence motives and algorithms used by this predictor are adjusted to ascomycetes, they are not necessarily as well suitable for basidiomycetous GPI motives, which were not extensively studied up to now. For this reason a prediction of GPI proteins in *C. cinerea* might not be reliable with the available tools.

3.5.4 Conclusions and Future Outlook

In conclusion, this study showed that the freely secreted proteome differs significantly from the cell wall proteome. Low overlapping of the freely secreted, the hyphal sheath and the cell wall proteins indicates directed and often selective secretion of proteins to the extracellular medium and to the cell wall of *C. cinerea*. The composition of the freely secreted and the hyphal sheath proteome is most probably highly dependent on the respective culture conditions (including a liquid environment and the unnatural shaking of the fungus) and the given nutrient sources. As these fractions of the proteome are in direct contact with the nutrient for degradation, they are most likely more flexible in protein composition than the cell wall proteome, which consists of many proteins thought to be responsible for the formation and restructuring of the cell wall and which should therefore by function be less dependent on the environmental conditions.

The liquid environment provided in this study probably caused despite of shaking, an oxygen deficiency and a too high humidity, resulting in additional stress for the fungus. Therefore, the proteome possibly shows stress responses of the fungus especially in the freely secreted and the hyphal sheath proteins.

Also as a reaction on the stressing culture conditions, the formation of a hyphal sheath for example could result from the high glucose content in the culture medium. On the other hand, it might be a structure which in nature retains the degradative enzymes which were found in the culture supernatant during this study. Future studies

have to show how these extracellular structures conduct under more natural growth conditions.

However, the freely secreted and hyphal sheath proteins consisted of degradative enzymes most likely for nutrient supply such as peptidases, glycoside hydrolases and several different oxidoreductases, these enzymes mainly have a classical signal peptide directing them to the extracellular space. In contrast, the cell wall proteome revealed several enzymes most likely responsible for the formation or restructuring of the cell wall, such as a Gas1p related glycoside hydrolases and as well several proteins without a predicted enzymatic function possibly having a structural function in the cell wall polysaccharide network. This as well shows a clear compartmentation in the sense of allocating tasks such as nutrient supply and cell wall formation.

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4 Post-translational Modifications in the Freely Secreted and the Cell Wall Proteome of *Coprinopsis cinerea*

4.1 Abstract

Post-translational modifications (PTMs) are very common for all kind of proteins in all organisms. Two well studied PTMs are glycosylation and phosphorylation of proteins. While protein glycosylation in fungi is well known to extensively occur on extracellular proteins, extracellular protein phosphorylation in fungi is less studied. We used ^{33}P-labeled phosphate in a phosphate-reduced medium to label the freely secreted, the hyphal sheath and the extractable cell wall proteins from *Coprinopsis cinerea* monokaryon Okayama 7 grown to the exponential phase. To differentiate between the protein phosphorylation and possible phosphorylation of sugars on glycosylated proteins, we applied the endoglycosidase PNGaseF to deglycosylate the extracellular proteins from *C. cinerea* prior to ^{33}P-detection. The study shows that proteins in the free secretome and the hyphal sheath of *C. cinerea* are highly glycosylated and that deglycosylation changes the protein profiles of 2-DE gels drastically. In contrast, the 2-DE profile of the extractable cell wall proteins is less effected by deglycosylation, indicating that the majority of those proteins is possibly not glycosylated. Further, we present evidence that some proteins in the cell wall of *C. cinerea*, the hyphal sheath and as well the free secretome are phosphorylated. Phosphorylation seems to occur only on specific proteins. We identified seven different proteins showing a positive signal in autoradiography, three of them lost the incorporated labeled phosphate after deglycosylation (with PNGaseF removing the N-glycosylations). The other four proteins the phosphate group is e.g. part of a prosthetic group, or of a GPI anchor, linked to an amino acid or to the sugars attached by O-glycosylation to the protein.

4.2 Introduction

Posttranslational modifications (PTMs) in general are considered to be one of the major reasons for organisms' protein complexity (Delom & Chevet, 2006). At least 200 different PTMs are known up to date, but only a few of them are known to be reversible and this was shown to play crucial roles in the regulation of biological processes (Delom & Chevet, 2006). Protein glycosylation is one of the major post-translational modifications occurring in all eukaryotic organisms and also in many prokaryotes (Geysens et al., 2009; Kukuruzinska & Lennon-Hopkins, 1999). Glycosylations are known to play an important role in protection of the proteins against protease digestion (Marinaro et al., 2000). Further, it assists in protein folding, targets proteins to specific locations, and regulates protein activity. Analysis of glycoproteins is challenging due to the fact that they can form isoforms of polypeptide chains varying in pI and molecular weight, differences that are revealed on 2-DE gels (Fryksdale et al., 2002). Two main types of glycosylation can be differentiated, N- and O-glycosylation, meaning that the sugar residues are either attached to the β-amide group of asparagine or to the β-hydroxyl group of serine or threonine residues. An example for a typical N-glycosylation as it occurs in *Saccharomyces cerevisiae* is shown in Figure 4.1 (Wildt & Gerngross, 2005). Glycosylation is a well examined PTM for fungal proteins (Wildt & Gerngross, 2005) and was shown to be responsible for the large variety of enzyme isoforms in e.g. *Phanerochaete chrysosporium* (Leisola et al., 1987; Tien & Kirk, 1984). Heterogeneity of glycoproteins is not only visible in their molecular weights but also causes charged heterogeneity as result of charged groups attached to the monosaccharides of glycosylated proteins such as phosphate or sulfate groups (Freeze, 1985; Ivatt et al., 1984; Leisola et al., 1987). Phosphate groups can as well be directly attached to the amino acids serine, threonine or tyrosine. This reversible protein phosphorylation acts generally as a common intracellular tool for signal transduction, cell differentiation and development, cell cycle control and metabolism (Delom & Chevet, 2006). Extracellular functions for protein phosphorylation in fungi are so far rarely studied (Kuan & Tien, 1989).

The fungal cell wall is a complex network of polysaccharides presenting a barrier and simultaneously a contact zone to the fungal environment. Therefore, this extracellular structure of the fungal cell contains a variety of different proteins responsible for structuring and restructuring the cell wall, substrate degradation or host infection (Rast et al., 2003). A large number of cell wall proteins as well as freely secreted proteins

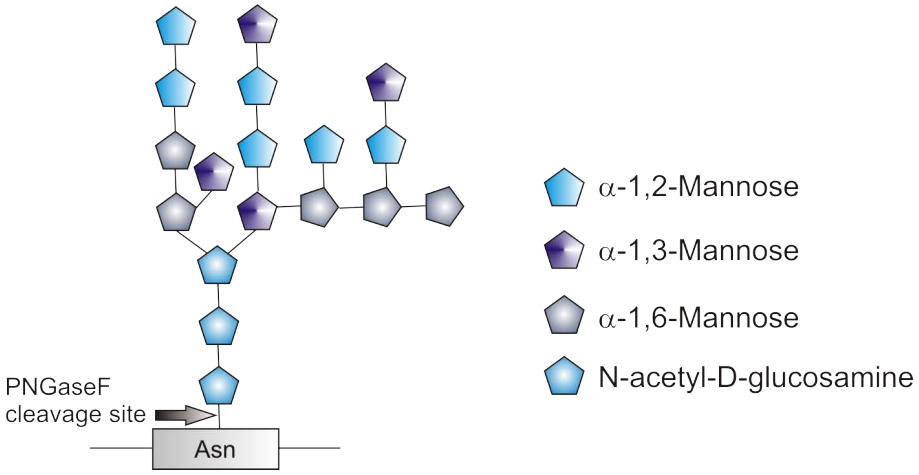

Figure 4.1: Typical N-glycosylation on the amino group of asparagine in protein chains as it occurs in the yeast *S. cerevisiae* (Wildt & Gerngross, 2005); the arrow indicates the cleavage site of the endoglycosidase PNGaseF.

from fungi were found to be highly glycosylated (Wildt & Gerngross, 2005). In contrast, phosphorus was shown to be a minor component of the cell wall in *S. cerevisiae* (Ruiz-Herrera, 1992) where it is involved in the phosphodiester linkages between mannose residues of the glycan network (Kollár et al., 1995, 1997). Phosphate groups were as well found to be present in phosphomannan protein complexes (Shibata et al., 1986, 1989) showing that they are not only a part of the cell wall structure but also attached to cell wall glycoproteins. Casanova & Chaffin (1991) suggested that the phosphorus present in the cell wall of *Candida albicans* is distributed between the phosphomannan of the cell wall structure, phospho-mannoproteins and phosphoproteins (proteins without glycosylation but with phosphate groups). This implicates also a phosphorylation of cell wall proteins in *Candida albicans*. In *P. chrysosporium*, association of phosphate groups to lignin peroxidase glycosylations was demonstrated already in 1989 by Kuan & Tien (1989), revealing that phosphorylation and dephosphorylation of this protein is one possible reason for changes in isoform patterns during fungal growth (Rothschild et al., 1999).

In this study, we analyze phosphorylation of particular proteins within the free secretome, the hyphal sheath and the extractable cell wall proteome from the basidiomycete *Coprinopsis cinerea* monokaryon Okayama 7 during growth in liquid culture.

4.3 Material and Methods

4.3.1 Chemicals

Yeast extract and malt extract were obtained from Oxoid (Hampshire, UK) and agar was supplied by Serva Electrophoresis (Heidelberg, Germany). All other chemicals were of p.A. quality unless otherwise noted. ^{33}P-labeled phosphoric acid was obtained from Hartmann Analytic (Braunschweig, Germany).

4.3.2 Fungal cultures

Coprinopsis cinerea Okayama 7 (#130), (*Coprinus* research community, FGSC #9003) was cultivated at 37 °C on YMG (yeast, malt, glucose) medium (Rao & Niederpruem, 1969) containing 4 g glucose, 4 g yeast extract, 10 g malt extract, and 1.5% agar per liter. Three 10 mm diameter pieces of a 6-days-old agar culture were used for inoculation of 100 ml liquid precultures. After three days of growth, liquid precultures were homogenized (1 min at 8000 min^{-1}, Ultraturrax T25, Janke & Kunkel, IKA Labortechnik, Staufen, Germany) and 5 ml aliquots were used as inoculum for 50 ml YMG experimental cultures. The anorganic phosphate present in the normal YMG medium was precipitated with 200 mM $CaCl_2$ and the precipitated Ca_2PO_4 was removed by centrifugation. After fungal inoculation 1 mCi ^{33}P-labeled phosphate was added to each culture flask. All liquid cultures were incubated under shaking conditions (120 rpm/min). Experimental cultures were grown to the early exponential phase (3 days of growth) at 37 °C in the dark for either 3 or 6 days. The experiment was repeated twice with normal YMG medium (without phosphate reduction) and once with YMG medium with reduced phosphate content. Determination of the phosphate content in the YMG medium was done by the KOSI (Center for Stable Isotope Research and Analysis; Faculty of Forest Sciences and Forest Ecology, University of Göttingen).

4.3.3 Protein Extraction and Identification

Extracellular proteins from *C. cinerea* were fractionated into freely secreted and hyphal sheath proteins as described in Chapter 3 (Section 3.3.3). Non-covalently bound cell wall proteins and proteins bound by disulphide bridges were extracted by four times boiling with SDS (Sodium dodecyl sulfate) -extraction buffer [Tris-base pH 7.5 60 mM,

EDTA (Ethylene diamine tetraacetic acid) 50 mM, SDS 2%, β-mercaptoethanol 0.5%, PMSF (Phenylmethylsulfonyl fluoride) 1 mM (1 ml/10 mg cell wall) (Masuoka et al., 2002)]. Intracellular proteins were extracted in course of the isolation of the cell wall. The supernatant of the two first extraction steps, containing the intracellular proteins, was further processed as intracellular fraction. Precipitation and quantification of the fractionated proteins were performed as in Chapter 3 (Section 3.3.4 and 3.3.5). Deglycosylation of the precipitated proteins with PNGaseF was performed using the deglycosylation kit from Sigma-Aldrich (Seelze, Germany) according to the manufacturers' recommendations.

The proteins were separated by 2-DE (2-dimensional gel-electrophoresis) as described in Chapter 3 (Section 3.3.6) and detected spots of interest were identified and labeled with ID-numbers as described in Chapter 3 (Section 3.3.8) and documented in the Appendix to Chapter 3 (Tables A.1 and A.2). After scanning, the gels were dried between cellophane films (gel-dryer from Zabona AG, Basel, Switzerland). The dry gels were placed on imager plates (Fujifilm, Düsseldorf, Germany) with an exposure time of 3 days for the gels from the extracellular fractions and 1 day for the gels of the intracellular proteins (see Appendix; Figure A.1). After exposure the imager plates were scanned using an FLA-5100 fluorescence reader (Fujifilm, Düsseldorf, Germany) with 50 µm resolution.

4.4 Results

4.4.1 Phosphorylated Proteins Occur in the Secretome of *C. cinerea*

In first experiments, *C. cinerea* monokaryon Okayama 7 was grown in shaking cultures for 3 and 6 days in standard YMG medium with 1 mCi ^{33}P per culture. The secretome of the experimental cultures was isolated and fractionated in freely secreted proteins of the culture supernatant, hyphal sheath proteins and extractable cell wall proteins (including non-covalently bound proteins and proteins bound with disulphide bridges). Proteins of the three different fractions were separated by 2-DE and radioactively labeled spots were detected by exposure of the gels to imager plates (Figures 4.2 to 4.4). In total, 91, 94, and 243 protein spots were visible on the 2-DE gels of the free secretome, the hyphal sheath and the extractable cell wall proteome, respectively. Less then 1% of these spots were specifically labeled with ^{33}P. In contrast, on the 2-DE gels of the intracellular

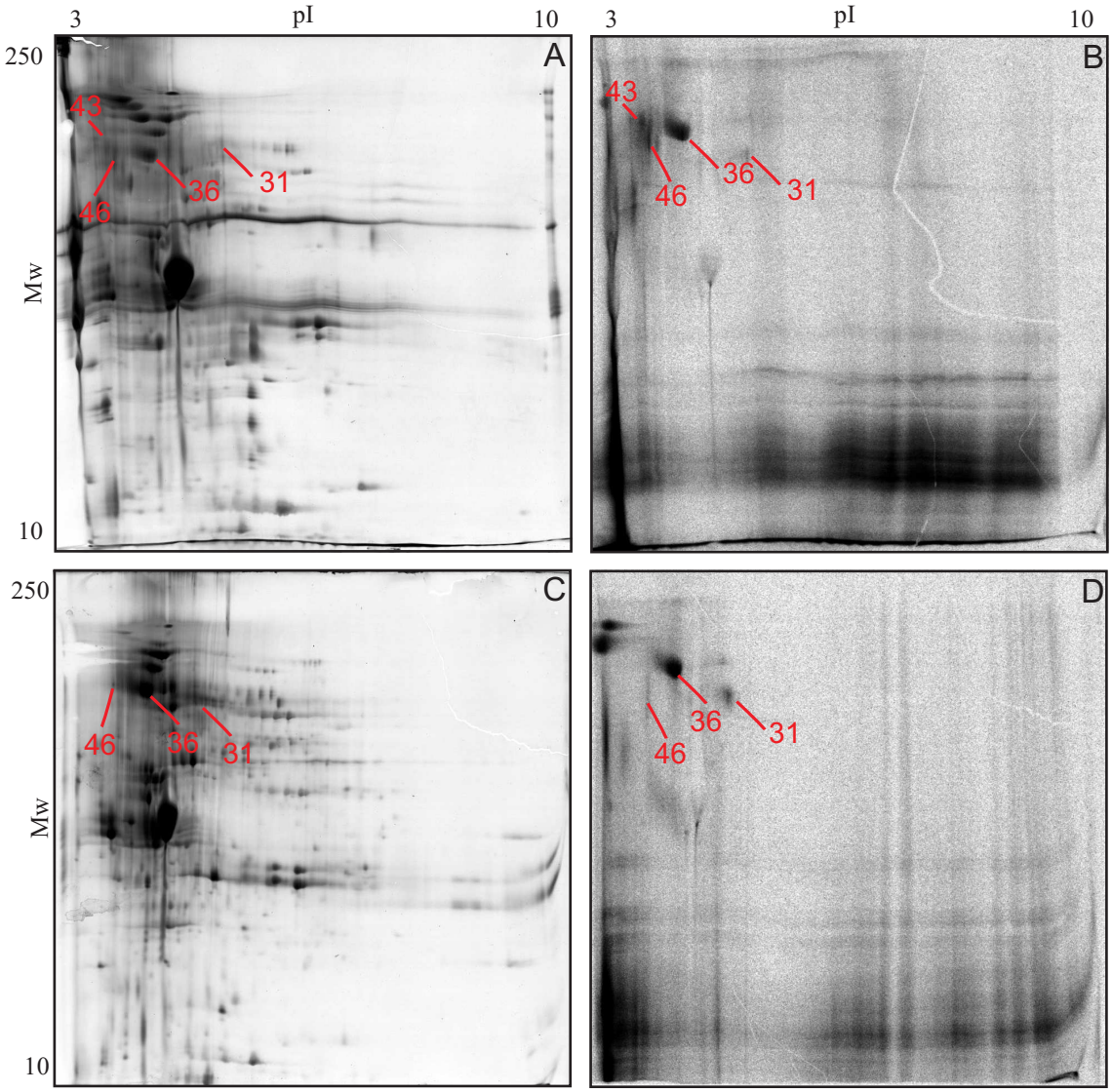

Figure 4.2: 2-DE of the freely secreted proteins from *Coprinopsis cinerea* grown at 37 °C in standard YMG medium (supplemented with 1 mCi ^{33}P-containing phosphate) on day 3 (A) and day 6 (C) of cultivation and corresponding autoradiographies (B) and (D), respectively. Proteins were separated on 18 cm Immobiline DryStrip pH 3-10 (Amersham Biosciences) in the first dimension and on a 12% SDS-PAGE in the second dimension. Gels were stained with RuBP. The imager plate for autoradiography was exposed to the gel for 3 days and subsequently scanned using a fluorescence reader FLA-5100 (Fujifilm, Düsseldorf, Germany) with 50 μm resolution.

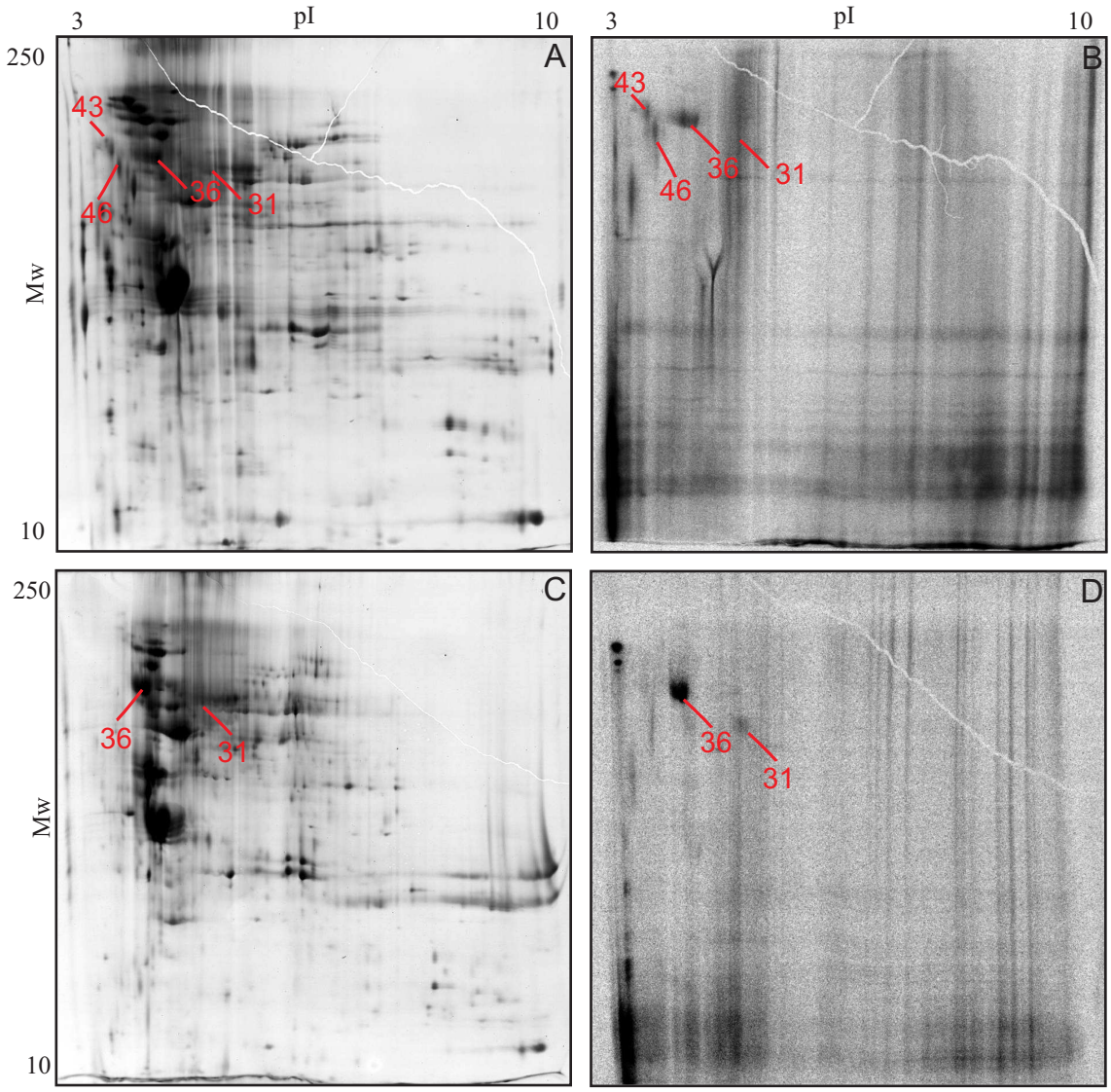

Figure 4.3: 2-DE of the hyphal sheath proteins from *C. cinerea* grown at 37 °C in standard YMG medium (supplemented with 1 mCi ^{33}P-containing phosphate) on day 3 (A) and day 6 (C) of cultivation and corresponding autoradiographies (B) and (D), respectively. For further experimental details compare legend of Figure 4.2.

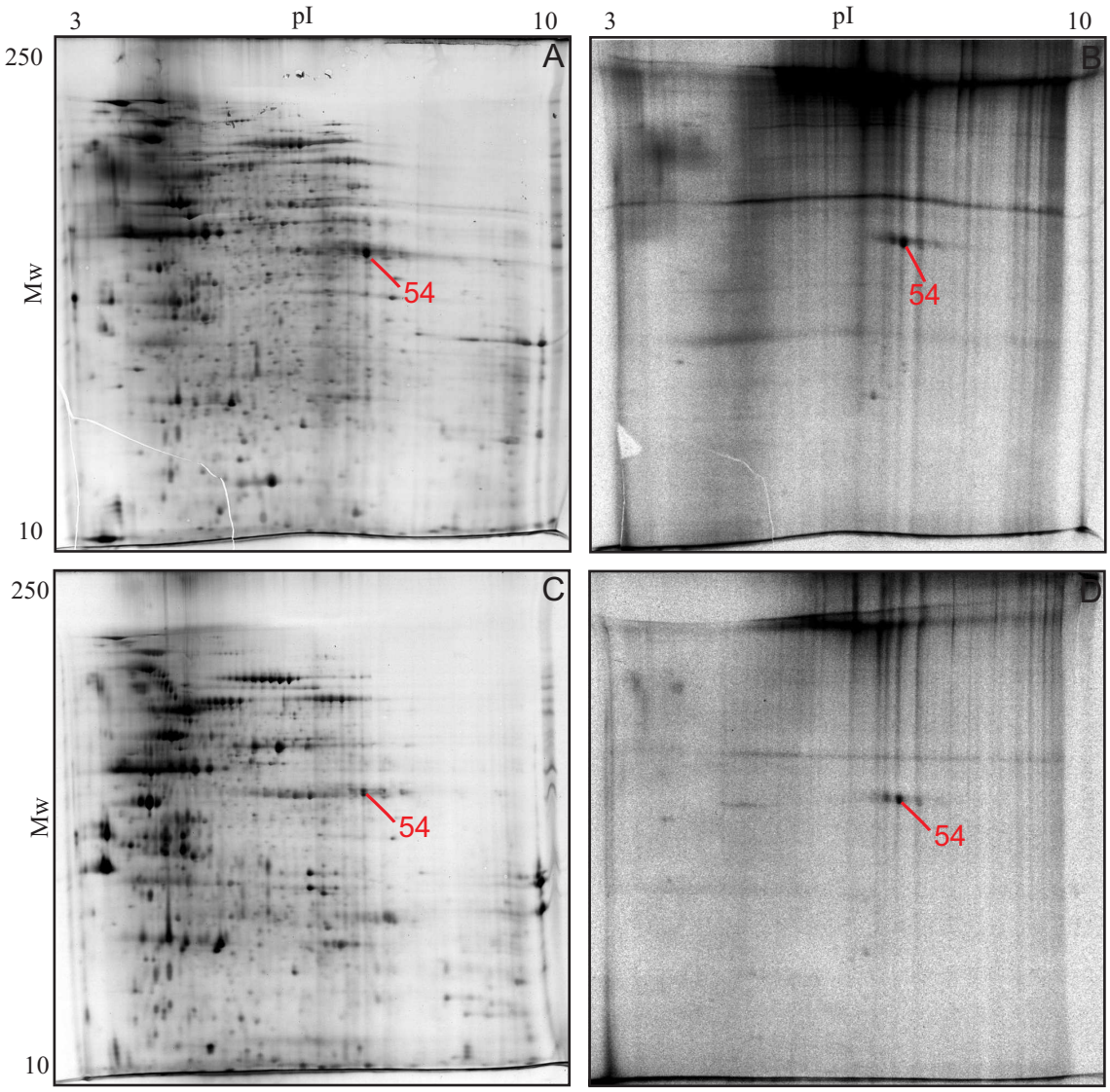

Figure 4.4: 2-DE of the extractable cell wall proteins from *C. cinerea* grown at 37 °C in standard YMG medium (supplemented with 1 mCi ^{33}P-containing phosphate) on day 3 (A) and day 6 (C) of cultivation and corresponding autoradiographies (B) and (D), respectively. For further experimental details compare legend of Figure 4.2.

protein fraction about 700 spots were detected and more than 40% are labeled with ^{33}P (see Figure A.1 in the Appendix to Chapter 4). The resulting autoradiographies from the different fractions of the secretome showed weak signals of specific ^{33}P-labeled spots (ID-numbers 31, 36, 43, 46, 54) in all fractions of the 3-days- and 6-days-old cultures with a high background on the imager plates. The gels and autoradiographies revealed that in the freely secreted protein fraction and the hyphal sheath fraction one spot (ID-number 43) and two spots (ID-numbers 43 and 46), respectively, disappeared from day 3 to day 6 (compare Figures 4.2 and 4.3). The extractable cell wall fraction shows no difference in the number and position of the ^{33}P-labeled spots on the analyzed days. In the following experiments, only day 3 was further examined.

Determination of the overall phosphorus content revealed that the *C. cinerea* standard growth medium YMG had a phosphorus concentration of 1.9 mM (3.37 mg/50 ml). In order to obtain stronger and clearer signals upon radioactive labeling on the imager plates for future experiments, inorganic phosphate of the standard YMG medium was precipitated with $CaCl_2$ prior to fungal inoculation. Due to this precipitation, the total phosphorus concentration was reduced to 1.0 mM (0.69 mg/50 ml) (see Table A.6 in the Appendix). 1 mCi ^{33}P-containing phosphate was added and the monokaryon Okayama 7 was cultivated in the phosphate-reduced medium for 3 days at 37 °C in shaking cultures. The reduced phosphate concentration in the culture medium had a general influence on the morphology of the fungus. The fungal pellets were observed to be smaller and more compact compared to the fungal pellets in standard YMG medium. Also a drastic decrease of mucilagous material, representing most likely soluble polysaccharides, in the culture supernatant was observed for the cultures with reduced phosphate content. ^{33}P-labeled extracellular proteins from 3-days-old phosphate reduced cultures were fractionated into the freely secreted proteins, the hyphal sheath proteins and the extractable cell wall proteins as in Chapter 2. Previously, polysaccharide material in supernatant samples were shown to hamper 2-DE of the free secretome (Fragner et al., 2009) and, in accordance, the resolution of the protein pattern on the 2-DE gels was drastically improved for the secretome of the phosphate-reduced cultures. Most importantly, protein profiles were only little changed compared to the fractionated secretome of cultures grown in standard YMG medium. Also autoradiography of the 2-DE gels from the fractionated secretome of phosphate-reduced cultures showed ^{33}P-labeled spots in the secretome, the hyphal sheath proteome and amongst the extractable cell wall proteins (Figures 4.5, 4.6 and 4.7). However, the signal intensity of ^{33}P-labeled proteins was

drastically enhanced showing strong and clear signals of ^{33}P-labeled spots.

In the autoradiography of the 2-DE gels of the free secretome, six spots with a relatively strong signal were detected with corresponding spots on the RuBP-stained 2-DE gel. In addition, three spots with rather weak signals were visible on the imager plate; however, these signals had no corresponding protein spots stained with RuBP [Ruthenium(II) tris(bathophenanthroline disulfonate); Figures 4.5 to 4.7]. Autoradiography of the hyphal sheath fraction showed a similar protein profile with also six ^{33}P-labeled protein spots and autoradiography of the extractable cell wall fraction revealed three ^{33}P-labeled protein spots. Compared to the previous gels performed with the fractionated secretome of standard YMG cultures, two additional proteins (ID-numbers 4, 62) were detected to be ^{33}P-labeled on the autoradiography of the phosphate-reduced YMG medium.

The spots corresponding to the autoradiography signals were picked from the 2-DE gels from the free secretome, the hyphal sheath proteome and the extractable cell wall proteome of the phosphate-reduced YMG cultures and analyzed by LC-MS^2 (Liquid chromatography coupled mass spectrometry). In total from all gels, seven different proteins were positively identified (Table 4.1). Two FAD/FMN-containing oxidoreductases (ID-numbers 31 and 36), a copper radical oxidase (ID-number 4), an unknown mannoprotein (ID-number 46) and two different glycoside hydrolases from the families 37 and 72 (ID-numbers 62 and 43) were detected from the free secretome and the hyphal sheath proteome, respectively. All of them were already identified previously when analyzing the different fractions of the *C. cinerea* secretome (Chapter 3; Section 3.4). Of these, the glycoside hydrolase from family 72 and the CBM (carbohydrate binding module) -containing protein were detected in two different spots.

Previously, the copper radical oxidase was shown to be present in three different spots (Chapter 3; Section 3.4.1) of more or less the same protein amount. The same multiple spot pattern was detected in this study (compare Figures 3.3 (A) and 4.5 (A)). However, only one of the three spots was found to have an incorporated phosphate group. Further in the hyphal sheath fraction, a glycoside hydrolase of the family 37 (ID-number 62) was found. The unknown mannoprotein (ID-number 46) was detected on the imager plates of the free secretome and the hyphal sheath as well as in the extractable cell wall proteome. Besides this unknown mannoprotein, also an uncharacterized protein with a CBM (ID-number 54) was detected in two radioactively labeled spots in the cell wall proteome.

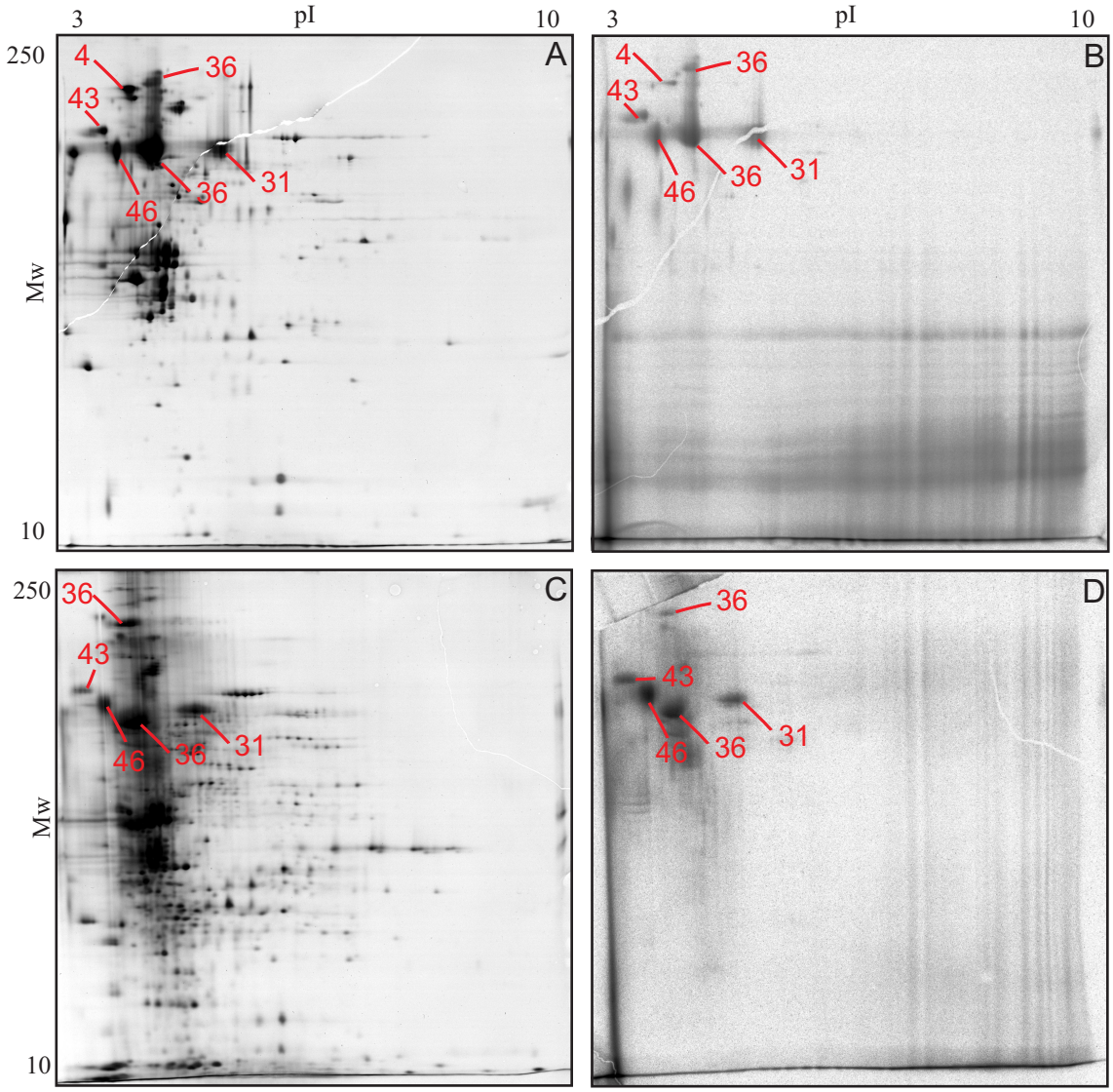

Figure 4.5: 2-DE (A) and corresponding autoradiography (B) of the freely secreted proteins and the deglycosylated proteins of this fraction (C and D) from 3 days old shaking cultures of *Coprinopsis cinerea* grown at 37 °C in phosphate reduced YMG medium. Proteins were separated on 18 cm Immobiline DryStrip pH 3-10 (Amersham Biosciences) in the first dimension and on a 12% SDS-PAGE in the second dimension. Gels were stained with RuBP. The imager plate for autoradiography was exposed to the gel for 3 days and subsequently scanned using a fluorescence reader FLA-5100 (Fujifilm, Düsseldorf, Germany) with 50 μm resolution. Protein deglycosylation was performed with PNGaseF according to the manufacturers recommendations (Sigma-Aldrich; Seelze, Germany).

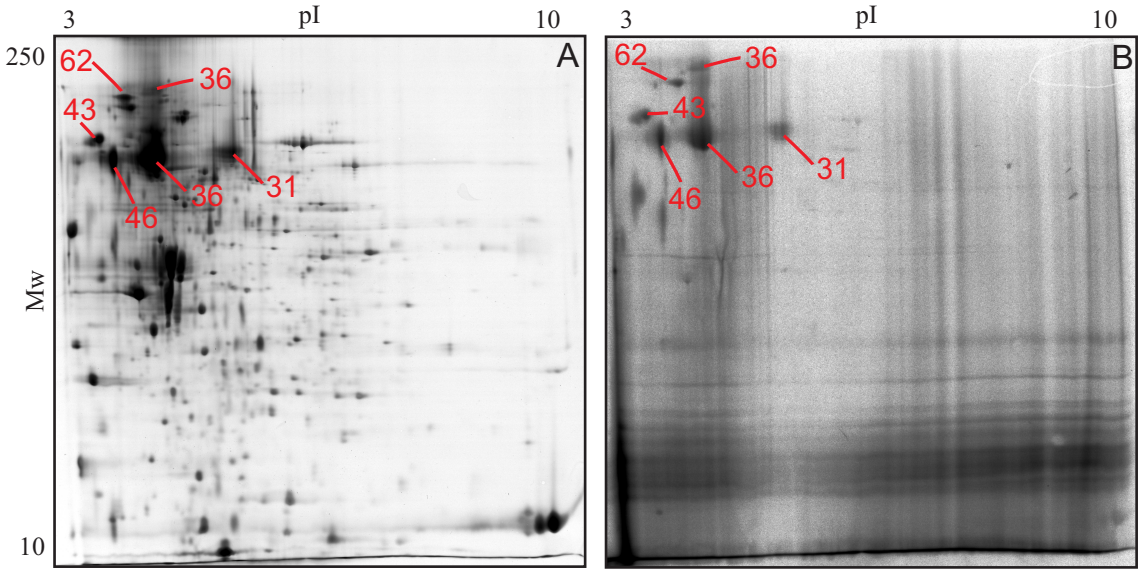

Figure 4.6: 2-DE (A) and corresponding autoradiography (B) of the hyphal sheath proteins from 3-days-old cultures of *C. cinerea*. For further experimental details compare legend of Figure 4.5.

Table 4.1: Identified spots from 2-DE gels of the fractionated secretome of *C. cinerea* grown in liquid YMG medium at 37 °C with a corresponding signal in autoradiography.

ID	Protein accession number	Proposed protein function*	Fraction**	Number of different spots	Labeled after deglycosylation
4	EAU83456	Copper radical oxidase	S	1	-
31	EAU80813	Oxidoreductase (2)	S, HS	1	+
36	EAU82165	Oxidoreductase (3)	S, HS	1	+
43	EAU90116	Glycoside hydrolase fam 72 (1)	S, HS	2	+
46	EAU83394	Unknown mannoprotein (3)	S, HS, CW	1	+/-***
54	EAU91343	CBM-containing protein (1)	CW	2	-
62	EAU86196	Glycoside hydrolase fam 37	HS	1	n.a.

* The proposed protein function is assigned after the protein analysis documented in Chapter 3 (Section 3.4.2.1). Numbers in brackets refer to the order in which these proteins were found, see Chapter 3 (Section 3.4.2.1)
** S: supernatant proteins; HS: hyphal sheath proteins; CW: cell wall proteins
*** Upon deglycosylation, this spot lost radioactive labeling only in the cell wall fraction, but not amongst the freely secreted proteins.

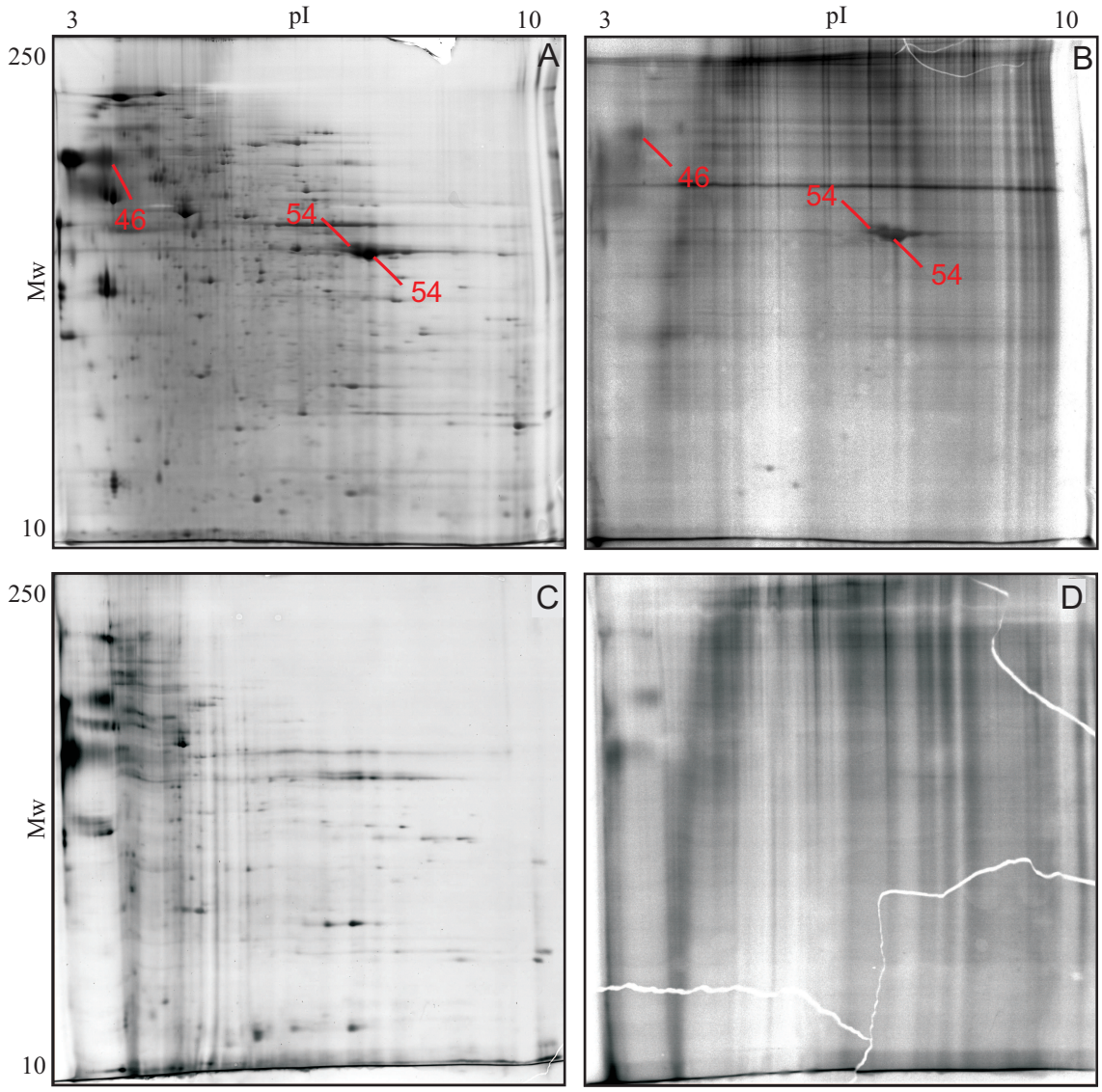

Figure 4.7: 2-DE (A) and corresponding autoradiography (B) of the extractable cell wall proteins and the deglycosylated cell wall proteins (C and D) from 3-days-old cultures of *C. cinerea*. For further experimental details compare legend of Figure 4.5.

4.4.2 The Nature of Phosphorylation of Secreted Proteins

Proteins from the freely secreted fraction and the extractable cell wall fraction were deglycosylated by treatment with the endoglycosidase PNGaseF. Unfortunately, analysis of the hyphal sheath proteins through PNGaseF by deglycosylation was not possible due to the low total protein amount obtained from the hyphal sheath fraction of the ^{33}P-labeled cultures and allowed only one 2-DE analysis.

Deglycosylation of the freely secreted proteins (Figure 4.5 C and D) resulted in a strongly altered protein profile on the 2-DE gels. In general, a decrease in the molecular weight of about 70% of the proteins was observed for this fraction (Figure 4.5 A and C). Furthermore, upon deglycosylation, the 2-DE profile showed up a high number of spot trains in horizontal direction but at positions of lower molecular weights than the original spots. In contrast, deglycosylation of the extractable cell wall proteins resulted in only a slightly changed 2-DE profile because most of the proteins retained in their molecular weight (about 80%; Figure 4.7 A and C).

Deglycosylation of the free secretome and the extractable cell wall proteins labeled with ^{33}P resulted in the loss of the labeled phosphate of three protein spots (Table 4.1). The copper radical oxidase spots (ID-number 4) in the free secretome and the hyphal sheath, and the spot of the CBM-containing protein (ID-number 54) from the extractable cell wall proteome gave no signal anymore upon PNGaseF treatment. At the same time the spots of these two proteins disappeared as well in the RuBP-stained 2-DE gels. Also the spot of the unknown mannoprotein (ID-number 46) lost the radioactive signal in the cell wall, while the protein was still radioactively labeled in the free secretome after deglycosylation. Also the intensity of the spot was not changed and thus no potential deglycosylation occurred (Figure 4.5 B and D). Such loss of the phosphate groups upon deglycosylation indicates a possible incorporation of phosphate groups on the sugars of the N-glycosylation of these proteins (Plummer et al., 1984). The other proteins (ID-numbers 31, 36, 43) were still radioactively labeled, indicating that the phosphate groups are linked likely in other ways such as to the proteins' amino acids or to the sugars attached by O-glycosylation to the proteins.

4.5 Discussion

In this study, a few specific extracellular proteins within the more complex free secretome, the hyphal sheath proteome and the extractable cell wall proteome from

C. cinerea were found to be phosphorylated. In first experiments of 3 and 6-days-old cultures, ^{33}P-labeled spots, indicating a possible phosphorylation of proteins, were detected in all fractions of the secretome from *C. cinerea*. Radioactively labeled spots of the free secretome and the hyphal sheath decreased in number from day 3 to day 6, which is obviously not due to a loss of phosphorylation but rather due to the fact that these specific proteins [glycoside hydrolase from family 72 (ID-number 43) and an unknown mannoprotein (ID-number 46)] were not present on day 6 of cultivation (Figures 4.2 and 4.4). The autoradiography of the cell wall fraction showed a similar picture on day 3 and on day 6 with one clearly radioactively labeled spot (Figure 4.4). These first experiments with standard YMG medium revealed clear but rather weak and diffuse signals of phosphorylated spots in the autoradiography (Figures 4.2 to 4.4), likely as the result of a high phosphate content of the standard YMG medium (Table A.6 in the Appendix), being in competition with the ^{33}P-labeled phosphate. In order to obtain stronger signals in the autoradiography, the phosphate content of the standard YMG medium was subsequently reduced. Upon phosphate reduction, stronger signals of the ^{33}P-labeled spots were indeed obtained. In the phosphate-reduced medium, the mycelial pellets of *C. cinerea* were smaller in size and of a more compact consistence, compared to standard YMG cultures, and also less mucilagous material was formed under these conditions. Probably, the massive reduction of the phosphate content and other co-precipitated compounds e.g. various minerals (Table A.6) prevented considerably the formation of a more or less complex soluble polysaccharides. A possible reason therefore might be the that these polysaccharides contain a high number of phosphodiester bridges, interlinking polysaccharide chains to a network (Shibata et al., 1995). Accordingly in the phosphate-reduced *C. cinerea* cultures, the reduction of the phosphate concentration probably led to less interlinking of polysaccharides and probably to a less complex network of glucan chains in the liquid medium. Furthermore, the reduction of the phosphate content with $CaCl_2$ also resulted in a considerably higher calcium concentration in the medium (See Table A.6 in the Appendix to Chapter 4). In *S. cerevisiae*, calcium is known to initiate the flocculation of yeast cells (Bester et al., 2006). A similar effect might be responsible for the formation of more compact mycelial pellets of *C. cinerea*.

The protein composition in the different secretome fractions however, was only little effected by the reduced phosphate content. Nevertheless, the 2-DE gels show a slightly changed profile due to a better resolution of the proteins. This is most probably resulting from the reduced amount of complex soluble polysaccharides in the culture medium.

4.5.1 Deglycosylation of the Secreted Proteins

Deglycosylation of the freely secreted proteins resulted in a reduction of the molecular weight of various proteins (Figure 4.5). However, the removal of N-glycosylations did not simplify the 2-DE profile but instead multiplied the spot number due to the formation of spot trains in horizontal direction and due to the decreased molecular weight of most proteins. The appearance of additional spot trains on gels upon deglycosylation of proteins was previously reported for the free secretome of *Trichoderma reesei* (Fryksdale et al., 2002) and was explained by the authors by an ineffective enzymatic deglycosylation of the proteins. In contrast, an acid deglycosylation with TFMS (trifluoromethanesulfonic acid) of the *T. reesei* secretome did not result in the formation of multiple spot trains. A similar approach for *C. cinerea* using TFMS for the deglycosylation of proteins, however, was not successful since, instead of deglycosylation, the proteins of the sample were completely degraded. Thus, the potentially less reliable deglycosylation with PNGaseF was applied for the secretome and the cell wall proteins of *C. cinerea*. However, an incomplete protein deglycosylation would most likely result in vertical and diagonal spot trains due to different molecular weights of the same protein. For the freely secreted proteins from *C. cinerea* deglycosylated with PNGaseF this was, however, not the case.

A possible explanation for the occurrence of such a complex protein profile after deglycosylation as obtained in this study for the freely secreted proteins of *C. cinerea* are other charged posttranslational modifications that were previously shielded by the sugar chains of the proteins' glycosylation and might upon elimination of sugar chains subsequently be projected to the surface (Gutiérrez et al., 1996; Scherba & Babitskaya, 2008; Wagner et al., 2004).

4.5.2 Phosphorylated Proteins Occur in the Secretome of *C. cinerea*

Phosphorylation of extracellular proteins is to our knowledge a rarely studied field, not only in fungi but as well in plants and animals. However, phosphorus was previously shown to be a component of the fungal cell wall, occurring in the phosphodiester linkages between mannose residues that are interlaced in the glucan network of the cell wall of yeasts (Kollár et al., 1997) and interconnect the phosphomannan protein complexes (Shibata et al., 1989). However, Casanova & Chaffin (1991) suggested that in the cell walls of *C. albicans* phosphate groups either occur in from of phosphomannan in

the cell wall structure, as phospho-mannoproteins and as phosphoproteins (without glycosylation).

In the here presented study, the occurrence of phosphorylated proteins in the free secretome, the hyphal sheath proteome and the cell wall proteome were examined. Seven different protein spots of the fractionated secretome from *C. cinerea* showed a positive signal in the autoradiography, suggesting an associated phosphate group either on the amino acids of the protein chains or on a sugar residue of the proteins' glycosylation (Casanova & Chaffin, 1991). In three independent experimental setups of 3-days-old as well as 6-days old cultures either in standard YMG medium or in phosphate reduced YMG medium almost the same proteins were found to be radioactively labeled. In the experimental setup with the phosphate-reduced YMG medium, two additional proteins were detected in autoradiography compared to the previous experimental setups with standard YMG medium. These proteins showed no affiliation to special protein classes. However, five are assigned as enzymes while the two others are uncharacterized proteins. In order to analyze the nature of phosphorylation occurring on these proteins, the freely secreted proteins and the extractable cell wall proteins from *C. cinerea* were deglycosylated with PNGaseF (Plummer et al., 1984). Deglycosylation removed the phosphate groups from three of the seven identified proteins, the putative copper radical oxidase (ID-number 4) found in the freely secreted and the hyphal sheath fraction, the CBM-containing protein (ID-number 54) and the unknown mannoprotein (ID-number 46) lost the linked phosphate group in the cell wall fraction. In conclusion, these three proteins seem to have an phosphate group linked to the sugars attached by N-glycosylation to the protein. Obviously, these proteins have a phosphate group on the sugars attached by N-glycosylation to the proteins and possibly serve in the linkage of the protein to the cell wall glucans via phosphodiester-linkages as previously suggested for *C. albicans* cell wall proteins (Casanova & Chaffin, 1991).

The identified copper radical oxidase appeared in this study also to be represented by three different spots of similar intensity in the RuBP stained 2-DE gels as previously shown in Chapter 3 (Section 3.4.2). However, only one spot representing the copper radical oxidase was detected in autoradiography, indicating a phosphorylation on only one of the possible isoforms of the copper radical oxidase. Deglycosylation of the freely secreted proteins suggested that the copper radical oxidase has a phosphate group linked to the N-glycosylation of the protein (Plummer et al., 1984). The incorporation of phosphate groups to only one of several possible isoforms might indicate that the

glycosylation of different isoforms is strongly dissimilar. Analysis of the deduced protein sequence of the putative copper radical oxidase (ID-number 4; EAU83456) predicts eight possible N-glycosylation sites (http://www.expasy.ch/tools/glycomod/). Different glycosylation patterns of the possible isoforms might therefore apply for this protein. For fungal proteins it is not uncommon to show a variety of different glycosylations on the same protein as it was recently documented for laccases in a literature review by Rodgers et al. (2009).

Four other proteins were still radioactively labeled upon deglycosylation of the proteins. However, different other explanations can be given for the radioactive signal of these four proteins. Two different FAD/FMN-containing oxidoreductases from *C. cinerea* with a putative flavin adenine dinucleotide (FAD) binding domain (Ozimek et al., 2005) were found in this study to have attached phosphate groups. FAD/FMN-containing oxidoreductases commonly possess a highly conserved pocket for the binding of FAD that is embedded deeply in the protein matrix (Boteva et al., 1999; Zlateva et al., 2001). FAD contains two phosphate groups linking the flavin to the adenine nucleotide (Ozimek et al., 2005). FAD as expected to be present in the FAD/FMN-containing oxidoreductases detected in the secretome of *C. cinerea* is therefore an obvious explanation for the radioactive labeling of these proteins.

A glycoside hydrolase from family 72 of *S. cerevisiae* (Mouyna et al., 2000a,b), being homologous to the enzyme identified here for *C. cinerea* (ID-number 43), was shown in yeast to appear in a glycosylphosphatidylinositol (GPI)-anchored form and a freely secreted form without GPI-anchor (Mouyna et al., 2000a). Also the glycoside hydrolase from family 72 found in the free secretome and the hyphal sheath proteome of *C. cinerea* has a putative GPI binding site predicted by the BIG-Π-predictor (Eisenhaber et al., 2004). Therefore, the signal obtained in the autoradiography could be due to a GPI anchor linked to the glycoside hydrolase from family 72, which was possibly released from the cell wall with its GPI anchor by degradation processes of the fungus.

A glycoside hydrolase from family 37 (ID-number 62) from the hyphal sheath fraction was also detected as a labeled spot in the autoradiography. However, deglycosylation of the hyphal sheath fraction was not possible to perform due to the low protein amount in this fraction which allowed only one 2-DE analysis. Therefore, it remains unclear whether the phosphate group is linked to the N-glycosylation of this protein. However, this glycoside hydrolase from family 37 has no putative GPI anchor site and a GPI-anchor can therefore not be responsible for the radioactive labeling with ^{33}P.

For three of the seven detected proteins (ID-numbers 4, 46, 54) labeled with ^{33}P in the secretome of *C. cinerea* it was shown that the phosphate group is part of the sugars attached by N-glycosylation to the protein. For the four remaining ^{33}P-labeled proteins (ID-numbers 31, 36, 43, 62) in the secretome of *C. cinerea*, the radioactive signals might derive from a prosthetic group, from a GPI anchor, a possible phosphorylation at an amino acid or from the presence of phosphate groups linked to the sugars attached by O-glycosylation to the protein. In any case, further studies have to show the biological relevance of these phosphorylations.

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5 Dynamics of the Free Secretome and the Cell Wall Proteome of *Coprinopsis cinerea* During Growth

5.1 Abstract

In order to obtain deeper insights into possible changes occurring in the secretome of *Coprinopsis cinerea* during growth, the free secretome, the proteome of the hyphal sheath and the extractable cell wall proteome were analyzed in different growth phases. The early exponential phase, the late exponential phase, the stationary phase and the phase of cell lysis were chosen for a 2-DE analysis of the fractionated secretome. The secretome of *C. cinerea* was shown to be reduced in its complexity with progression of cultivation, especially the extractable cell wall proteome. Various single proteins such as peptidases, glycoside hydrolases and oxidoreductases changed significantly in protein concentration during the time of cultivation. In total from all 2-DE gels of all time points and fractions, 70 spots were identified by LC-MS^2, representing 31 single proteins. This study revealed a dynamic secretome, concerning the variation of concentrations of single proteins changing with the environmental conditions and fungal age during the different growth phases. The results confirm a strict allocation of proteins to distinct extracellular compartments of the fungus also over the time of cultivation.

5.2 Introduction

Secretome analyzes for higher basidiomycetes are rare and were until now only performed for a few species. Most of the proteomic studies from higher basidiomyctes were done for the white rot fungus *Phanerochaete chrysosporium* (Kersten & Cullen, 2007;

Ravalason et al., 2008; Sato et al., 2007; Shary et al., 2008; Vanden Wymelenberg et al., 2009). These studies focus on the free secretome of *P. chrysosporium* in reaction to different growth substrates such as different carbon sources in liquid media (e.g. cellulose), wood chips and saw dust. A focus on nutrition was also given in a proteomic study of *Pleurotus sapidus* grown in submerged cultures with peanut shells or glass wool (with glucose as carbon source) as carrier material (Zorn et al., 2005). However, only one of these studies describes secretomes in different growth phases of a fungus. This study on *P. chrysosporium* showed that most but not all spots were present over the whole examined growth period but changed in their intensities over the time, indicating varying concentrations of the proteins representing these spots (Sato et al., 2007).

The few studies mentioned above focused on the freely secreted proteins of basidiomycetes. However, also the cell wall as outer layer of the fungal cell contains a multitude of different proteins as it was documented for a number of ascomycetous fungi such as *Trichoderma reseii* (Lim et al., 2001), *Aspergillus fumigatus* (Bruneau et al., 2001) and *Candida albicans* (Pitarch et al., 2002; Rast et al., 2003) and, in this thesis, also for the basidiomycete *C. cinerea* (Chapter 3 and 4).

The cell wall is a crucial structure for fungi and consists of a three-dimensional network of mainly glycans and glycoproteins (Bowman & Free, 2006), which provides the shape of the cell and protects it from osmotic and physical stress as well as other environmental threats (Klis et al., 2002). The cell wall structure presents the contact zone between the fungus and its environment and can contain a diversity of enzymes necessary for the structuring and restructuring of the cell wall as well as for self-defence and the interaction with the environment, as shown in experimental studies for *Saccharomyces cerevisiae* (Chaffin et al., 1998). Amongst the proteins attached to the fungal cell wall of the various fungal species are oxidoreductases, phosphatases, glucosidases, chitinases, and chitin synthases. Such enzymes were detected in the cell wall in specific fungal species by specific enzyme tests and/or EM-localization studies [for an extensive review see Rast et al. (2003)].

In addition to the rigid cell wall, several fungi are known to produce an extracellular glycan layer associated with the cell wall, the so called hyphal sheath (Bes et al., 1987). The hyphal sheath is surrounding the fungal hyphae (Gutiérrez et al., 1995) and covalently linked to the cell wall glucans (Sietsma & Wessels, 1981). Though the role of the hyphal sheath is not completely understood, various functions have been proposed in previous studies. This structure possibly acts as a protective layer against

toxic molecules or radicals (Vesentini et al., 2007). Storing of nutrient in the form of extracellular glucans, used by the fungus under starvation conditions, was shown by Stahmann et al. (1992). Since the hyphal sheath forms the contact between the fungus and its substrate, it mediates the attachment to foreign surfaces (Stahmann et al., 1992) and it could also act as a reaction space for extracellular degradation enzymes (Gutiérrez et al., 1995).

In this study, the dynamics of the secretome, fractionated into the freely secreted proteins, the proteins of the hyphal sheath and the extractable cell wall proteins, during the growth of the fungus *C. cinerea* in liquid YMG medium was examined by a 2-DE approach.

5.3 Material and Methods

5.3.1 Chemicals

Yeast extract and malt extract were obtained from Oxoid (Hampshire, UK) and agar was supplied by Serva Electrophoresis (Heidelberg, Germany). All other chemicals were of p.A. quality unless otherwise noted.

5.3.2 Culture Conditions

C. cinerea Okayama 7 (#130), (*Coprinus* research community, FGSC #9003) was cultivated at 37 °C on YMG medium (Rao & Niederpruem, 1969) containing 4 g glucose, 4 g yeast extract, 10 g malt extract, and 1.5% agar per liter. Three 10 mm diameter pieces of a 6-days-old agar culture were used for inoculation of 100 ml liquid precultures. After three days of growth, liquid precultures were homogenized (1 min at 8000 min^{-1}, Ultraturrax T25, Janke & Kunkel, IKA Labortechnik, Staufen, Germany) and 5 ml aliquots were used as inoculum for 100 ml YMG experimental cultures. All liquid cultures were incubated under shaking conditions (120 rpm/min). Experimental cultures were grown at 37 °C and harvested after 3, 6, 9, and 12 days of growth, respectively. Per culture age, 3 parallel cultures were analyzed.

5.3.3 Determination of the Fungal Biomass

The fungal mycelium was separated from the culture liquid by vacuum filtration with a Büchner funnel using Whatman filterpaper No 1, ⌀ 90 mm (Schleicher & Schuell, Munich, Germany). Mycelium was placed together with the filter of known weight in a 50 ml Falcon tube deep frozen at −80 °C and submitted to a freeze drier (Zirbus Technology, Bad Grund, Germany). The samples were weighted after 16 h freeze drying. For the determination of the ergosterol content, 40 ml of 10% KOH in methanol with 200 mg/l BHT (2,6-di-tetra-butyl-4-methyl-phenol) and 10 μg/ml cholesterol as an internal standard were added to the tubes with dry mycelium, which were tightly sealed and incubated for 3 h at 60 °C (Nielsen & Madsen, 2000). After cooling to room temperature, the samples were centrifuged at 1700g (centrifuge 5810R, Eppendorf, Hamburg, Germany) for 20 min and 4 ml of the clear supernatant was transferred into a new tube. A volume of two ml hexane was added to the supernatant and the samples were mixed thoroughly by vortexing. Subsequently, 4 ml H_2O was added to the samples and the tubes were shaken at room temperature for at least 30 min. The samples were centrifuged at 1700g for 10 min and 1 ml of the hexane phase was transferred into a Eppendorf tube, dried in a vacuum centrifuge (Concentrator 5301, Eppendorf, Wesseling-Berzdorf, Germany) and stored at −20 °C until further proceeding. Derivatization with pyridine and BSTFA (bis(trimethylsilyl)trifluoroacetamide) was performed according to Nielsen & Madsen (2000) and samples were analyzed by GC-MS (gas chromatography coupled masspectrometry; 6890N, Agilent Techn., Network GC Systems, Santa Clara CA, USA).

5.3.4 Determination of the Protein and Glucose Concentration

Protein concentration in the single fractions was determined as already previously described in Chapter 3 (Section 3.3.5) after Fragner et al. (2009).

Glucose concentration in the culture medium was determined using the the Glucose (HK) Assay Kit (Sigma-Aldrich Chemie GmbH, München, Germany) according to the manufactures manual.

5.3.5 Protein Extraction and Identification

Fungal cultures were harvested and the freely secreted proteins, the hyphal sheath proteins, the cell wall proteins and the intracellular proteins were extracted, separated by 2-DE and analyzed by LC-MS^2 as described in Chapter 4 (Section 4.3.3). From each fraction and time point, three technical replicates were analyzed by 2-DE and for the identification of low abundant protein spots, the three corresponding spots of the 2-DE gel replicates were pooled and further analyzed by LC-MS^2 as described in Chapter 3 (Sections 3.3.6 and 3.3.7; analysis and identification of protein spots from 2-DE gels).

Spot detection and gel matching was done with Proteomweaver (Version 3.1.0.7 Definiens AG München, Germany). The parameters for spot detection and normalization were set as follows: radius limit 16.0; intensity limit 174857; contrast limit 100.0; border contrast 0.2; normalization point 95. The minimal significant factor for the spot detection against the background was calculated as 1.59. Three technical replications were used for the statistical analysis of the 2DE-gels. Relative changes of the spot intensities of the free secretome and the other fractions were determined by comparing the spot-background ratio of the gels of the free secretome of day 3 to all the other gels.

The identified spots were labeled in the gel pictures with their corresponding arbitrary ID-numbers as already used previously in Chapter 3 and 4 and which are listed in the Appendix (Table A.7).

5.4 Results

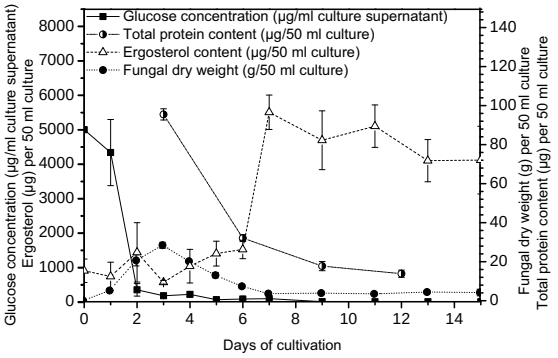
5.4.1 Growth of *C. cinerea* in Liquid Culture Over the Time

C. cinerea monokaryotic strain Okayama 7 was cultivated in shaking YMG cultures at 37 °C for 15 days. Every second day, ergosterol and total dry biomass was determined from a subset of parallel cultures (3/day). Ergosterol content, indicative for living fungal cells (Nielsen & Madsen, 2000) raised strongly after six days of cultivation to stay high until the end of the experiments (Figure 5.1 a). However, total dry biomass did not show a parallel development to the ergosterol content in the cultures (Figure 5.1 a and b). Total dry weight was high in the beginning of cultivation with the highest biomass amount (28.44 g/50 ml culture) at day 3 of cultivation. Subsequently, it decreased rapidly to reach the lowest amount (3.66 g/50 ml culture) at day 7 of cultivation (Figure

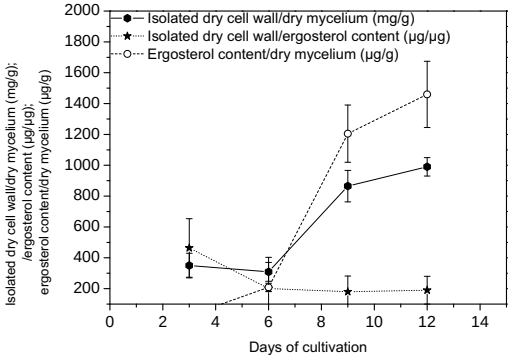
5.1 a). In relation, this leads to low ergosterol/dry biomass quotients early in cultivation and to high quotients at later cultivation times (Figure 5.1 b), indicating that the absolute cell numbers increase with time in the dry biomass. Corresponding to the increase of the dry biomass with the maximum at day 3, the glucose concentration in the medium decreased and glucose was already used up at day 3 after inoculation (Figure 5.1 a). All in all, growth of *C. cinerea* in YMG cultures over the time was followed up in three independent sets of experiments. Biomass amounts, ergosterol content and glucose concentration was in all comparable (not further shown).

Four points of cultivation were chosen for further analysis (Figure 5.1 b and c): day 3 from the early logarithmic phase with highest biomass amounts and low ergosterol content, day 6 from the late logarithmic phase prior to increase of the ergosterol content, day 9 from the stationary phase and day 12 from aging cultures both with high ergosterol content (Figure 5.1 a). Total biomass and from the biomass the cell walls were harvested from each three representative cultures per day of cultivation. The amount of isolated cell wall per gram dry mycelium was measured at each point of harvest. The amounts of isolated cell walls over the time followed the curve of ergosterol content with lower values in the logarithmic growth phase and highest values in the stationary and aging phase (Figure 5.1 b). On day 3 and day 6 of cultivation, in total 350 $\pm$ 80 mg and 309 $\pm$ 61 mg of dry cell wall material were extracted per 1 g dry mycelium, respectively (Figure 5.1 b). On day 9 and day 12 of cultivation, 865 $\pm$ 102 mg and 990 $\pm$ 97 mg of dry cell wall material were isolated from 1 g dry mycelium, respectively (Figure 5.1 b). However, as the total fungal dry weight, the yield of cell walls did not correlate over the time in an equal relation with the ergosterol content (Figure 5.1 b). The total protein content of the cultures was also not well correlated with first the ergosterol content and second somewhat better with the dry biomass but third comparably well with the dry cell wall amount of the cultures (Figure 5.1 c; for the absolute protein concentrations see Figure 5.1 c). This data showed nearly steady total protein concentrations in relation to the total dry cell wall amount and somewhat less to total biomass, while the protein content correlated with the ergosterol content shows a considerable decrease from day 3 to day 9 of cultivation (Figure 5.1 c).

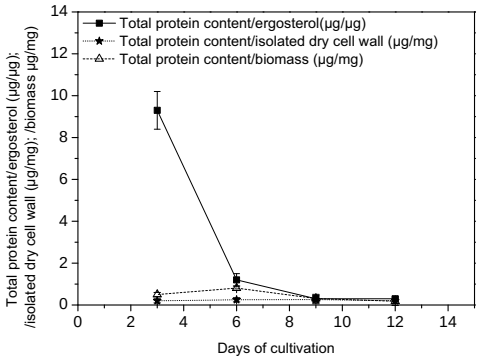
In order to examine the development of the fractionated secretome, the freely secreted proteins, the hyphal sheath proteins and the cell wall extractable proteins were isolated from each three representative cultures at day 3, 6, 9 and 12 of cultivation. Measurements of the protein concentration of the different fractions as well as of the in-



(a)



(b)



(c)

Figure 5.1: Ergosterol content, fungal dry weight, glucose concentration and total protein content (a) and amount of isolated dry cell wall from 1 g dry mycelium and relative to the ergosterol content as well as the fungal dry weight relative to the ergosterol content (b) and total protein contents of the liquid cultures relative to the ergosterol content, the fungal dry biomass and the isolated dry cell wall (c) of shaken cultures of *Coprinopsis cinerea* grown at 37 °C in liquid YMG medium.

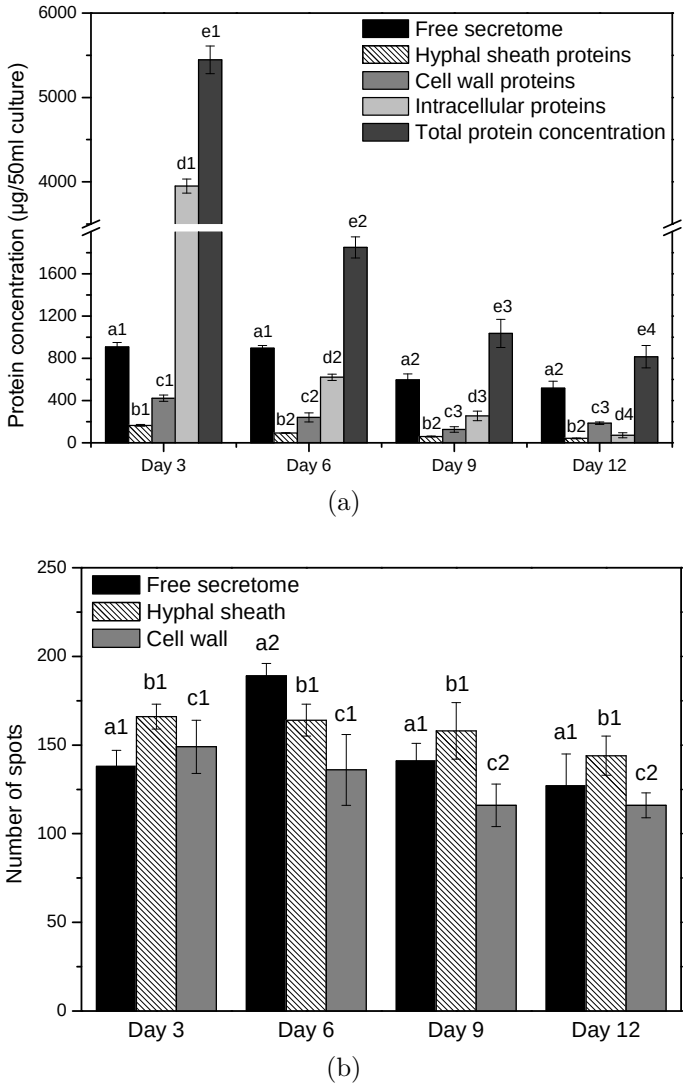


Figure 5.2: Protein content (a) of the different secretome fractions, the intracellular proteins and the total protein content (summed up from all the different fractions of the secretome) and total number of detected spots on the 2-DE gels (b) of the different secretome fractions from 50 ml *C. cinerea* cultures at day 3, 6, 9 and 12 of cultivation (grown at 37 °C in liquid YMG medium in shaking cultures). The protein concentration was measured after Fragner et al. (2009); average values and standard deviations were calculated from each three replicates; statistical analysis (ANOVA-test) was done with Origin 8.1 software (OriginLab, Northhampton, USA); letters above the samples indicate those that were compared with each other, numbers at the letters indicate significant differences between two values of one group of compared samples.

tracellular proteins (Figure 5.2 a) showed that the protein concentration of all observed fractions was highest at day 3 of cultivation. Therefore, also the total protein amount was highest at day 3 of cultivation and decreased drastically during the examined time of cultivation (Figure 5.2 a).

5.4.2 Comparison of the Fractionated Secretome of the Different Growth Phases

Proteins of the free secretome, the hyphal sheath proteome and the extractable cell wall proteome (NaCl- and SDS-extractable proteins; isolation methods are further described in Chapter 3) of day 3, 6, 9 and 12 of cultivation were separated by 2-DE (Figure 5.3 - 5.5) and 2-DE gels were analyzed with the Proteomweaver software (Version 3.1.0.7 Definiens AG München, Germany), thereby determining the spot numbers and matching the gels. All gels from the four different time points obtained from one fraction (three gels per day and fraction) were matched against each other in order to evaluate the similarities and changes in the protein profile over the time of growth.

The 2-DE profiles of the different fractions showed in most instances a slight decrease in the number of spots from day 3 to day 12 in all analyzed fractions (Figure 5.2 b). In the hyphal sheath fraction, the absolute number of spots was however almost stable during the examined growth period showing no significant difference between the observed days of fungal growth. In contrast, the absolute number of spots in the free secretome increased from day 3 to day 6 of cultivation and decreased in the following from day 6 to day 12 of cultivation. In the cell wall fraction, the absolute spots number decreased slightly from day 3 to day 12 (Figure 5.2 b).

The development of the spot profiles within one fraction was monitored by matching the gels of the four time points from one type of fraction against each other (Table 5.1, 5.2 and 5.4 and Figures 5.6, 5.7 and 5.8). In general, the number of spots overlapping between the gels of the four analyzed time points of one type of fraction increased with the time of growth (Table 5.1, 5.2 and 5.4). Further, the greater part of spots was shared between all time points of growth (3, 6, 9 and 12 days of cultivation) in all fractions (Figures 5.6, 5.7 and 5.8).

Further, the gels of the freely secreted and the hyphal sheath proteins from one time point (e.g. free secretome from day 3 to the hyphal sheath proteome of the same day of cultivation) were as well matched, due to their similarity in spot distribution (Table

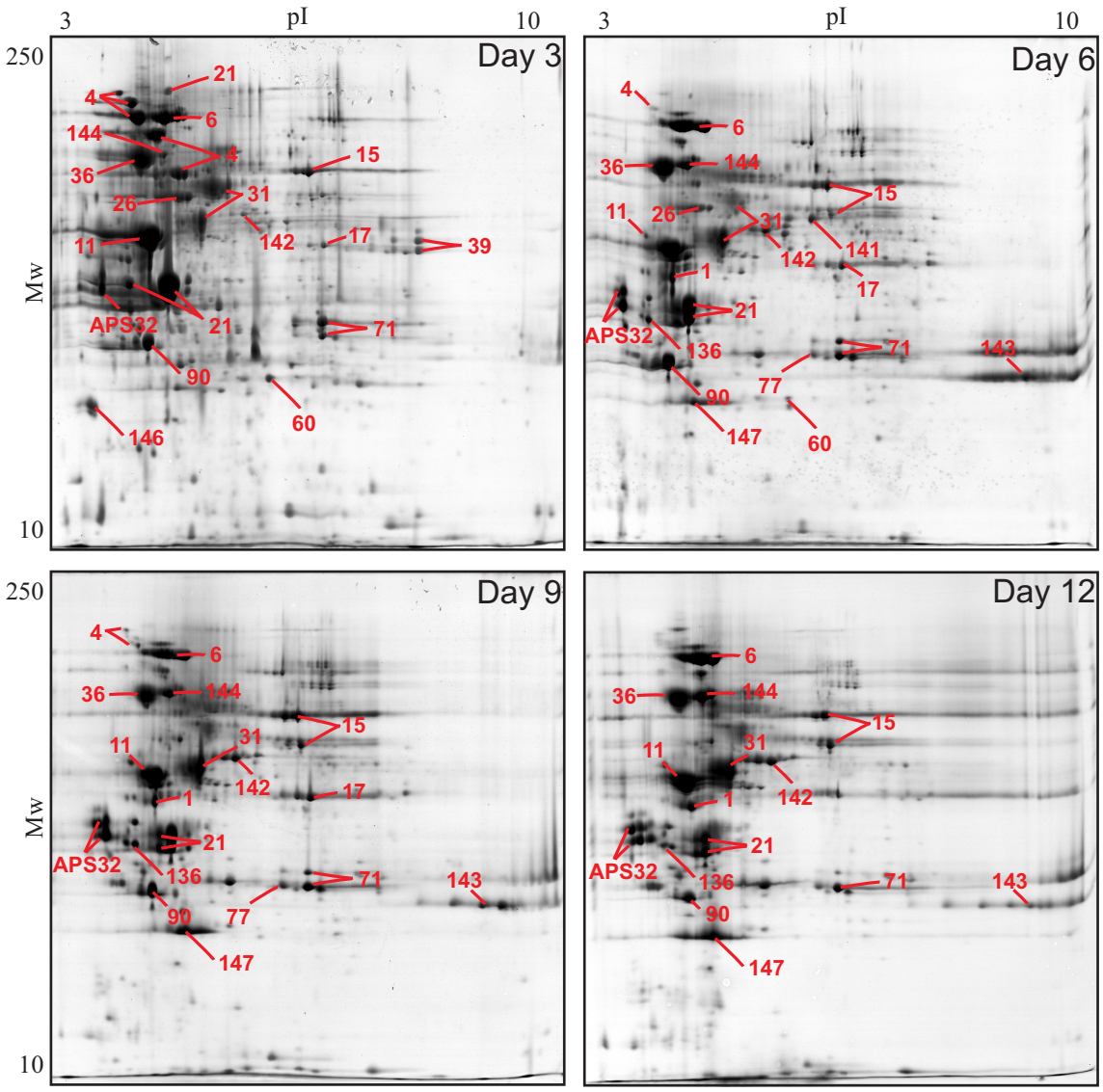


Figure 5.3: 2-DE-gels of the freely secreted proteins from day 3, 6, 9 and 12 of cultivation of *C. cinerea* grown at 37 °C in shaking cultures in liquid YMG medium. Day 3 of cultivation is according to the ergosterol and the cell wall amount (Figure 5.1) the early exponential phase, day 6 the late exponential phase, day 9 the stationary phase and day 12 the phase of cell aging (see Figure 5.1 a). Proteins were separated on 18 cm Immobiline DryStrips pH 3-10 (Amersham Biosciences) in the first dimension and on a 12% SDS-PAGE in the second dimension. Gels were stained with RuBP. The labeled spots were positively identified by LC-MS^2.

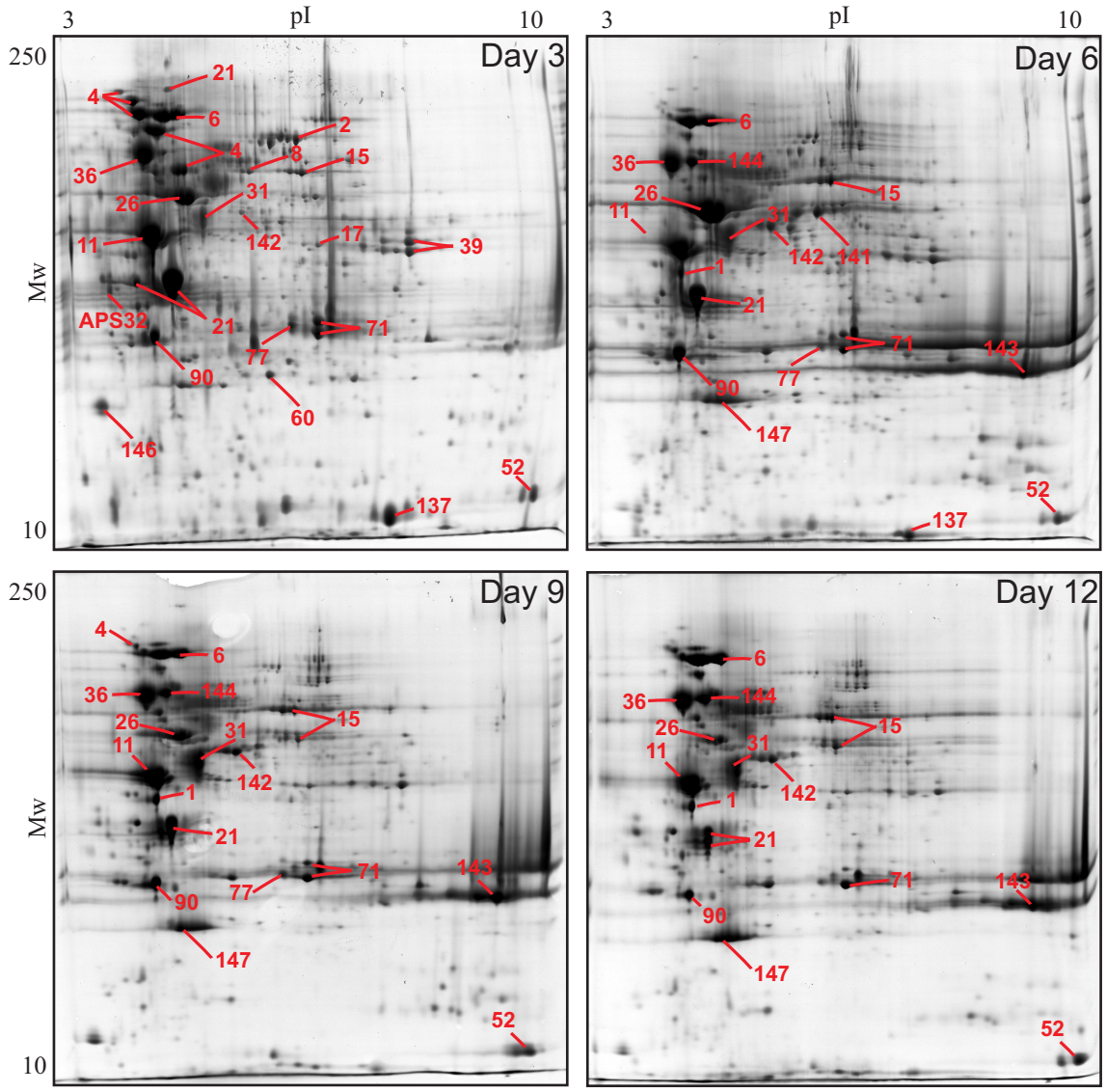


Figure 5.4: 2-DE-gels of the hyphal sheath proteins extracted with Tris-buffer containing 2% Tween 80 from day 3, 6, 9 and 12 of cultivation of *C. cinerea* grown at 37 °C in shaking cultures in liquid YMG medium. Day 3 of cultivation is according to the ergosterol and the cell wall amount (Figure 5.1) the early exponential phase, day 6 the late exponential phase, day 9 the stationary phase and day 12 the phase of cell aging (see Figure 5.1 a). For further experimental details compare legend of Figure 5.3.

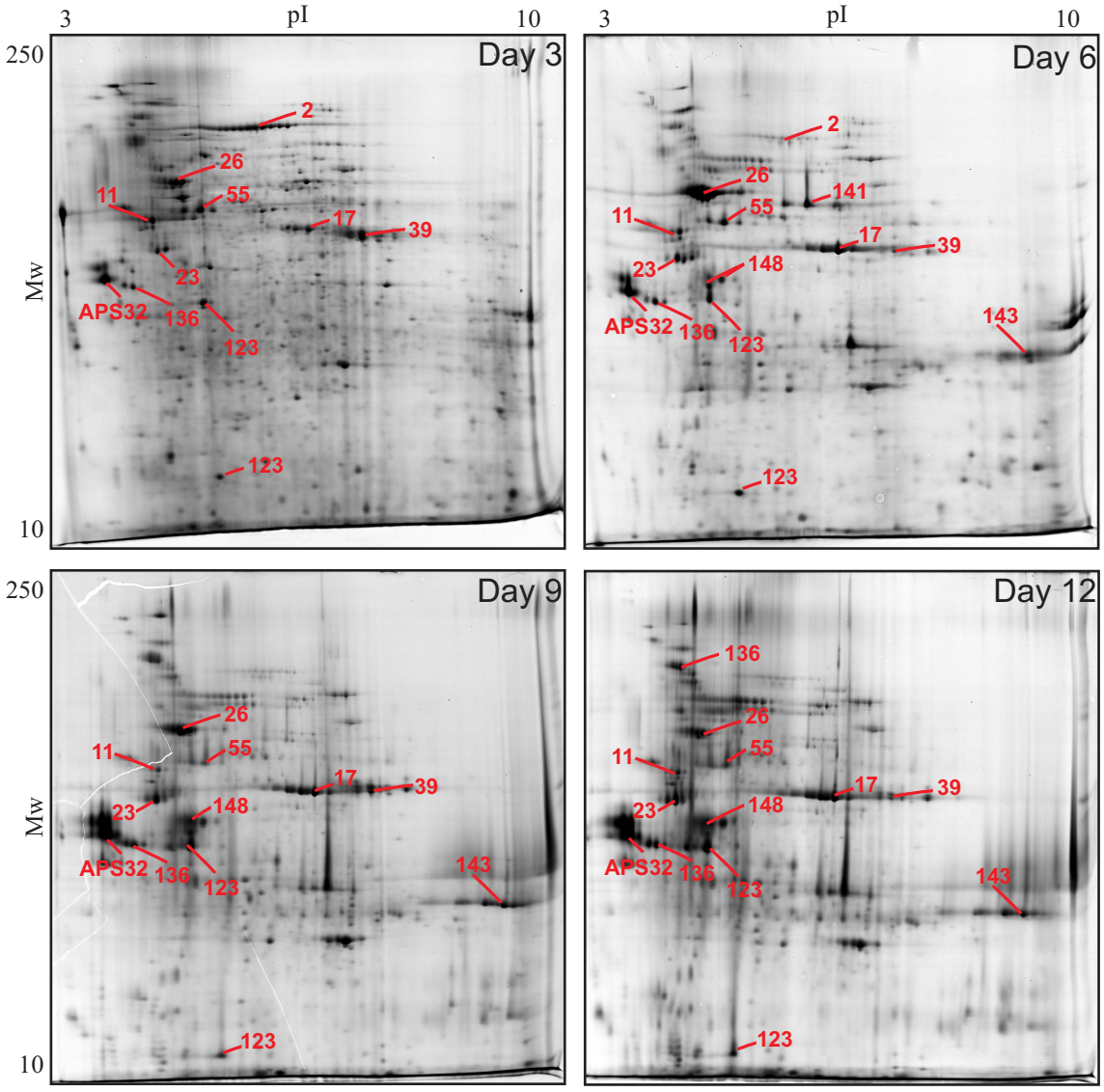


Figure 5.5: 2-DE-gels of the extractable cell wall proteins extracted from the isolated cell wall fraction with hot SDS under reducing conditions from day 3, 6, 9 and 12 of cultivation of *C. cinerea* grown at 37 °C in shaking cultures in liquid YMG medium. Day 3 of cultivation is according to the ergosterol and the cell wall amount (Figure 5.1) the early exponential phase, day 6 the late exponential phase, day 9 the stationary phase and day 12 the phase of cell aging (see Figure 5.1 a). For further experimental details compare legend of Figure 5.3.

Table 5.1: Number of spots matching between the 2-DE gels of the days 3, 6, 9 and 12 of cultivation from the free secretome of *C. cinerea* shaking cultures grown at 37 °C in liquid YMG medium as deduced by analysis with Proteomweaver software (Version 3.1.0.7 Definiens AG München, Germany).

Sample	S3*	S6	S9	S12
S3	**138 ± 9**			
S6	61 ± 3	**189 ± 7**		
S9	47 ± 3	94 ± 4	**141 ± 10**	
S12	42 ± 4	81 ± 8	90 ± 8	**127 ± 18**

S: freely secreted proteins; numbers stand for the day of growth of the fungus
Values in bold represent the absolute spot numbers.

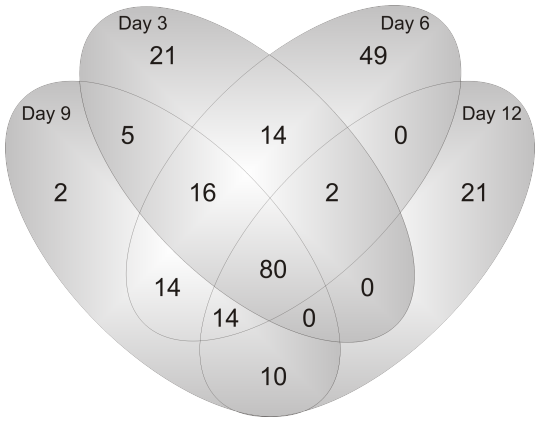


Figure 5.6: Venn diagram showing the spots overlapping between the days 3, 6, 9 and 12 of cultivation in the free secretome from *C. cinerea* grown at 37 °C in shaking cultures in liquid YMG medium (see also Table 5.1).

5.3). About half of the spots were shared at all time points between the free secretome and the hyphal sheath proteome. The analysis revealed that the percentage of spots in the free secretome matching to spots in the hyphal sheath varies only slightly during growth with 62.2% of total spots on day 3, 41.7% on day 6, 55.2% and 59.5% on the days 9 and 12, respectively.

Matching of the gels between the four time points of cultivation from each of the different fractions revealed alterations of single protein spots in their intensities from one of the different secretome fractions during the examined time of cultivation. Representative spots changing significantly in their intensity during cultivation were analyzed with respect to the changes over the time (Figure 5.9). For example, metallopeptidase MH/M28 (ID-number 26) showed the highest spot intensity in the hyphal sheath at

Table 5.2: Number of spots matching between the 2-DE gels of the days 3, 6, 9 and 12 of cultivation from the hyphal sheath proteome of *C. cinerea* shaking cultures grown at 37 °C in liquid YMG medium as deduced by analysis with Proteomweaver software (Version 3.1.0.7 Definiens AG München, Germany).

Sample	HS3	HS6	HS9	HS12
HS3	**166 ± 7**			
HS6	74 ± 5	**164 ± 9**		
HS9	63 ± 5	96 ± 6	**158 ± 16**	
HS12	51 ± 5	78 ± 3	99 ± 3	**144 ± 11**

HS: hyphal sheath proteins; numbers stand for the day of growth of the fungus
Values in bold represent the absolute spot numbers.

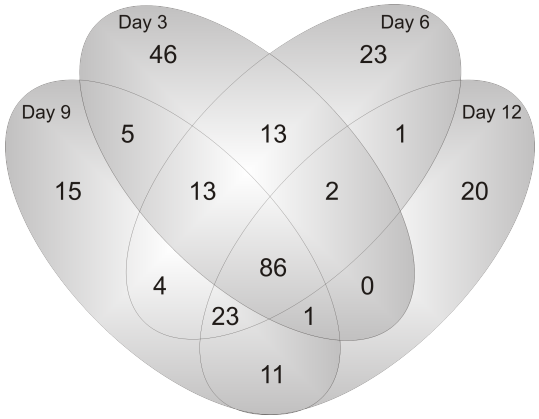


Figure 5.7: Venn diagram showing the spots overlapping between the days 3, 6, 9 and 12 of cultivation in the hyphal sheath from *C. cinerea* grown at 37 °C in shaking cultures in liquid YMG medium (see also Table 5.2).

Table 5.3: Number of spots matching between the 2-DE gels of the days 3, 6, 9 and 12 of cultivation between the free secretome and the hyphal sheath proteome of *C. cinerea* shaking cultures grown at 37 °C in liquid YMG medium as deduced by analysis with Proteomweaver software (Version 3.1.0.7 Definiens AG München, Germany).

Sample	S3	S6	S9	S12
HS3	86 ± 5			
HS6		79 ± 8		
HS9			78 ± 6	
HS12				76 ± 8

S: freely secreted proteins; HS: hyphal sheath proteins; the numbers stand for the day of growth of the fungus

Table 5.4: Number of spots matching between the 2-DE gels of the days 3, 6, 9 and 12 of cultivation from the extractable cell wall proteome of *C. cinerea* shaking cultures grown at 37 °C in liquid YMG medium as deduced by analysis with Proteomweaver software (Version 3.1.0.7 Definiens AG München, Germany).

Sample	CW3	CW6	CW9	CW12
CW3	**149 ± 5**			
CW6	49 ± 15	**136 ± 4**		
CW9	30 ± 12	62 ± 12	**116 ± 12**	
CW12	29 ± 8	58 ± 10	72 ± 4	**116 ± 7**

CW: cell wall fraction; numbers stand for the day of growth of the fungus
Values in bold represent the absolute spot numbers.

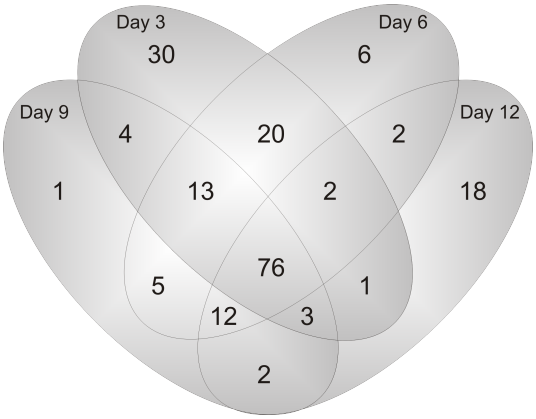


Figure 5.8: Venn diagram showing the spots overlapping between the days 3, 6, 9 and 12 of cultivation in the cell wall from *C. cinerea* grown at 37 °C in shaking cultures in liquid YMG medium (see also Table 5.4).

day 6 of cultivation but the concentration decreased with progression of cultivation (Figure 5.9 a). In contrast, the spot intensity of the unknown protein 29 (ID-number 147) showed a steady increase from day 3 of cultivation to the highest intensity on day 12 of cultivation (Figure 5.9 d).

5.4.3 Protein Identification

In order to identify proteins from the 2-DE gels, 87 spots from all fractions and time points were picked and analyzed by LC-MS^2. In total, 70 picked spots were positively identified, representing 31 different proteins (Table 5.5). The majority of identified proteins (18 of 31) was detected on every examined day of cultivation or at least at three of the four time points of cultivation (compare Figure 5.10). In contrast, only few were present at only one of the examined days of growth (Figure 5.10). Amongst these latter proteins were a metallophosphoesterase (ID-number 8, on day 3 in the hyphal sheath), a glycoside hydrolase from family 18 (ID-number 141, on day 6 in all three fractions) and an unknown protein (ID-number 146, on day 3 in the supernatant and the hyphal sheath).

Amongst the positively identified protein spots ten peptidases from all samples, distributed over 23 different spots in all fractions and over all time points, were detected, most of them in high abundant spots (Figures 5.3 to 5.5). Three of the putative peptidases (ID-numbers 6, 26, 39) changed in all fractions significantly in their concentration during growth (Figure 5.9 a-c).

Table 5.5: Identified protein spots from the 2-DE gels of all analyzed *C. cinerea* secretome fractions and of all examined time points of cultivation.

ID	Predicted protein function	Signal peptide	Protein accession	Fraction	Days of growth 3	6	9	12
Peptidases								
6	Serine peptidase SB/S8 (1)	+	EAU90899	S	+	+	+	+
				HS	+	+	+	+
				CW	-	-	-	-
71	Serine peptidase SB/S8 (2)	+	EAU91794	S	+	+	+	+
				HS	+	+	+	+
				CW	-	-	-	-
23	Aspartic type peptidase AA/A1	+	EAU84813	S	-	-	-	-

Table 5.5: continued

ID	Predicted protein function	Signal peptide	Protein accession	Fraction	Days of growth 3	6	9	12
				HS	-	-	-	-
				CW	+	+	+	+
8	Metallophosphoesterase	+	EAU93605	S	-	-	-	-
				HS	+	-	-	-
				CW	-	-	-	-
11	Metallopeptidase MA/M36 (1)	+	EAU82511	S	+	+	+	+
				HS	+	+	+	+
				CW	+	+	+	+
21	Metallopeptidase MA/M43 (1)	+	EAU90085	S	+	+	+	+
				HS	+	+	+	+
				CW	-	-	-	-
26	Metallopeptidase MH/M28 (2)	+	EAU87896	S	+	+	-	-
				HS	+	+	+	+
				CW	+	+	+	+
39	Metallopeptidase MA/M36 (2)	+	EAU86463	S	+	-	-	-
				HS	+	-	-	-
				CW	+	+	+	+
77	Metallopeptidase MH/M28 (1)	+	EAU92866	S	-	+	+	-
				HS	+	+	+	-
				CW	-	-	-	-
90	Metallopeptidase MA/M43 (3)	+	EAU83013	S	+	+	+	+
				HS	+	+	+	+
				CW	-	-	-	-
Glycoside hydrolases								
1	Glycoside hydrolase family 3 (1)	+	EAU87688	S	-	+	+	+
				HS	-	+	+	+
				CW	-	-	-	-
17	Glycoside hydrolase family 5	+	EAU92553	S	+	+	+	-
				HS	+	-	-	-
				CW	+	+	+	+
142	Glycoside hydrolase family 7	+	EAU85436	S	+	+	+	+
				HS	+	+	+	+
				CW	-	-	-	-
15	Glycoside hydrolase family 15 (1)	+	EAU86023	S	+	+	+	+
				HS	+	+	+	+
				CW	-	-	-	-

Table 5.5: continued

ID	Predicted protein function	Signal peptide	Protein accession	Fraction	Days of growth 3	6	9	12
143	Glycoside hydrolase family 17	+	EAU88679	S	-	+	+	+
				HS	-	+	+	+
				CW	-	+	+	+
141	Glycoside hydrolase family 18	+	EAU86796	S	-	+	-	-
				HS	-	+	-	-
				CW	-	+	-	-
Other hydrolases								
2	Alkaline phosphatase	+	EAU83081	S	-	-	-	-
				HS	+	-	-	-
				CW	+	+	-	-
Oxidoreductases								
4	Copper radical oxidase	+	EAU83456	S	+	+	+	-
				HS	+	-	+	-
				CW	-	-	-	-
31	Oxidoreductase (2)	+	EAU80813	S	+	+	+	+
				HS	+	+	+	+
				CW	-	-	-	-
36	Oxidoreductase (3)	+	EAU82165	S	+	+	+	+
				HS	+	+	+	+
				CW	-	-	-	-
144	Pyranose dehydrogenase	+	EAU87901	S	+	+	+	+
				HS	-	+	+	+
				CW	-	-	-	-
Other enzymes								
52	Peptidyl-prolyl-cis-trans-isomerase (1)	-	EAU82113	S	-	-	-	-
				HS	+	+	+	+
				CW	-	-	-	-
123	ATP-synthase subunit	-	EAU88709	S	-	-	-	-
				HS	-	-	-	-
				CW	+	+	+	+
Proteins without predicted enzymatic function								
APS32	Ricin-B-lectin	+	EAU89191	S	+	+	+	+
				HS	+	-	-	-
				CW	+	+	+	+
55	CBM-containing protein (2)	+	EAU85085	S	-	-	-	-

Table 5.5: continued

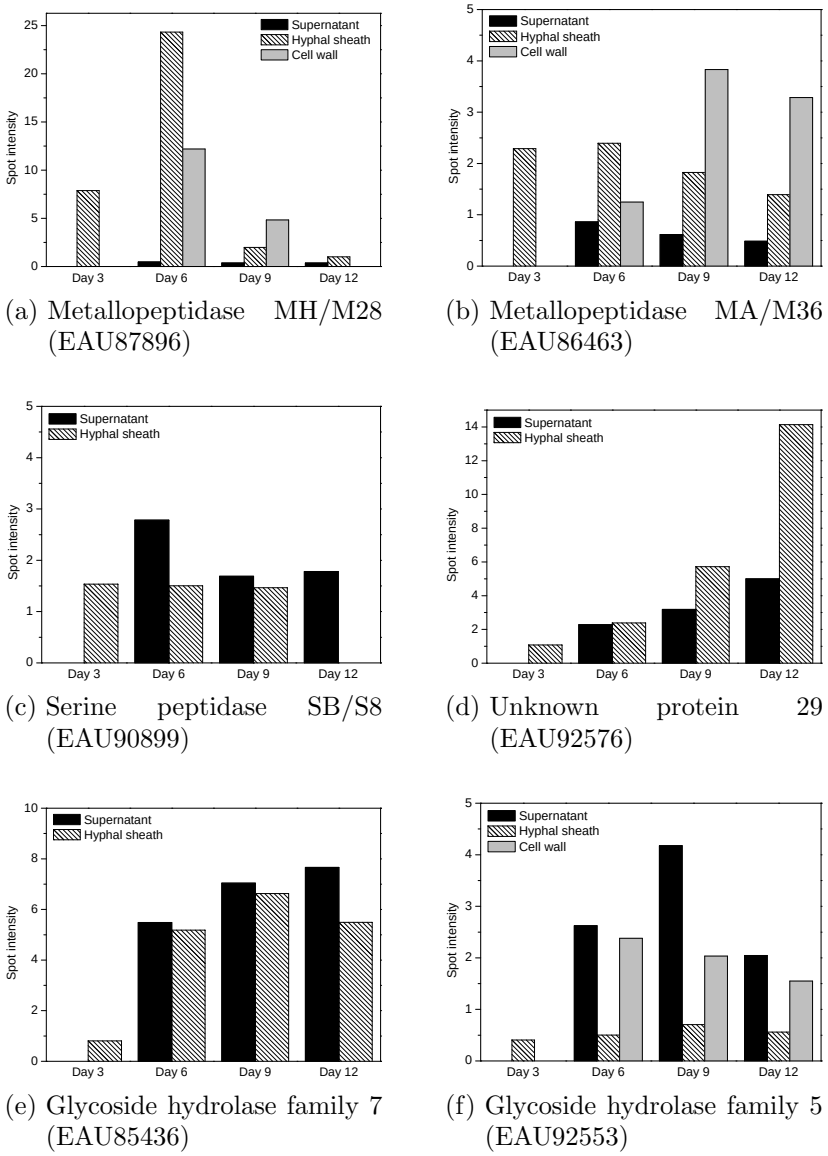
ID	Predicted protein function	Signal peptide	Protein accession	Fraction	Days of growth 3	6	9	12
				HS	-	-	-	-
				CW	+	+	+	+
60	Heat shock 31-like	-	EAU84741	S	+	+	-	-
				HS	+	-	-	-
				CW	-	-	-	-
137	Ceratoplatanin (2)	+	EAU81969	S	-	-	-	-
				HS	+	+	-	-
				CW	-	-	-	-
Unknown proteins								
136	Unknown protein (15)	+	EAU88001	S	-	+	+	+
				HS	-	-	-	-
				CW	-	-	-	-
146	Unknown protein (28)	+	EAU87171	S	+	-	-	-
				HS	+	-	-	-
				CW	-	-	-	-
147	Unknown protein (29)	-	EAU92576	S	-	+	+	+
				HS	-	+	+	+
				CW	-	-	-	-
148	Unknown protein (30)	+	EAU89154	S	-	-	-	-
				HS	-	-	-	-
				CW	-	+	+	+

* S: Freely secreted proteins, HS: Hyphal sheath proteins, CW: Extractable cell wall proteins
Protein spots marked with + were picked and analyzed by LC-MS^2 as described in Material and Methods. Spots with low intensities were pooled from the three replicated gels.

Further, six different glycoside hydrolases were identified in totally 13 different spots of all fractions and time points. Except of the two glycoside hydrolases from family 5 and 7 (ID-numbers 17, 142; Figure 5.9), the glycoside hydrolases seem to be secreted constantly during the whole examined growth period in the cell wall (ID-number 17) and the supernatant and the hyphal sheath (ID-number 142), respectively (Table 5.5).

In addition, also proteins without enzymatic function such as a ricin-B-lectin (ID-number APS32) and a protein with a carbohydrate binding domain (ID-number 55) were detected at all time points in the extractable cell wall fraction (Table 5.5).

Compared to the 1-DE shotgun analysis of the fractionated secretome of *C. cinerea*



(a) Metallopeptidase MH/M28 (EAU87896)

(b) Metallopeptidase MA/M36 (EAU86463)

(c) Serine peptidase SB/S8 (EAU90899)

(d) Unknown protein 29 (EAU92576)

(e) Glycoside hydrolase family 7 (EAU85436)

(f) Glycoside hydrolase family 5 (EAU92553)

Figure 5.9: Relative intensities (intensity minus background) of identified spots in the 2-DE gels of the different fractions of the secretome of *C. cinerea* cultures of day 3, 6, 9 and 12 of cultivation (at 37 °C in shaking cultures with liquid YMG medium). For identification of the proteins, NCBI accession numbers are given. Spot intensities measured by Proteomweaver software (Version 3.1.0.7 Definiens AG München, Germany) were multiplied by a factor (protein concentration of one fraction divided through the protein concentration of the free secretome as reference value) reflecting the differences in the protein amount of the different protein fractions.

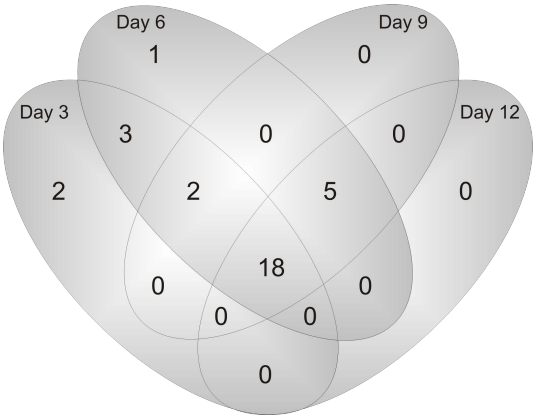


Figure 5.10: Venn diagram showing overlaps of all the identified proteins of all types of fractions between the day 3, 6, 9 and 12 of cultivation of *C. cinerea* grown at 37 °C in shaking cultures in liquid YMG medium.

analyzed at day 3 of cultivation (Chapter 3; Section 3.5.2), only seven new proteins were detected in the here presented study (Table 5.5). Amongst them were three different glycoside hydrolases (ID-numbers 141, 142, 143), two of them appearing at day 6 of cultivation in all fractions. Further three unknown proteins (ID-numbers 146, 147, 148) are amongst the newly detected proteins, one of which (ID-number 146) is only present at day 3 of cultivation in the free secretome and the hyphal sheath and two of them (ID-numbers 147, 148) appearing as well at day 6 of cultivation continuing to be present till day 12 of cultivation. The last newly detected protein is a pyranose dehydrogenase (ID-number 144) detected at all days of cultivation in the free secretome and from day 6 to day 12 also in the hyphal sheath.

5.5 Discussion

A previous study (Chapter 3) revealed a complex secretome for *C. cinerea* monokaryon Okayama7 cultures in the early logarithmic growth phase. Proteins in the free secretome and the hyphal sheath were to considerable parts overlapping whereas in the cell wall proteome mostly other proteins occurred. In the study presented in this chapter, the secretome and its compartmentation was further followed up over different growth phases during cultivation of *C. cinerea* monokaryon Okayama7 in shaking cultures at 37 °C. Therefore, the secretome of *C. cinerea* was fractionated into freely secreted proteins, proteins of the hyphal sheath and extractable cell wall proteins, bound to the

cell wall by noncovalent linkages and disulphide bridges. The different fractions of the fungal secretome were analyzed at day 3, 6, 9 and 12 of growth, which are representative time points for the fungal growth according to the overall biomass dry weight, the ergosterol concentration, the cell wall amounts isolated from the fungal cultures and the total protein content of the cultures (Figure 5.1). According to the ergosterol, the cell wall values and the total protein amount correlated to the ergosterol content, day 3 represents the early exponential phase, day 6 the late exponential phase, day 9 the stationary phase and day 12 the stage of beginning of cell lysis (Figure 5.1).

The values obtained from the biomass dry weight measurement on the one hand and the ergosterol measurements on the other hand were however, not corresponding, as the dry weight was highest on day 3 while the ergosterol concentration and the cell wall amounts raised in later stages of cultivation with a peak of ergosterol at day 7 of cultivation and highest cell wall amounts at days 9 and 12 of cultivation (Figure 5.1 a). The same discrepancy was therefore observed for the total protein content related to the ergosterol content on the one hand and the bio dry mass and the dry cell wall on the other hand (Figure 5.1 b and c). The discrepancy between the bio dry mass and the ergosterol values is possibly caused by high molecular weight polysaccharides produced by the fungus in form of soluble polysaccharides (remaining in the mycelial fraction after filtration of the culture) and in form of the hyphal sheath. The dry weight of both kinds of polysaccharides might hinder the correct determination of the dry weight of the fungal cells in the mycelium. The hypothesis of a massive occurrence of polysaccharides is supported by the glucose concentrations measured in the liquid medium (Figure 5.1 a). On day 3 of cultivation, the glucose is almost used up in parallel to the massive increase of polysaccharides. Thus, one can postulate that *C. cinerea* is first forming a hyphal sheath as well as soluble polysaccharides from the available glucose of the culture medium. This idea is supported by the observations that upon filtration of the cultures (with a Büchner funnel and filterpaper; see Material and Methods of this Chapter) from early days of cultivation (day 2 to day 5) yellowish material accumulating on the filter paper (not shown). Under natural conditions, fast production of polysaccharide material possibly makes easily available carbon sources, such as glucose, inaccessible for competitive organisms (e.g. bacteria, able to enter the space between the fungal hyphae) as these might not possess enzymes able to degrade the linkages between the complex glucans formed by the fungus.

On day 3 of cultivation, the absolute protein concentration was highest in all se-

creted protein fractions as well as in the intracellular protein fraction. This result is in agreement with the results of the ergosterol measurement which indicate that day 3 of cultivation is the early exponential phase (Figure 5.2 a). Also with a low amount of fungal mycelium, the protein content is the highest of all examined days of cultivation, most probably due to logarithmic growth of mycelium which needs high amounts of resources provided by the enzymes. During growth, especially the content of intracellular proteins decreased significantly, but also the protein content of the secretome fractions is decreasing although with a time shift from day 6 to day 9 of cultivation (Figure 5.2 a). This might be due to the fact that the size of the mycelial pellets formed in shaking cultures is increasing. Possibly only the cells on the pellet surface are physically active while the cells present in the center of the pellet may serve only the structure of the fungal pellet (Grimm et al., 2005; Rühl, 2010). This hypothesis of reduction of physiologically active cells explains well the decrease of the intracellular protein content and as well the significant increase of the amount of cell wall isolated from the dry mycelium of *C. cinerea* (Figure 5.1 b). In contrast, the amount of the secreted proteins is delayed in decrease compared to the amount of the intracellular proteins. One can postulate that this is due to a higher stability of extracellular proteins compared to intracellular proteins or that the extracellular proteins are produced and secreted constantly. However, future studies have to show whether the extracellular proteins are stable or continuously newly secreted, possibly by the analysis of the transcriptome from *C. cinerea*.

5.5.1 The Fractionated Secretome of *C. cinerea* Is Reduced in Complexity During Growth

The gels of the four time points of cultivation from the freely secreted, the hyphal sheath and the extractable cell wall fraction were compared to each other by matching them with the Proteomweaver software (Tables 5.1 to 5.4 and Figures 5.6, 5.7 and 5.8). Further, the gels of the freely secreted and the hyphal sheath fraction from each time point (day 3 of harvest during cultivation of the freely secreted fraction against day 3 of the hyphal sheath fraction and so on) were matched against each other (Table 5.3), due to the obvious high similarities between these fractions (Figures 5.3 and 5.4). A similar analysis for the extractable cell wall proteins was without much positive result due to the fact that this fraction is strongly divergent in spot pattern to the other two

fractions.

Matching of the gels between the four time points of one given compartment (free secretome or hyphal sheath proteome or extractable cell wall proteome, respectively) showed that all gels from day 3 of cultivation differed most significantly from the gels of the three other analyzed days (Tables 5.1, 5.2 and 5.4 and Figures 5.6, 5.7 and 5.8). The general complexity of the gels, represented by the spot number, was reduced over the time (Tables 5.1, 5.2 and 5.4). As day 3 represents the early exponential growth phase, it can be assumed that the fungus has strong needs for enzymes involved in nutrient supply as well as in formation of the cell wall and formation of the hyphal sheath. Due to the dramatic decrease in biomass on day 7 of cultivation (Figure 5.1), one can postulate that the hyphal sheath is degraded and used as carbon source (Stahmann et al., 1992). This option is possibly reducing the need for a high number of different enzymes because the glucans of the hyphal sheath are most probably linked by only a small number of different linkages as shown for *Pleurotus* species (Gutiérrez et al., 1995). In consequence, in *C. cinerea* this should lead to a reduced complexity of the secretome. A similar observation concerning a reduced complexity of the free secretome over the time was made by Sato et al. (2007) with *P. chrysosporium* when grown on sawdust as carbon source for 30 days and harvested for secretome analysis at day 6, 12, 18 and 30 of cultivation, respectively.

The relative differences of the protein profiles from the freely secreted and the hyphal sheath proteins at the same time points showed a constant overlapping of protein spots over the whole time of cultivation (Table 5.3). This shows that despite of a possible reduction of the hyphal sheath and of the total protein concentration during the time of cultivation (Figures 5.1 and 5.2), the hyphal sheath proteins still discriminate from the freely secreted proteins. The strict compartmentation previously described in Chapter 3 is further maintained during the later growth phases.

5.5.2 Identification of Protein Spots

In total, 70 spots from all 2-DE gels analyzed in this study were positively identified by LC-MS^2 and the analysis revealed 31 different proteins. All except of seven proteins were already detected previously within the study of 3-days-old *C. cinerea* cultures described in Chapter 3 (Tables 3.3 to 3.10). Amongst the seven newly detected proteins are three glycoside hydrolases (ID-numbers 141, 142, 143), three unknown proteins (ID-numbers

146, 147, 148) and a pyranose dehydrogenase (ID-number 144). Only 3 of these (ID-numbers 142, 144, 146) were positively identified in the fractionated secretome of day 3 of cultivation (Table 5.5). The detection of additional proteins compared to the 1-DE shotgun approach of samples with the same age (Chapter 3; Section 3.5.2) can be considered as biological variance which is with only 3 newly detected proteins on day 3 of cultivation very low. Four newly detected proteins appeared first at day 6 and continued to be present till day 12 of cultivation. It can not be fully excluded that the rather low number of newly detected proteins after day 3 of cultivation might be a result of the different techniques used for protein identification in this experimental setup and the 1-DE shotgun approach presented in Chapter 3 (Section 3.5.2). However, the much more sensitive 1-DE shotgun approach lead to the identification of also low abundant proteins due to the fact that protein concentration of the sample is about 30 times higher than for 2-DE analysis as performed in Chapter 3 [see Chapter 3; Section 3.5.2; Wolters et al. (2001)] and possibly 10 times higher as performed here with pooling of parallel spots from replicate 2-DE gels. Therefore it can be speculated that many more proteins were already present at day 3, in many cases only in low concentrations. Such low abundant proteins are not detectable by a 2-DE approach and possibly had even a concentration under the detection limit of the RuBP-staining [about 0.5 ng protein; (Rabilloud et al., 2001)]. Nevertheless, the spot picking was optimized in this experimental setup in comparison to the 2-DE analysis presented in Chapter 3 (Section 3.4.1) due to pooling parallel spots. However, the 2-DE analysis is still far from a complete documentation of the secretome. With the shotgun approach (Chapter 3, Section 3.5.2) 99 more proteins were positively identified on day 3 of cultivation than with the 2-DE analysis presented here.

Overlapping of the identified spots between the examined days of cultivation of *C. cinerea* showed that the majority of proteins was detected on all of the examined days (Figure 5.10). Only few proteins were found to be present on only two or three days of cultivation (ID-numbers 2, 60, 137 on two days and ID-numbers 1, 4, 77, 136, 143, 147, 148 on three days) and even fewer were detected at only one day of cultivation (ID-numbers 8, 141, 146). The dynamics of the secretome of *C. cinerea* is thus not represented by a high variety of different proteins but rather reflects a change of concentration of single proteins. Figure 5.9 shows a selection of proteins changing significantly in their concentration during the time of cultivation (for an analysis of all positively identified spots see Tables A.8 and A.9 in the Appendix). At this place, a selection of proteins

changing in amounts during cultivation is discussed in further detail.

Several of the identified peptidases secreted by *C. cinerea* are changing significantly in their concentration during the time of cultivation (for representative examples see Figure 5.9 a, b and c; see also Tables A.8 and A.9, ID-numbers 6, 8, 11, 21, 23, 26, 39, 71, 77, 90 in the Appendix to Chapter 5), indicating that they are possibly specific in their functions and are possibly also responsible for different regulative events occurring in the extracellular space. For example, the concentration of serine peptidase SB/S8, present in the free secretome and the hyphal sheath (ID-number 6) showed a significant increase from day 3 to day 6 (Figure 5.9 c). In contrast, the concentration of the omnipresent metallopeptidase MA/M36 (ID-number 11; Figure 5.9 b) is almost stable over the time in the freely secreted and the hyphal sheath fraction but he concentration of the protein increases significantly from day 3 to day 6 in the cell wall fraction. Functionally, there is no obvious difference between serine and metallopeptidases (Rao et al., 1998) and there is no striking explanation for the compartmentation. Still, the pH value of the different compartments (free secretome and the hyphal sheath in contrast to the cell wall) might be one possible explanation for the compartmentation of the peptidases as they might have different pH optima for enzyme activity.

Generally, this study revealed a high number of peptidases represented by the most abundant spots of the free secretome and the hyphal sheath proteome. These findings confirm the results obtained previously in Chapter 3 (Section 3.5.2.3). As already discussed in Chapter 3, extracellular peptidases are known to provide nitrogen in form of amino acids by degradation of peptides and proteins in the substrate for microorganisms (Rao et al., 1998).

The change in the protein concentration of several peptidases (Figures 5.9 a to c) over the time of cultivation indicates a regulation of the peptidases possibly due to the decrease of nitrogen in the medium during growth, which is known to be a regulation factor for the expression of peptidases (Rao et al., 1998). However, also regulative functions of extracellular peptidases in fungi were shown in literature. For example, different laccase isoforms of *Pleurotus ostreatus* were shown to be regulated positively and/or negatively by a peptidase from the culture supernatant (Palmieri et al., 2000, 2001). Also, lignin peroxidase activity from *P. chrysosporium* showed a negative correlation with two specific peptidases in the culture supernatant (Dosoretz et al., 1990).

A similar time pattern as for the peptidases was observed for the six identified glycoside hydrolases (ID-numbers 1, 15, 17, 141, 142, 143; Table 5.5). For exam-

ple, glycoside hydrolase from family 5 (ID-number 17) and glycoside hydrolase from family 7 (ID-number 142) increase in their protein concentration from day 3 to day 9 in the free secretome and the extractable cell wall fraction and from day 3 to day 12 of cultivation in the free secretome and the hyphal sheath, respectively (Figures 5.9 f and e). Both glycoside hydrolases families are known to contain different endoglycosidases, amongst others 1,3-β-glucosidase, known to hydrolyze 1,3-β-glucans (http://www.cazy.org/fam/acc_fam.html). In *C. cinerea*, they might be involved in the degradation of the glucans forming the hyphal sheath or the restructuring of the cell wall as it was shown previously for other fungal species (e.g. *Aspergillus* species and *S. cerevisiae*) (Bowman & Free, 2006).

In addition, several putative oxidoreductases were detected amongst the freely secreted and the hyphal sheath proteins. Copper radical oxidase (ID-number 4), similar to glyoxal oxidases, catalyzing the oxidization of aldehydes to carboxylic acids, and most likely involved in the supply of H_2O_2 (Whittaker et al., 1996) occurs in three different isoforms in the free secretome and the hyphal sheath. Two of the three putative isoforms disappeared over the time of cultivation, indicating either a selective degradation or a selective secretion of a single isoform. For interest, a similar occurrence of degradation of specific isoforms was shown for lignin peroxidase in *P. chrysosporium* (Glumoff et al., 1990). However, the one long-lasting isoform of the copper radical oxidase found here over the time in the *C. cinerea* secretome is not identical with the isoform shown to be phosphorylated on sugar chains originating from the N-glycosylation of the protein (Chapter 4; Section 4.4.2).

Pyranose dehydrogenase (Pyranose dehydrogenases (PDHs)), a sugar oxidoreductase from the family of pyranose oxidases (Volc et al., 2000) is oxidizing aldo-pyranoses (e.g. glucose) to 2-aldo-ketoses by using different kinds of benzoquinones as electron acceptor (Peterbauer & Volc, 2010; Volc et al., 2000). In *C. cinerea*, the pyranose dehydrogenase (ID-number 144) was present on all examined days of cultivation in the free secretome but only appeared on day 6 of cultivation in the hyphal sheath and continued to be present till day 12 of cultivation. PDH is stated to be an enzyme specific for litter decomposing fungi such as *Agaricus bisporus* (Sygmund et al., 2008). In this work is proposed that PDH is involved in the breakdown of lignocellulose by litter decomposing fungi such as *A. bisporus* as the enzyme is catalytically related to fungal pyranose oxidase and cellobiose dehydrogenase (Peterbauer & Volc, 2010). Also in *C. cinerea*, PDH might play a similar role as in *A. bisporus* in the degradation of

lignocellulose contained in horse dung, the natural substrate of *C. cinerea* (Kües, 2000). This, however, explains not the occurrence of PDH in the artificial YMG medium used for the cultivation of the fungus in this experimental setup and a function for this enzyme is currently not visible.

Two putative FAD/FMN-containing oxidoreductases (ID-numbers 31 and 36) appeared in a constant amount during the whole time of growth in the free secretome and the hyphal sheath. This might be due to constant secretion over the whole growth period or due to extreme stability of the enzyme and resistance against degradation. FAD/FMN-containing oxidoreductases are known to provide H_2O_2 for peroxidases as already stated in Chapter 3 (Section 3.5.2.1). However, as they might use sugars as substrate they might as well be involved in the supply of C-sources.

5.5.3 *C. cinerea* Possesses a Dynamic Secretome

In general, the results of this study show that several proteins are increasing or decreasing in their relative concentration in the secretome over the time such as putative isoforms from the predicted copper radical oxidase (ID-number 4). The obtained results from this study confirm again a clear compartmentation of the secretomes from the different compartments of the extracellular space as can be seen in the analyzed 2-DE gels of the three fractions (Figures 5.3 to 5.5). The results in these Figures also indicate that the proteins of the different compartments are dynamic in their concentration. However, the majority of the identified proteins is found during the whole time of observation (Figures 5.6 to 5.8). Only few proteins were identified at only one time point. Although artificial culture conditions, such as shaking cultures and liquid medium, were used in this experimental setup and presence of not all of the proteins can be explained by current knowledge, the obtained results for other proteins suggest that the secretome of *C. cinerea* is dynamic concerning amounts of specific proteins probably as an adaption to the prevailing environmental conditions.

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6 General Discussion

The fungal cell wall is a complex network of polysaccharides and proteins surrounding the fungal cell. It serves not only as a protection layer, but as well it provides the shape of the fungal cell and establishes the contact of the fungus with its environment (Bowman & Free, 2006). From model organisms such as *Saccharomyces cerevisiae* and *Candida albicans*, the fungal cell wall structure is known to consist of glucans as load bearing construct, chitin as a minor component (1-2%) and glycoproteins (5-20%) attached to the cell wall through different kinds of linkages (Chaffin et al., 1998; Klis et al., 2006; Kollár et al., 1995, 1997; Lipke & Ovalle, 1998). The cell wall of higher basidiomycetous fungi in contrast was not as much studied up to now (Bottom & Siehr, 1979, 1980; Gooday, 1979; Kamada et al., 1993; Schaefer, 1977; Wessels & Sietsma, 1979) but it is known to contain also glucans as a main component and to have a higher chitin content with about 10 to 20% of the wall dry weight (Gooday, 1979; Kamada et al., 1993). Nevertheless, the cell wall of basidiomycetes is most probably a crucial structure for the substrate degradation of these fungi, as it provides the contact zone between the fungal cell and its substrate. In addition to the cell wall, several higher basidiomycetes are known to produce an additional extracellular polysaccharide layer, the so called hyphal sheath, distributed over the surface of the hyphae in the mycelial network (Bes et al., 1987; Gutiérrez et al., 1995). As discussed in Chapter 5, this additional polysaccharide layer might serve as a storage of carbohydrates (Stahmann et al., 1992). Beside the cell wall, also this polysaccharide structure was shown in different fungi to incorporate several enzymes for substrate degradation (Rast et al., 2003).

The aim of this thesis was to perform an analysis of the secretome, fractionated into freely secreted proteins, proteins of the hyphal sheath and cell wall proteins (non-covalently and covalently linked) from the model basidiomycete *Coprinopsis cinerea* with proteomic methods and techniques. The secretome was defined as a compendium of freely secreted proteins (supernatant proteins), proteins of the hyphal sheath and

cell wall proteins. Cell wall proteins are attached to the fungal cell wall by different linkages: non-covalently by ionic bonds, by hydrogen bridges, by van der Waals forces and covalently by disulphide bridges (easy to destroy), by alkali-sensitive linkages or by GPI anchors (De Groot et al., 2005; Pitarch et al., 2002). On the basis of these linkages, the secretome was fractionated into the freely secreted proteins, the proteins of the hyphal sheath, the NaCl-, SDS- and NaOH-extractable cell wall proteins. The remaining unextractable proteins (possibly 5 - 10% of the total cell wall proteins) were attempted to be isolated for subsequent identification by digestion of the remaining cell walls with CNBr and trypsin, a treatment known to reveal GPI-anchored cell wall proteins (Ebanks et al., 2006). Unfortunately, no such GPI-anchored proteins were positively identified.

6.1 Strategies for Protein Identification

During the experimental work for this thesis, proteins of the fractionated secretome from *C. cinerea* were identified by LC-MS^2 in four independent experimental series (Chapter 3 to 5). Two different strategies with slightly different emphasis were used: one type of analysis appointed 2-DE gels, which revealed 31 positively identified proteins distributed over 70 different spots taken all experiments together (Chapter 3 to 5) and a 1-DE shotgun approach (Dowell et al., 2008; Merrihew et al., 2008; Wolters et al., 2001) described in Chapter 3. The later revealed 162 positively identified proteins, more than five times more compared to the more specific 2-DE approach. This is due to the fact that the analyzed protein amount is by 30 times larger than the amount loaded on a 2-DE gel (Chapter 3; Sections 3.3.6 and 3.3.8). Thus, a great advantage of the shotgun approach over the 2-DE approach is that also low abundant proteins were identified. Another advantage is that the 1-DE shotgun approach is less laborious in identification of a large number of different proteins (Wolters et al., 2001). In contrast, the analysis of the fractionated secretome by 2-DE reveals insight into the characteristics and the relative quantity of the single proteins which is not possible by a 1-DE shotgun approach. Combining both strategies, as it was done in this thesis (see Chapter 3), gives a more complete picture of the fractionated secretome of *C. cinerea*.

All four experimental setups (2-DE approach and 1-DE approach shown in Chapter 3; 2-DE approach for the analysis of posttranslational modifications presented in Chapter 4

and the 2-DE approach over the time of cultivation shown in Chapter 5) were performed under the same culture conditions and resulted in the identification of mainly the same proteins. The 1-DE shotgun approach was performed with 3-days-old cultures and revealed most proteins. The spot analysis of 2-DE gels from cultures with the same age as performed in Chapter 3 (Section 3.4.1) revealed in comparison no new proteins. In Chapter 5, however, an optimized identification of protein spots from 2-DE gels (due to pooling of parallel spots; see Chapter 5, Section 5.3.4) of the fractionated secretome of *C. cinerea* of day 3, 6, 9 and 12 of cultivation was performed (Chapter 5). This analysis revealed only three new proteins on day 3 of cultivation, which is a rather low number for biological variance between two experimental setups. The identification of protein spots from cultures older than 3 days revealed only four extra proteins in a 2-DE approach (Chapter 5; Table 5.5). This might be due to the fact that the shotgun identification was able to detect also low abundant proteins, as a result of the 30 times larger protein concentration used for this analysis (Dowell et al., 2008; Merrihew et al., 2008; Wolters et al., 2001), which might not be visible on 2-DE gels stained with RuBP [0.5 ng per spot detection limit (Rabilloud et al., 2001)]. Thus, proteins which were identified in 3-days-old cultures within the shotgun approach might only be detectable on the 2-DE gels of day 6 or later. This outlines the great advantage of the shotgun identification of proteins.

The identification of almost the same sets of proteins within four independent experimental sets with the same culture conditions (Chapters 3, 4 and 5) shows that the biological variance between these four different experimental setups was comparably low. Accordingly, the culture conditions and protein extraction methods are reproducible and also the response of *C. cinerea* to the artificial culture conditions (such as shaking conditions and liquid medium) is reproducible. Although the liquid medium and the given carbon source (glucose) are not close to the natural growth conditions of *C. cinerea* [substrate horse dung; (Kües, 2000)], the fungus seems to have a specific strategy to deal with the prevalent environment.

6.1.1 Putative Intracellular Enzymes Were Detected in the Cell Wall

The isolation of a pure, contamination-free fungal cell wall is challenging when sensitive protein identification methods such as the 1-DE shotgun approach are used. The contamination of the isolated cell wall with intracellular proteins was controlled by en-

zyme assays (Ibraheem et al., 2005; Sassoon & Mooibroek, 2001; Soundar et al., 2006) during purification of cell wall fractions (Figure 3.1). Although an activity of the tested intracellular enzymes was not detectable on the isolated cell wall fractions, various typically intracellular proteins were later identified by LC-MS^2, especially in the 1-DE shotgun approach; e.g. formate dehydrogenase, malate dehydrogenase, and succinate dehydrogenase (Tables 3.4 and 3.10). Homologues of some of the identified putative intracellular proteins found in the cell wall of *C. cinerea* were shown to occur in the cell wall of ascomycetes, possibly secreted by the fungi by an yet unknown mechanism (Alloush et al., 1997; Eroles et al., 1997; Gil-Navarro et al., 1997; López-Ribot & Chaffin, 1996; López-Ribot et al., 1996). In *C. cinerea*, some of these typically intracellular enzymes such as enolase (Edwards et al., 1999) or proteins of the heat shock protein family 70 (Hsp 70) (Edwards et al., 1999) were detected in the cell wall and in course of the shotgun experiment even in the free secretome. One of these proteins was an ATPase (ID-number 123) that was not only identified by the 1-DE shotgun approach but also on the 2-DE gels of the cell wall fraction over the whole observed growth period (Chapter 5; Table 5.5). However, ATPases are known to be high abundant plasma membrane enzymes, responsible for the ATP synthesis (Ohta et al., 1997), and because of the plasma membrane location of these enzymes, the ATPase (ID-number 123) was possibly extracted together with the cell wall proteins.

Nevertheless, some of the putative intracellular contaminations might be so called moonlighting enzymes, proteins performing multiple functions depending on their location (Nombela et al., 2006), as already outlined in Chapter 3. An deeper analysis of these proteins and localization studies could give more information about the nature and biological function of possible moonlighting enzymes in *C. cinerea*.

6.2 The Secretome of *Coprinopsis cinerea* Shows a Distinct Compartmentation

The secretome of *C. cinerea* was fractionated into the freely secreted proteins, proteins of the hyphal sheath and cell wall proteins. The latter were further fractionated into NaCl, SDS and NaOH-extractable proteins. Further, an attempt was undertaken to identify GPI-anchored proteins, detectable by a CNBr-trypsin digest of the cell wall remaining after the extraction of the other fraction (Ebanks et al., 2006).

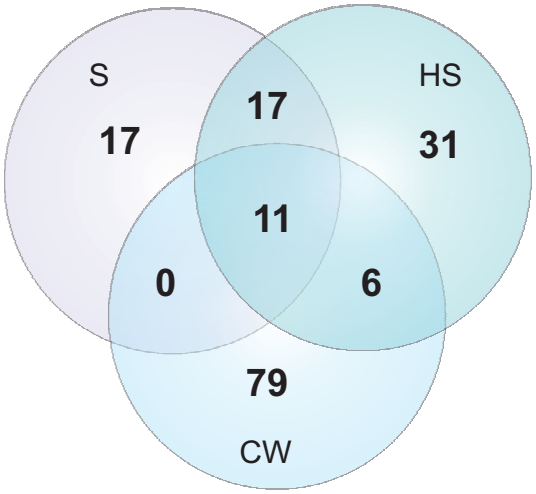


Figure 6.1: Venn diagram showing the overlap of all identified proteins from *C. cinerea* (grown at 37 °C in shaking cultures in liquid YMG medium) in course of this study between the freely secreted protein fraction (S), the hyphal sheath fraction (HS) and the combined cell wall fractions (CW).

Indeed, it was shown by 2-DE and LC-MS^2 analysis of individual proteins that the secretome of *C. cinerea* is clearly compartmented into freely secreted and hyphal sheath proteins on the one hand and extractable cell wall proteins on the other hand (Chapter 3; Figure 3.3). This compartmentation is kept up also in later growth phases (Chapter 5). This is shown by the low overlapping between the extractable cell wall proteins on the one hand and the freely secreted and hyphal sheath proteins on the other hand considering all proteins identified in course of this study (Figure 5.10 and 6.1). The overlapping proteins were glycoside hydrolases, peptidases and an alkaline phosphatase, respectively. These are enzymes involved in various reactions possibly concerning the gain of nutrients. It can also be postulated that the glycoside hydrolases detected in all fractions of the secretome are possibly involved in both the formation of the cell wall and the gain of nutrient.

Fractionation of the cell wall proteins according to their chemical binding showed as well only a small overlap between proteins shared by all or at least by one fraction, assuming that the individual cell wall proteins are attached to the wall polysaccharides by specific linkages (De Groot et al., 2005; Pitarch et al., 2002). In contrast, regarding the overlapping of the free secretome and the proteome of the hyphal sheath, these two fractions are obviously not well separated in respect to the protein content (Figure 6.1).

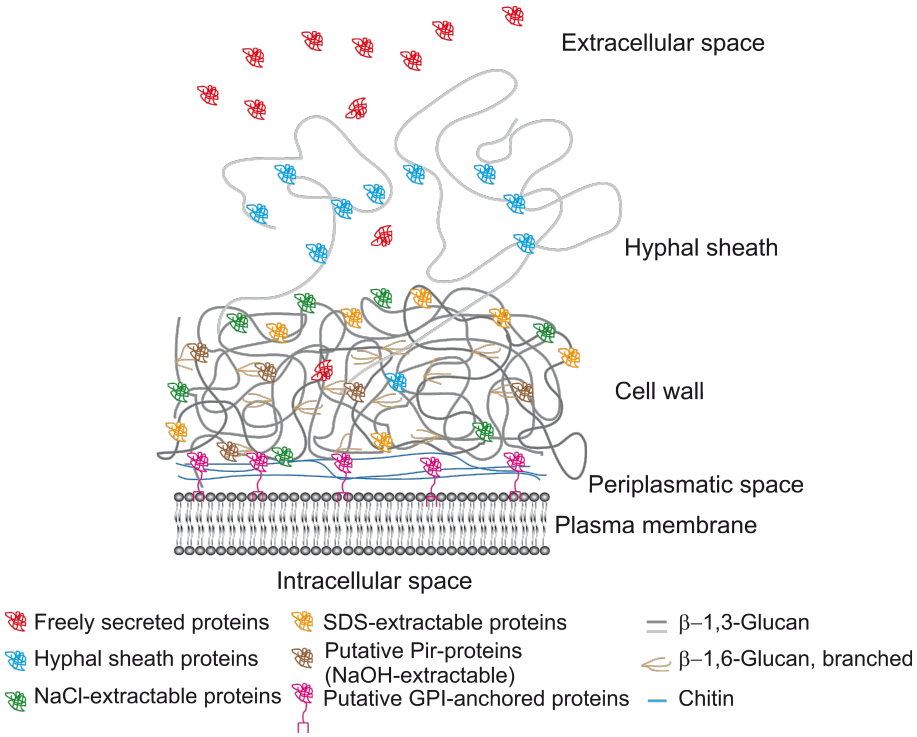


Figure 6.2: Schematic composition of the cell wall from *C. cinerea*.

However, the occurrence of freely secreted proteins is possibly not as much a natural occurring event when the fungus grows outside on horse dung but may be rather restricted to artificial growth in liquid medium. In contrast, the hyphal sheath observed in liquid cultures of *C. cinerea* might resemble a more natural structure. On natural substrate, it might act as reaction space for substrate-degrading enzymes. In the liquid laboratory cultures, the free secretome in contrast might just result from massive diffusion of proteins, normally loosely connected to the hyphal sheath glucans, from the hyphal sheath. Diffusion of loosely connected proteins might even be supported by shaking of the cultures as done in this study.

In conclusion, the study of the total secretome of *C. cinerea* grown in liquid cultures reveals a division of the extracellular compartments as follows: the liquid culture medium containing the freely secreted proteins, the hyphal sheath containing proteins incorporated into the hyphal sheath glucans by weak linkages and the cell wall containing proteins which are bound by different linkages to the cell wall polysaccharides. Figure 6.2 shows a model of how proteins might interlink with different compartments. The freely secreted proteins are present in the culture medium of liquid cultures. The

hyphal sheath proteins are loosely bound to the loose glucan network of the hyphal sheath extractable with nonionic surfactant such as Tween 80. These two fractions of the secretome are obviously locally separated. In contrast, the different fractions of the cell wall proteome are separated by the different chemical binding of the proteins: Ionically bound proteins extractable with salt-containing buffer, proteins bound with disulfide bridges extractable with SDS-containing buffer (Pitarch et al., 2002) and covalently linked proteins, extractable by mild alkaline conditions. Finally, GPI-anchored proteins are most likely anchored in the cell wall and the cell membrane as known from ascomycetous fungi (De Groot et al., 2005). Such proteins could unfortunately not be detected in *C. cinerea* by the applied methods. However, a computational analysis of the *C. cinerea* genome performed after Eisenhaber et al. (2004) revealed 652 putative GPI-anchored proteins.

6.3 Enzymes Involved in Nutrient Supply

C. cinerea naturally occurs on horse dung (Kües, 2000), an already by large digested but still lignocellulose-containing substrate. However, straw in horse dung is not anymore as compact in structure as for example wooden materials. Thus, *C. cinerea* has apparently no need for very aggressive enzymes involved in the degradation of wood, particularly in the attack of the very compact lignocellulosic cell walls of wood fibres. In agreement to this, *C. cinerea* has no genes for lignin peroxidase, manganese peroxidase and alike peroxidases, respectively (Stajich et al., 2010). There are however, 17 genes for different laccases (Kilaru et al., 2006) but the enzyme properties, as much as known, do not point to a function evolved specifically for the attack of lignin in wooden substrates (Hoegger et al., 2006; Kilaru et al., 2006; Rühl, 2010). However, the fungus could nevertheless still have a need for various glycoside hydrolases for the degradation of cellulose structures present in the crushed plant material present in horse dung (Igarashi et al., 2008; Kawai et al., 2006; Yoshida et al., 2009). The secretomic study of *C. cinerea* performed here confirmed the principle ability for the secretion of multiple types of these cellulose degrading enzymes.

Numerous different glycoside hydrolases were identified not only from exponentially growing cultures but as well in stationary cultures. Most of the identified glycoside hydrolases in this study were detected from the hyphal sheath fraction, indicating that

they are possibly involved in the formation and restructuring of the hyphal sheath (Table 6.1; glycoside hydrolases involved in the cell wall formation are further discussed in the following Section). Massive formation of the hyphal sheath obviously occurs during the early growth of the fungus in liquid culture. This is indicated by the high biomass dry weight likely resulting from a high polysaccharide content simultaneously to the total disappearance of glucose from the culture medium. Possibly, the glycoside hydrolases in the secretome of *C. cinerea* act in both, formation of the hyphal sheath and substrate degradation. However, the hyphal sheath is an external structure of the fungus which is possibly also produced as carbohydrate storage and can therefore act as a crucial structure for the fungal nutrition (Stahmann et al., 1992).

The broad spectrum of different glycoside hydrolases indicates a complex glucan network, forming the hyphal sheath linked by a variety of different linkages. Examining the glycoside hydrolases during the different growth phases showed that all but two of them (ID-number 17 and 142) are present in equal concentration over the whole growth period (Table 5.5). The stable concentration of most glycoside hydrolases might on the one hand be due to the possible role that many of them catalyze the formation as well as the hydrolysis of glycosidic linkages. Thus, the detected glycoside hydrolases might be involved in the formation and in the subsequent degradation of the hyphal sheath (Stahmann et al., 1992). Also, a constitutive expression of several glycoside hydrolases as an adaption to the natural substrate of *C. cinerea* can not be excluded. *C. cinerea* is found to inhabit a very specific substrate, horse dung (Kües, 2000), and it is possibly adapted the expression of substrate degrading enzymes to the horse dung, probably resulting in a constitutive expression of these substrate degrading enzymes such as glycoside hydrolases also in unnatural media such as YMG.

As another group of enzymes, a multitude of peptidases was detected in the secretome of *C. cinerea*. Most of them are from the class of metalloproteases and serine peptidases. Functionally, there are no predicted differences between these two groups of peptidases (Rao et al., 1998). Such types of peptidases are known to provide nitrogen for the fungus gained from the degradation of proteins in the fungal substrate (Rao et al., 1998). However, horse dung as the natural substrate of *C. cinerea* is unlikely to contain high amounts of proteins and amino acids that could serve as nitrogen sources for the fungus. Instead, urea is probably the prevalent nitrogen source on this natural substrate. Thus, the large number as well as the high concentration of single peptidases might be a response to the artificial culture conditions with a medium containing yeast

Table 6.1: Glycoside hydrolase families (GH) detected in the fractionated secretome of *C. cinerea* combined from all experimental setups in this thesis (http://www.cazy.org/fam/acc_fam.html).

Family	Representative enzyme	Reaction	Compartment
GH 3	Family of β-glucosidases	Hydrolysis of terminal, non-reducing β-D-glucose	S HS CW
GH 5	Cellulases and β-mannosidase	Endohydrolysis of 1,4- β -D-glucosidic linkages	S HS CW
GH 6	Cellobiohydrolase	Hydrolysis of 1,4- β -D-glucosidic	S
GH 7	Endo-β-1,3-1,4-glucanase; cellulases	Acting on reducing ends of cellulose chains generating cellobiose	S HS
GH 15	Glucoamylase, α-trehalase	Hydrolysis of 1,4-α-D-glucose from non-reducing ends	S HS
GH 17	Glucan 1,3-β-glucosidase	Acting on β-1,3-glucan	S HS CW
GH 18	Endo-β-N-acetylglucosaminidase; chitinase	Acting on chitin	S HS CW
GH 24	Lysozyme	Hydrolysis of 1,4- β -l linkages	HS
GH 31	α-glucosidases	Hydrolysis of non-reducing 1,4-α-D-glucose residues	S HS
GH 47	α-mannosidase	Hydrolysis of the terminal 1,2-α-D-mannose in oligo-mannose	CW
GH 62	α-L-arabino-furanosidase	Hydrolysis of non-reducing α-L-arabinofuranoside in α-L-arabinosides	S HS
GH 72	β-1,3-glucanosyl-transglycosylase	Hydrolyses of β-1,3-glucan; transfer of this reducing end to non-reducing ends of β-1,3-glucan Mouyna et al. (2000)**	HS CW

* S: Free secretome; HS: hyphal sheath proteome; CW: cell wall proteome

** This group of glycoside hydrolases was extensively studied in *Saccharomyces cerevisiae*

and malt extract with a large amount of peptides and amino acids. These unnatural nitrogen sources possibly cause an overexpression of peptidases. On the other hand, in nature the peptidases could be responsible for the degradation of proteins from other organisms such as bacteria and other fungi growing on the same substrate. This idea is supplemented by the fact that *C. cinerea* is a late colonizer of horse dung following in colonization of horse dung at the end of a relatively defined succession of other organisms displacing each other (Richardson, 2002) and the fungus might thus benefit from the proteins released from lysed bacterial and fungal cells (Kües, 2000).

Phosphate is as well an essential part of nutrition and is provided by phosphatases acting on organo-phosphates (e.g. phosphoester compounds, phosphorylated amino acids) (Guimaraes et al., 2003). In the secretome studies of *C. cinerea*, two putative phosphatases were detected, one alkaline phosphatase found in the hyphal sheath and the cell wall and an acidic phosphatase found in the free secretome (Chapter 3; Table 3.7). This strongly supports the hypothesis of formation of enclosed environments in close distance to the fungal mycelium that deviate in pH from the larger outer environment.

Another larger part of the identified enzymes were oxidoreductases which might supply H_2O_2 either for substrate degradation or for self-defence of the fungus in a manner as known from plants (Salzer et al., 1999). Totally eight different enzymes for the potential supply of H_2O_2 were detected in either the free secretome, the hyphal sheath and the cell wall. In contrast, only one putative likely intracellular peroxidase as potential consumer of H_2O_2 was detected (ID-number 118; Chapter 3, Table 3.3). However, this peroxidase was not the well known *C. cinerea* fungal class II peroxidase (Cip) (Baunsgaard et al., 1993), but rather a peroxidase related to a group of mitochondrial cytochrome C oxidases (pfam.sanger.ac.uk/).

Although *C. cinerea* is in nature not obviously involved in the degradation of wooden substrates (Kües, 2000), evidence lately rose that it nevertheless has certain wood colonizing and degrading abilities (Navarro-González, 2008). *C. cinerea* was found to grow on straw of e.g. wheat but also on fresh wood chips as they are found nowadays by modern gardening quite often in flower beds. It is possible that the fast growing fungus makes use of storage compounds in the freshly chipped wood. However, on the other hand, the detection of oxidoreductases such as the putative copper radical oxidase, homologous to enzymes from white rot fungi such as *P. chrysosporium* (Whittaker et al., 1996), allow speculations on some degree of wood degrading abilities of *C. cinerea*.

C. cinerea might possess at least potentially an enzymatic system for the degradation of lignocellulose which might for example be induced under stress. Normally in nature, this might not as often be appointed but such systems would allow flexibility to conquer new biotopes such as the human created wood chips in flower beds (Navarro-González, 2008). *C. cinerea* is a strongly aerobic fungus and the compact structure of larger wood blocks may normally restrict *C. cinerea* in occupying wood as substrate. Further future studies have to reveal the extend of the wood-degrading potential of *C. cinerea*.

6.4 Putative Proteins Involved in the Construction of the Cell Wall and the Hyphal Sheath

Enzymes involved in the degradation of the fungal substrate are considered to be freely secreted or to be attached to the outer surface of the hyphae in order to be in contact with the substrate. Indeed in this thesis, the enzymes putatively involved in nutrient supply, such as peptidases, phosphatases, oxidoreductases and glycoside hydrolases, were predominantly detected in the free secretome and the hyphal sheath confirming a strict compartmentation of the extracellular space of the fungus (Chapters 3 and 5). The cell wall of *C. cinerea* in contrast revealed proteins probably involved in the formation and restructuring of the cell wall such as specific glycoside hydrolases (e.g. glucanosyl-transglycosylases, chitinases and mannosidases; Table 6.1).

For the formation of the cell wall glycoside hydrolases, such as β-1,3- and β-1,6-glucanases and chitinases, are essential as they contribute to the formation and remodeling of the polysaccharide structures (Rast et al., 2003). Several of these enzymes have been detected and analyzed in different fungi for example in *Aspergillus fumigatus* (Bernard & Latgé, 2001). Also in *C. cinerea*, various different glycoside hydrolases were detected in this thesis in the cell wall, three of these (ID-numbers 25, 43, 58) were identified exclusively from the hyphal sheath and the cell wall. This is also the case for a glycoside hydrolase from a family of α-mannosidases (ID-number 25), possibly involved in the formation of galactomannan (Bernard & Latgé, 2001) and two β-1,3-glucanosyl-transglycosylase (ID-numbers 43, 58) homologous to the Gas-protein family known from *S. cerevisiae* (Popolo & Vai, 1999), as extensively described in Chapter 3 (Section 3.5.2.2; see also Table 6.1).

Further, proteins without predicted enzymatic activity were exclusively found in the

cell wall. Lectin-like proteins with carbohydrate binding domains were detected in the extractable cell wall fractions (ID-numbers 49, APS12, APS32; Chapter 3, Table 3.9). Fungal lectins are known to bind specifically to cell surface carbohydrates (Singh et al., 2010) and thus the finding of lectin-like proteins in the cell wall of *C. cinerea* makes sense.

Further, two so far functionally uncharacterized proteins (ID-numbers 54, 55; Chapter 3, Table 3.9) are predicted to contain carbohydrate domains (CMB), which fits well with the location in the cell wall. Possibly such proteins with CMBs present in the fungal cell wall contribute to the structure of the cell wall, by interlinking glucan chains and stabilizing the polysaccharide network (Bowman & Free, 2006).

A ceratoplatanin-like protein (ID-number 137; Chapter 3, Table 3.9), with a homolog in *Ceratocystis fimbriata* acting as a phytotoxin (Pazzagli et al., 1999), resembles in some properties the hydrophobins, commonly found fungal proteins. For hydrophobins, it is well known that these secreted cell wall-attached proteins do not cover the hyphae as long as they are present in liquids but only when hyphae enter the aerial space (Linder et al., 2005).

The fractionated secretome of *C. cinerea* was in this thesis examined under artificial conditions, such as liquid medium with high amounts of glucose as carbon source and high amounts of organic nitrogen in form of peptides and proteins and shaking conditions, conditions which represent only very limited the natural conditions of the fungus. Most probably, these artificial culture conditions (liquid medium and shaking conditions and possibly the high glucose amount as well as the high amount of organic nitrogen) generated stress and therefore resulted in a secretome driven by the stress response. Especially, the free secretome might be affected by the stress, as this is possibly the most variable secretome in the sense of enzyme composition because of the proximity to substrate degradation and nutrient supply. Thus, the protein spectrum of the free secretome is most likely strongly effected by the predominant environmental conditions. Generally, a study of the secretome under natural conditions is challenging as the technical possibilities are limited and natural substrates are often very complex. The degradation of natural substrates of basidiomycetes such as wood and straw for *Pleurotus ostreatus* or *Ganoderma lucidum*, results in the formation of various secondary metabolites, low molecular weight molecules and soluble polysaccharides hampering protein sample preparation (Gutiérrez et al., 1996; Scherba & Babitskaya, 2008; Wagner et al., 2004).

A study presented in the following of the white rot fungus *P. ostreatus* strain PC9 (monokaryotic) grown on wheat straw showed that protein isolation methods, developed in this study on mycelium of *C. cinerea* grown in liquid medium, are also applicable for the secretome analysis for recalcitrant natural substrates. However, whilst a number of specific proteins could be identified from *P. ostreatus* by proteolytic digestion and peptide identification, the study also shows that in particular 2-DE-protocols have to be further optimized for obtaining the maximum range of data and the maximum quality data from 2-DE gels.

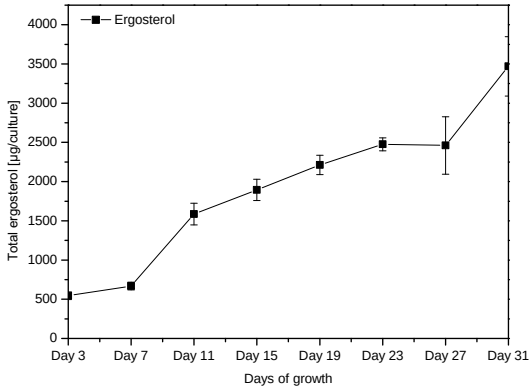
6.5 Analysis of the Secretome from *Pleurotus ostreatus* on Straw as Natural Substrate

A proteomic analysis of the secretome from the monokaryotic *P. ostreatus* strain PC9 (genome of the strain is in annotation process; deduced protein sequences were not available at the time of experimental performance; instead, protein sequences from the annotated genome of *P. ostreatus* strain PC15 were used) during the growth on wheat straw was performed (all details to the methods used in this study are given in the Appendix to Chapter 6). In order to obtain an insight into the change of growth phases, first the activities of typical lignocellulose degrading enzymes and the ergosterol content were measured during a growth period of totally 31 days: The fungus was inoculated from BMS (composition see Material and Methods in Appendix Chapter 6) agar plates into plastic bags (1 l volume) filled with 10 g wheat straw (two mycelium-agar pieces per bag). After about 7 days of cultivation, the mycelium was distributed within the total block of straw. During the following time, the mycelium became more dense and caused shrinking of the previously voluminous straw. Over the time, the ergosterol measurements showed a steadily increasing ergosterol amount, indicating an increase in biomass of the fungus (Figure 6.3 b). In parallel, the enzymatic activities relevant for lignocellulose degradation were measured from extracts of the straw cultures, obtained by washing the straw-mycelium composition with 50 ml Tris-buffer (0.5 M, pH 7.5) containing 2% (v/v) Tween 80 (see Material and Methods in Appendix Chapter 6), by six different enzyme tests: manganese-dependent peroxidase (MnP) with phenol red as substrate (Kuwahara et al., 1984; Michel et al., 1991), laccase with ABTS (2,2-azino-bis [3-ethylbenzthiazoline-6- sulfonate]) as substrate (Matsumura et al., 1986), phenol oxidases

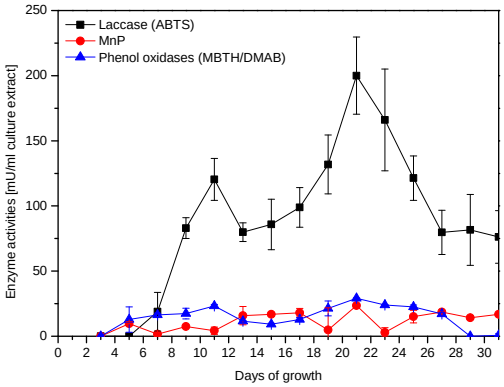
with MBTH (3-methyl-2-benzothiazolinon-hydrazone-hydrochloride, monohydrate) and DMAB (3-(dimethylamino)-benzoic acid) as substrate (Ngo & Lenhoff, 1980), cellobiose dehydrogenase (CDH) with DCPIP (dichlorophenolindophenol) as substrate (Rotsaert et al., 2003), and cellulase and xylanase with cellulose (CMC) and xylan (from birch wood) as substrates, respectively (Miller, 1959; Nair et al., 2008). The results are shown in Figure 6.3 b and c.

The enzyme activities for some of the different tested enzymes showed two main peaks: laccase activity measured with ABTS as substrate had peaks at day 11 and 21 of cultivation. Between these two peaks at day 15 and 29 of cultivation, the lowest enzyme activities of laccase and other phenol oxidases were detected (Figure 6.3 b and c). Also the activities of xylanase and cellobiose dehydrogenase showed two peaks, although at other days of cultivation than laccase activity; xylanase activity was highest at day 13 and 27 of cultivation and cellobiose dehydrogenase at day 9 and 19 of cultivation, respectively (Figure 6.3 b and c). Obvious fluctuations in enzymatic activity were not observed for manganese peroxidase measured with phenol red as substrate (Figure 6.3 b). In other studies, fluctuation of lignocellulose degrading enzyme activities over the time of cultivation was shown, e.g. in *P. ostreatus* grown on leaves (Elisashvili et al., 2003), in *Trametes trogii* grown on wood chips (Levin et al., 2008) and in *Phlebia radiata* grown on wood chips (Mäkelä, 2009). Rühl et al. (2008) demonstrated for a *P. ostreatus* dikaryon that the activity of lignocellulose degrading enzymes is only occurring during vegetative growth and is not detectable during fruiting body formation. In the here presented study, laccase activity showed peaks at day 11 and 21 of cultivation and in contrast, xylanase and cellulase show peaks at day 13 and 27 of cultivation. One can postulate that the fungus is first degrading lignin and subsequently the cellulosic structures of the straw, as in this experimental setup is possibly indicated by the time shift of enzyme activities between laccase on the one hand and cellulase and xylanase on the other hand (Figure 6.3). In dikaryotic strains, this change in the substrate might induce fruiting (Rühl et al., 2008).

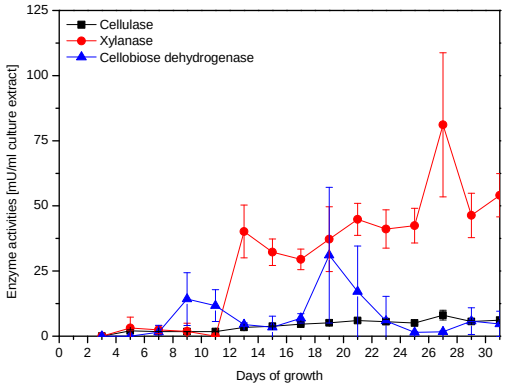
On natural substrates, a free secretome is not as easily obtainable as it is the case for liquid cultures. However, in this work the straw with the fungal mycelium of the cultures of *P. ostreatus* was extracted with Tween 80 containing buffer in a manner as it was done previously for the extraction of the hyphal sheath proteins in *C. cinerea* (Chapters 3 to 5). This Tween 80 treatment most probably extracted the fungal proteins involved in the degradation of the straw. Most importantly, the protein preparation



(a) Ergosterol values



(b) Enzyme activities



(c) Enzyme activities

Figure 6.3: Ergosterol values (a) and enzyme activities from laccase [ABTS (2,2-azino-bis [3-ethylbenzthiazoline-6- sulfonate]) as substrate], manganese-dependent peroxidase (MnP) with phenol red as substrate, and phenol oxidases [MBTH (3-methyl-2-benzothiazolinon-hydrazone-hydrochloride) and DMAB (3-(dimethylamino)-benzoic acid) as substrate] (b) as well as cellulase, xylanase (with cellulose and xylan as substrate, respectively) and cellobiose dehydrogenase with DCPIP (dichlorophenolindophenol) as substrate (c) from *Pleurotus ostreatus* monokaryotic strain PC9 measured over 31 days of cultivation grown on wheat straw at 25 °C in the dark. Average values and standard deviations were calculated from 3 repeats. Proteins were extracted from the straw cultures by washing the straw-mycelium composition with with 50 ml Tris-buffer (0.5 M, pH 7.5) containing 2% (v/v) Tween 80 (see Material and Methods in Appendix Chapter 6).

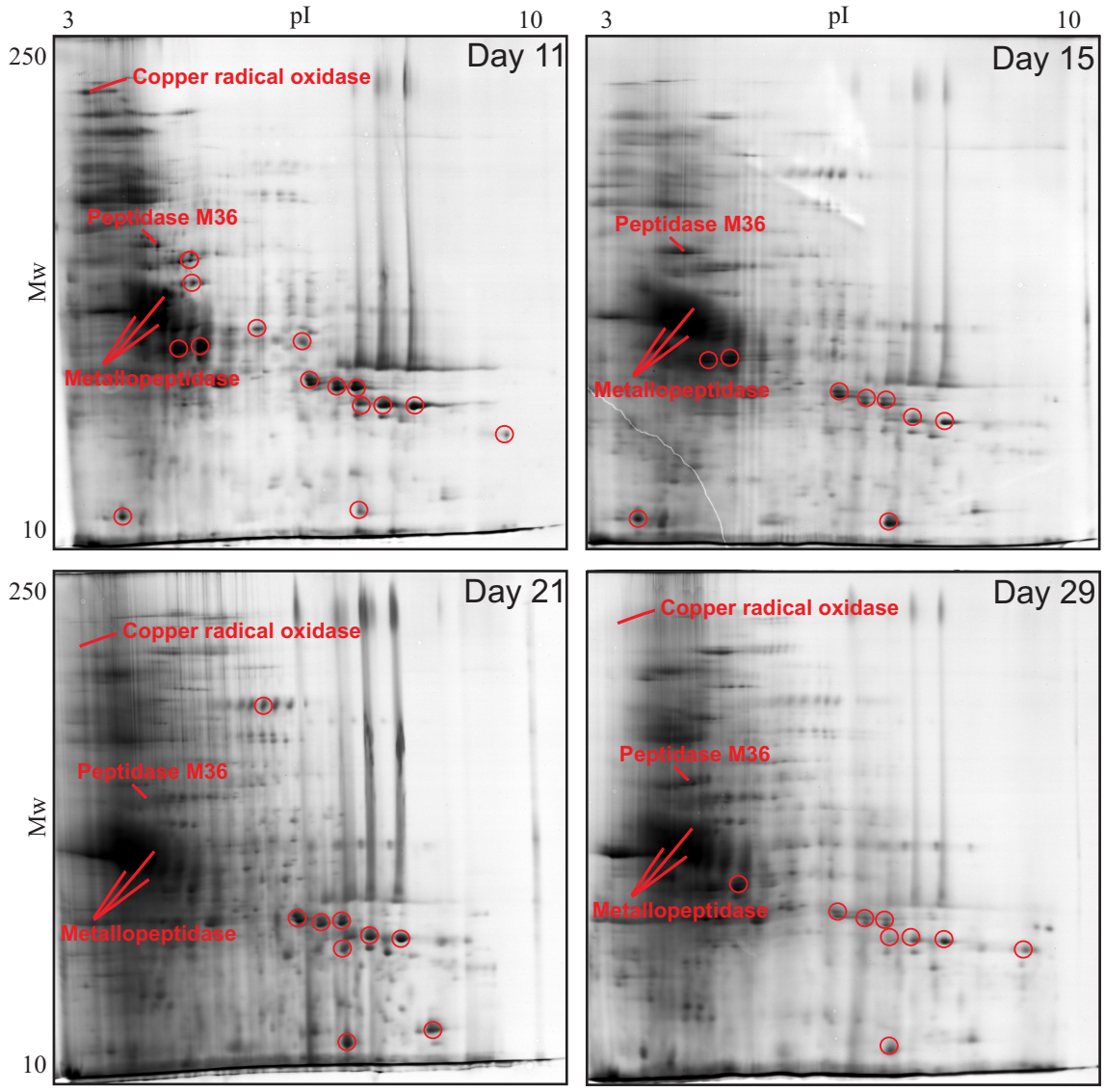


Figure 6.4: 2-DE-gels of the extracted proteins from day 11 (A), day 15 (B), day 21 (C) and day 27 (D) from *P. ostreatus* monokaryotic strain PC9 grown on wheat straw at 25 °C in the dark. Proteins were extracted from the straw cultures by washing the straw-mycelium composition with with 50 ml Tris-buffer (0.5 M, pH 7.5) containing 2% (v/v) Tween 80 (see Material and Methods in Appendix Chapter 6). Proteins were separated on 18 cm Immobiline DryStrips pH 3-10 (Amersham Biosciences) in the first dimension and on a 12% SDS-PAGE in the second dimension. Gels were stained with RuBP. The labeled spots were positively identified by LC-MS^2, the encircled spots were also picked but could not be identified likely due to insufficient databases (see discussion in the text).

methods used for the liquid cultures of *C. cinerea* turned out to be as well suitable for the cultures of *P. ostreatus* grown on straw with small modifications (see Material and Methods in the Appendix to Chapter 6). However, although the sample preparation and the isoelectric focusing performed well, the resolution on the 2-DE gels was hampered, possibly by polysaccharides and secondary metabolites derived from the degraded straw (Figure 6.4). Thus, the obtained 2-DE gels were not sufficient in quality for the computational analysis of triplicate gels with Proteomweaver software, a standard for proteomic analysis. This was also the case for 2-DE gels of culture extracts from *P. chrysosporium* grown on sawdust as shown by Sato et al. (2007). However, smearing of spots on the 2-DE gels should not necessarily hinder the identification of the protein by tryptic digest and LC-MS^2.

From the four chosen time points of cultivation, 2-DE of the extracted proteins presenting the proteins of the free secretome and the proteins of the hyphal sheath was performed (Figure 6.4). In this experiment, these two types of fractions occur in one because of the growth conditions that do not imply the release of protein into a liquid phase. Proteins that might have the qualities for free diffusion into liquids will in solid stage cultivation remain in close association to the hyphal sheath due to lack of much free liquid. A free secretome as it was described in the previous chapters could thus not be obtained from the *P. ostreatus* cultures on straw. Thus, in this experiment it can not be distinguished between the freely secreted proteins and the proteins attached to the hyphal sheath of the fungus. Surprisingly, all four time points resulted in a similar protein profile, although the enzyme activities indicated a change in physiological behavior (Figure 6.3 b and c) along with the continuously increasing production of biomass (Figure 6.3 a).

From the 2-DE gels of the different time points of cultivation, spots were picked and submitted to LC-MS^2 analysis. For protein identification, a Mascot database of the *C. cinerea* genome (used in Chapters 3, 4 and 5) was supplemented with deduced protein sequences from the genome of *P. ostreatus* monokaryotic strain PC15 and the translated amino acid sequences of the NCBI protein database. Obviously, neither enzymes available in the NCBI database nor entries of the completed *C. cinerea* proteome were similar enough to reveal more than five positive hits from totally 30 picked spots. However, our database contained proteins deduced from first computational annotations of the genome of the *P. ostreatus* strain PC15. Many more predicted protein hits from *P. ostreatus* strain PC9 might have been expected. Since this was not the case,

the following reasons for this failure have to be considered: First, the first annotation of the *P. ostreatus* strain PC15 is still preliminary and further emphasis has to be given by the *P. ostreatus* genome annotation group to optimize and complete the predictions as much as possible. Second, *P. ostreatus* strain PC15 might differ considerably in protein sequences from strain PC9 which would hinder identification of allelic proteins in the PC15 database. Experience for such situations of diverging sequences from two strains of a species is presented for example by two genomes of the dikaryotic *Postia placenta* strain sequenced by JGI (Martinez et al., 2009). In *P. ostreatus* with a now ongoing annotation of the genome of strain PC9, this situation should be hopefully overcome in the future. Another technical possibility for the failure of protein identification could be that the picked spots were too low in concentration for LC-MS^2 identification. Intensities of the picked spots from *P. ostreatus* gels argue against such failure, as the experience of the other experimental setups in course of this thesis showed that spots of such intensities as picked from the gels of the *P. ostreatus* secretome contain a sufficient concentration of protein for the identification by LC-MS^2. Nevertheless, optimizing of increasing amount by picking and pooling spots from parallel 2-DE gels or a 1-DE approach as performed with *C. cinerea* (Chapter 3; Section 3.4.2) should be performed to increase the possible chances for positive protein identification.

In conclusion, this study not only showed a restricted applicability (in the sense that spots on the 2-DE gels show extensive smearing) of the developed isolation methods for secretomes of fungi grown on natural substrates but as well the importance of a sequenced and well annotated genome of the studied organism for proteomic studies. More than 30 different spots were picked in order to identify proteins but the effort resulted in the identification of only 3 different proteins (from 5 different spots; Table 6.2), which are a putative copper radical oxidase, a putative peptidase from the clan M36 and a putative metalloprotease already previously characterized in *P. ostreatus* (Joh et al., 2004). Identification of the copper radical oxidase and the peptidase came from each one spot whereas three different spots represented the identified metalloprotease (Table 6.2). Of the positively identified enzymes, copper radical oxidase can, by analogy to other enzymes of other organisms, easily be linked to wood degradation as already postulated for *P. chrysosporium* (Vanden Wymelenberg et al., 2006; Whittaker et al., 1996).

Table 6.2: Positively identified spots from *P. ostreatus* monokaryotic strain PC9 grown on wheat straw for 31 days at 25 °C in the dark.

Protein name	Accession	Score	Signal peptide	Two best peptide hits (peptide score)
Copper radical oxidase	EAU83456	139	+	GAPEAENMEWVIER (71) ILDPVTFDTIK (43)
Peptidase M36	EAU82511	105	+	LTGGGTASCLQTTESR (52) YGFTEAAFNFQQDNFGK (41)
Metalloprotease	AAU94648	122		GAGPNNIQQADMK (63) TTGLLGYATFPGDYER (57)
		121	+	TTGLLGYATFPGDYER (64) RPDALNVYSVAFR (57)
		63		GAGPNNIQQADMK (53) TTGLLGYATFPGDYER (36)

The spots were obtained from 2-DE gels of culture extracts from day 11, 15, 21 and 27 of cultivation. For the 2-DE gels see Figure 6.4

6.6 Future Perspective

Proteomic studies performed on higher basidiomycetes are only at their beginning and, in future, such studies should be further considered as promising tool for the investigation of all different aspects of fungal biology (Kersten & Cullen, 2007; Ravalason et al., 2008; Sato et al., 2007; Shary et al., 2008; Vanden Wymelenberg et al., 2009; Zorn et al., 2005).

The cell wall and the hyphal sheath of basidiomycetous fungi is still a rather unknown field concerning the polysaccharide structure and also the formation and restructuring of the cell wall matrix under natural conditions (Bottom & Siehr, 1979, 1980; Schaefer, 1977; Wessels & Sietsma, 1979). Future studies on the cell wall enzymes found in course of this thesis in *C. cinerea*, possibly involved in the formation of the cell wall but also in the generation and in the degradation of the hyphal sheath, could give a deeper insight into the processes of cell wall formation. Such studies might as well show up the homologies and differences of the basidiomycetous cell walls compared to the already comparably well studied cell walls of ascomycetes (Klis et al., 2006; Kollár et al., 1995, 1997; Lipke & Ovalle, 1998).

Secretomic studies of *C. cinerea* grown on different substrates such as the natural horse dung or also fresh straw and wood chips could give insight into the degradative enzyme systems of *C. cinerea* and clarify the question to which extend *C. cinerea* is a

putative degrader of lignocellulosic materials.

Concerning the biology of *C. cinerea*, the analysis of the extracellular proteome of monokaryotic strains is as important as the analysis of dikaryotic strains. This gives the possibility to study not only the extracellular compartments of vegetative mycelium in terms of prevailing enzymes and other proteins and there functions but, in dikaryotic strains, as well the various tissues of fruiting bodies and the different stages of fruiting body development (for details in fruiting body formation in *C. cinerea* see Navarro-González (2008) and Chaisaena (2009)).

As the principle applicability of the developed methods for other fungi as well as natural substrates was shown in this thesis at least for *P. ostreatus*, studies on other straw- and wood-degrading fungi could add to the picture of degradation processes of lignocellulosic material and might reveal enzyme systems not only interesting for biology but as well for biotechnological applications. However, techniques for presenting fungal proteins obtained from lignocellulosic material on 2-DE gels have further to be improved.

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A Appendix

Chapter 3 - The Free and the Cell Wall Secretome in *Coprinopsis cinerea* Differ in Composition

Table A.1: Proteins identified within the performed 1-DE shotgun approach from liquid YMG cultures of *C. cinerea* grown at 37 °C. ID numbers were given according to the own Access database generated for data analysis. The UniProtKB code is obtained from the UniProt database (http://www.uniprot.org/). Molecular weight and pI value are predicted by EMBOSS (http://emboss.sourceforge.net/). The proteins listed here, have at least 2 peptides higher than the homology score given by Mascot. The protein accession number was obtained from the NCBI site http//www.ncbi.nlm.nih.gov/.

						LC-MS^2 data		
ID	UniProtKB	Protein accession	MW*	IP*	Signal peptide	Number of peptides	Sequence coverage	Total score
1	A8NIX3	EAU87688	96,699	4.47	+	29	69	1697
2	A8P4C5	EAU83081	72,212	6.31	+	24	78	1382
3	A8PAG1	EAU81840	97,271	5.11	+	22	57	1421
4	A8P297	EAU83456	104,983	4.24	+	21	39	1484
5	A8PFU7	EAU80752	102,879	5.40	+	20	49	1092
6	A8N7M9	EAU90899	94,639	4.73	+	19	53	1135
8	A8N066	EAU93605	63,456	5.39	+	17	51	943
9	A8NI75	EAU87957	45,888	5.51	-	16	76	816
11	A8P763	EAU82511	63,326	4.52	+	15	67	1078
12	A8NI40	EAU87930	41,365	7.17	+	15	63	847
14	A8N870	EAU90814	53,778	5.90	+	14	80	775
15	A8NRI4	EAU86023	61,178	6.38	+	14	56	729
16	A8NRJ6	EAU86031	31,565	9.32	-	14	80	756
17	A8N2Z2	EAU92553	45,249	6.51	+	13	85	672

Table A.1: continued

ID	UniProtKB	Protein accession	MW*	IP*	Signal peptide	LC-MS^2 data: Number of peptides	LC-MS^2 data: Sequence coverage	LC-MS^2 data: Total score
18	A8PFW6	EAU80766	100,734	5.13	+	13	24	637
19	A8NFG5	EAU88550	76,334	5.22	-	13	48	655
20	A8NNP9	EAU86655	85,418	5.25	+	12	41	604
21	A8NAA2	EAU90085	29,331	4.50	+	12	68	693
23	A8NXK7	EAU84813	43,159	4.88	+	11	50	637
24	A8N529	EAU91840	95,398	7.04	+	11	27	589
25	A8PDN7	EAU81201	59,613	4.86	+	11	40	527
26	A8NHZ4	EAU87896	52,468	4.86	+	11	48	588
27	A8P9Q2	EAU81960	76,136	6.37	+	10	28	468
28	A8NXM1	EAU84825	53,598	4.59	+	10	50	465
29	A8NVA5	EAU85209	50,495	4.60	+	9	38	503
30	A8N3D3	EAU92338	58,145	4.61	+	9	45	381
31	A8PFJ9	EAU80813	61,287	5.30	+	9	30	491
32	A8NHZ3	EAU87895	61,081	6.24	+	9	30	396
33	A8NPI3	EAU86468	28,824	4.71	+	8	64	632
34	A8N4R5	EAU91944	49,138	7.33	-	8	42	407
35	A8NPE3	EAU86523	18,724	5.21	+	8	80	335
36	A8P8V7	EAU82165	52,522	4.36	+	8	31	541
37	A8N184	EAU93188	37,466	7.96	+	8	35	441
38	A8N719	EAU91256	28,152	6.01	+	8	30	450
41	A8N392	EAU92297	93,705	6.57	-	7	36	386
42	A8NLH7	EAU87137	54,440	6.33	-	7	36	292
43	A8NA51	EAU90116	55,658	3.95	+	7	23	362
45	A8PBG2	EAU81622	44,367	7.17	-	7	29	367
46	A8P332	EAU83394	35,940	4.12	+	7	39	455
47	A8N4X3	EAU91906	17,642	5.77	-	7	73	415
49	A8NDU3	EAU88955	87,913	6.74	-	7	22	286
50	A8NCK1	EAU89294	22,432	7.25	-	7	66	482
52	A8P981	EAU82113	17,520	9.85	-	7	65	412
53	A8N988	EAU90579	33,790	9.55	-	7	49	398
54	A8N6K7	EAU91343	34,205	8.58	-	7	83	399
55	A8NVK7	EAU85085	42,690	6.81	+	7	50	460
56	A8N3J7	EAU92397	47,333	5.92	-	6	34	262
57	A8NJ67	EAU87654	34,793	4.98	-	6	35	286
58	A8N291	EAU92645	83,092	4.17	-	6	11	325

Table A.1: continued

ID	UniProtKB	Protein accession	MW*	IP*	Signal peptide	LC-MS^2 data: Number of peptides	LC-MS^2 data: Sequence coverage	LC-MS^2 data: Total score
59	A8NWJ7	EAU84527	86,576	4.75	-	6	18	253
60	A8NXB9	EAU84741	23,941	6.02	-	6	56	359
61	A8NN17	EAU86801	59,687	6.93	-	6	49	351
63	A8P7W1	EAU82343	39,126	6.01	-	6	51	335
64	A8NMI3	EAU86905	50,557	5.02	+	6	33	357
65	A8NVM1	EAU85095	17,939	8.31	+	6	68	350
66	A8NGZ5	EAU88183	16,632	7.76	-	6	68	324
67	A8N783	EAU91058	38,463	6.91	-	6	54	296
68	A8NNP4	EAU86652	54,017	7.66	-	6	56	335
69	A8NI11	EAU87908	43,225	8.61	-	6	27	353
70	A8NZ12	EAU84200	22,697	7.66	-	6	64	269
71	A8N5I4	EAU91794	35,665	7.79	+	6	50	442
72	A8P8P5	EAU82201	20,874	7.68	+	6	38	266
73	A8PC10	EAU81511	41,859	6.85	+	6	34	255
74	A8N212	EAU92918	53,651	6.59	-	5	29	305
75	A8N1C8	EAU93181	33,577	10.43	-	5	37	255
78	A8NJH5	EAU87630	20,891	7.28	+	5	36	265
79	A8PEQ3	EAU80873	26,178	8.26	+	5	36	219
81	A8PB28	EAU81725	36,558	5.71	-	5	48	224
83	Q12574	EAU86629	26,884	5.81	-	5	39	321
84	A8N7G9	EAU91144	29,349	8.29	+	5	29	262
85	A8P5L0	EAU82898	62,295	5.85	+	5	24	242
86	A8P7W6	EAU82346	35,124	9.51	-	5	47	223
87	A8PA35	EAU81873	70,252	6.72	-	5	22	211
88	A8P7X6	EAU82352	78,196	5.00	+	5	30	206
89	A8NFE7	EAU88538	50,299	5.73	+	4	32	206
90	A8P4Q2	EAU83013	29,926	4.38	+	4	18	188
91	A8NYS1	EAU84422	28,959	4.76	-	4	54	242
92	A8P8M5	EAU82190	37,201	9.50	+	4	23	274
93	A8N2B0	EAU92705	84,557	4.39	-	4	12	245
94	A8P1H4	EAU83673	23,150	4.81	-	4	26	184
95	A8PBK9	EAU81656	18,019	5.68	-	4	52	247
96	A8NFV0	EAU88450	25,614	4.48	-	4	57	245
97	A8NJP4	EAU87549	136,264	4.93	-	4	18	212
98	A8NLD8	EAU87109	51,678	4.14	+	4	11	226

Table A.1: continued

ID	UniProtKB	Protein accession	MW*	IP*	Signal peptide	LC-MS^2 data: Number of peptides	LC-MS^2 data: Sequence coverage	LC-MS^2 data: Total score
100	A8NXT9	EAU84882	84,601	6.61	-	4	29	209
101	A8NWI0	EAU84514	23,189	4.65	-	4	59	237
102	A8NI41	EAU87931	37,298	4.08	+	4	26	197
103	A8NHD1	EAU88102	46,806	9.18	-	4	17	175
104	A8NAG6	EAU90001	38,639	4.18	+	4	22	271
105	A8N076	EAU93615	66,033	5.32	+	4	20	160
106	A8NBX1	EAU89492	38,102	7.61	-	4	27	164
108	-	ccin_1.51-g27.1	31,777	6.38	+	4	20	173
110	A8N5N7	EAU91660	23,366	11.26	-	3	37	151
111	A8NRX3	EAU85945	39,825	4.05	+	3	14	221
112	A8P2Z0	EAU83363	27,420	6.72	+	3	19	132
113	A8N1H8	EAU93024	50,113	9.78	-	3	30	155
114	A8N3F6	EAU92441	22,192	11.25	-	3	27	128
115	A8N208	EAU92914	39,104	4.56	+	3	21	149
116	A8N7T4	EAU90954	18,216	7.68	-	3	65	158
117	A8NLJ6	EAU87151	46,478	4.04	-	3	23	158
118	A8N7K3	EAU90873	42,337	8.96	-	3	23	136
119	-	TS_1_199_191	48,830	6.08	+	3	23	130
122	A8N7C4	EAU91099	37,665	7.90	+	2	5	114
123	A8NEG5	EAU88709	53,885	4.86	+	2	25	121
124	A8N3X1	EAU92210	50,849	6.13	-	2	10	114
125	A8NXM3	EAU84827	34,515	9.62	-	2	24	106
126	A8NCR3	EAU89150	93,479	6.52	+	2	16	139
129	A8P9Q5	EAU81962	19,441	7.92	+	2	14	115
130	A8NE72	EAU88638	61,371	6.33	-	2	14	109
131	A8NRB5	EAU86088	17,906	8.76	+	2	13	104
132	A8PG31	EAU80670	42,782	4.48	+	2	10	120
133	A8N1J7	EAU93086	48,032	8.42	-	2	21	78
135	A8N592	EAU91702	50,991	6.84	+	2	21	115
137	A8P9R6	EAU81969	16,618	4.64	+	2	37	128
138	A8N472	EAU92199	30,625	3.20	+	2	11	160
139	A8N0P0	EAU93364	11,920	7.52	-	2	70	113
140	A8PCC0	EAU81416	29,593	4.55	+	2	36	139

*MW: predicted molecular weight; pI: predicted isoelectric point

Table A.2: Additionally obtained proteins from the 1-DE shotgun experiment by the average peptide score (APS) approach as described elsewhere (Chepanoske et al., 2005; Shadforth et al., 2005). For further experimental details compare the legend of Figure A.1.

						LC-MS^2 data		
ID	UniProtKB	Protein accession	Mw*	IP*	Signal peptide	Number of peptides	Sequence coverage	Total score
APS1	A8PFE6	EAU80782	63,184	6.98	+	5	24	173
APS2	A8PEP4	EAU80866	27,244	8.81	+	5	56	195
APS3	A8P9E0	EAU82052	126,313	7.11	-	4	14	178
APS6	-	retrain_ccin_ Contig112-snap.73	47,878	6.30	-	4	24	158
APS7	-	Jan06m300_ GLEAN 08660	100,749	5.37	+	4	18	157
APS8	A8N9Y3	EAU90172	56,055	4.79	-	3	18	107
APS9	A8N9T7	EAU90126	125,717	6.35	-	3	19	136
APS11	A8PH25	EAU80499	14,623	10.94	-	3	58	122
APS12	A8PCJ0	EAU81467	17,025	8.45	-	3	40	122
APS13	A8NQJ9	EAU86184	86,078	5.30	-	3	14	120
APS14	A8NRF9	EAU86122	62,298	4.53	+	3	16	113
APS15	A8N8Y4	EAU90475	57,012	6.50	-	3	20	124
APS16	A8PF48	EAU80978	22,552	3.85	+	3	12	135
APS17	A8NX92	EAU84719	29,717	10.61	-	3	43	126
APS19	A8NEL1	EAU88751	31,849	7.80	-	3	43	125
APS20	A8NKR9	EAU87254	31,938	4.95	+	3	19	133
APS21	A8NXY8	EAU84924	23,474	5.17	-	3	23	123
APS22	A8NAL2	EAU89899	82,560	7.76	+	2	12	90
APS24	A8NQS8	EAU86243	77,965	5.42	-	2	33	100
APS25	A8N7U4	EAU90964	15,435	4.95	+	2	26	89
APS26	A8NA16	EAU90205	35,787	7.13	+	2	15	81
APS27	A8N780	EAU91055	30,778	5.85	+	2	26	111
APS28	A8P338	EAU83399	33,456	6.73	+	2	24	84
APS29	A8N513	EAU91824	112,966	9.42	-	2	13	92
APS30	A8N3K2	EAU92402	26,879	4.84	-	2	34	83
APS31	A8P4R3	EAU83020	15,768	11.42	-	2	39	78
APS32	A8NCX0	EAU89191	34,777	4.30	+	2	15	109
APS33	A8N1Q5	EAU93015	72,099	8.80	-	2	23	105
APS34	A8N1J1	EAU93037	54,796	6.17	-	2	16	92

Table A.2: continued

ID	UniProtKB	Protein accession	Mw*	IP*	Signal peptide	LC-MS^2 data: Number of peptides	LC-MS^2 data: Sequence coverage	LC-MS^2 data: Total score
APS35	A8N0W9	EAU93299	23,031	6.45	+	2	33	80
APS36	A8NU53	EAU85448	64,862	6.94	+	2	10	94
APS37	A8PJ25	EAU80161	23,318	6.79	-	2	51	89
APS39	A8P6Q4	EAU82636	10,620	9.61	-	2	80	88
APS40	A8P3K1	EAU83254	24,587	5.90	-	2	33	83
APS41	A8NUE0	EAU85387	49,256	4.74	+	2	20	96
APS43	A8NMJ8	EAU86914	84,411	6.66	-	2	22	92
APS44	A8NME0	EAU86921	36,885	4.84	+	2	16	83
APS45	A8NIX0	EAU87685	77,469	5.25	+	2	14	85
APS48	A8NFE2	EAU88534	76,684	6.40	+	2	19	86
APS49	A8NE22	EAU88891	42,893	6.08	-	2	23	82
APS50	A8P4K9	EAU83052	16,853	9.53	-	2	46	80
APS51	A8NH85	TS.ccin_1.112.139	23,069	4.97	-	2	21	87
APS52	-	retrain_ccin_Contig167-snap.9	8,404	7.70	-	2	41	91
APS54	-	TS.ccin_1.174.103	47,416	8.24	+	2	18	85
APS57	A8N2W8	EAU92592	14,078	4.70	-	1	35	64

*MW: predicted molecular weight; pI: predicted isoelectric point

Table A.3: Two peptide hits with the highest peptide scores for the proteins listed in Table A.1 [after the HUPO guidelines (http://www.hupo.org/)]; for further experimental details compare the legend of Figure A.1.

ID	UniProtKB	Peptide sequence	Ion score
1	A8NIX3	ELGAASIVLLKNER	90
		IYGAVSSSDGDLVAAWER	127
2	A8P4C5	NSPTGDESDALDQPGLK	98
		NVLLFVGDGMTQAMITAAR	92
3	A8PAG1	SLVLDQPVIYVSINHR	97
		VYLPVSDAQLDEIAR	95
4	A8P297	VSLLAAASSTHGNTMGAR	111

Table A.3: continued

ID	UniProtKB	Peptide sequence	Ion score
		GAPEAENMEWVIER	103
5	A8PFU7	LLDYLYTALNK	83
		LIFTLDPQYFPLNR	83
6	A8N7M9	VSFPQNGGTVSFPNPR	109
		ISIANNGDAPK	91
8	A8N066	GLTSFDDDVCEGAVR	97
		VLALLGDGPFVGTFTAVCK	85
9	A8NI75	ITAADIWVATLIQK	78
		LFEGAAIAR	73
11	A8P763	LTGGGTASCLQTTESR	109
		TNAFYVVNAIHDFAYR	105
12	A8NI40	IDGSNEYLLLHEAIGSDGR	113
		DISHGELIR	77
14	A8N870	TLSVDTDKNTAVIGAGNR	107
		LGEGAPDLYFK	75
15	A8NRI4	AGVVANIGPDGAR	67
		GSATYTALTNAVR	74
16	A8NRJ6	GPTFTTDAVVGR	82
		SSNDLLSK	67
17	A8N2Z2	VIDEWTFGQYLDR	81
		SFTGPASGYSQSYK	73
18	A8PFW6	LIFTLDPQYFPLNR	83
		SEMAMTTAELR	77
19	A8NFG5	AEWEGFTAIVNK	79
		APTLIQDLPWGK	76
20	A8NNP9	DFGAVGDGIHDDTR	81
		FGIWGGNQQFTVR	77
21	A8NAA2	GAGPNNIQQADMK	93
		EDEGNIPDSQLDSQLDVLNK	84
23	A8NXK7	FDGILGLAYDTISVNR	88
		LIDSPVFAFR	85
24	A8N529	SPTAYLANPAAER	73
		FGAVIEDVSSSPYR	73
25	A8PDN7	VESGGYTGITDVNQLFGPR	90
		TNIAEAGTLLLEWSTLSK	60
26	A8NHZ4	FAADEGHR	60

Table A.3: continued

ID	UniProtKB	Peptide sequence	Ion score
		LPSAPTEFSGR	63
27	A8P9Q2	SEEHTALAR	66
		YGYSANMDDR	57
28	A8NXM1	AITTEAIGEFLESYVTGK	81
		VDCVEEADLCQSK	85
29	A8NVA5	NFQLGATAFADLPAK	82
		ITVFAAQGNAR	76
30	A8N3D3	LLEDGFQTSGK	54
		TPVGGETFSYVGK	85
31	A8PFJ9	TPSDIAAGIHFANEHHLR	72
		TTFFGSHYDR	68
32	A8NHZ3	SSAATAYLSQSVR	69
		TGPLTEAVGHQILWAR	70
33	A8NPI3	NVQSPLLLSLAETDSTFSTAASR	146
		ADPNDANAVWAK	91
34	A8N4R5	ILITQYDNPSR	67
		NLLLSSINEAIETGPLR	88
35	A8NPE3	ERDNAIER	66
		LYSPSAVDHFYTTDAK	51
36	A8P8V7	NPEDIQAAIEFANEHYLR	105
		SVDDGNAAFNTFFELAR	100
37	A8N184	IPTLTEVFDFAR	93
		SVDEFVNAQYR	69
38	A8N719	VAPNTAQQQAMK	76
		TTSVDWFQR	74
41	A8N392	AYLPVAESFGFNGELR	92
		ILADDYGWDVTDAR	89
42	A8NLH7	IGPALATGNTIVLKPSEFTPLTAIR	51
		ITGQTIETDER	64
43	A8NA51	IAPDWSTNLLDAYTR	70
		LVGYAAINGAPDFR	70
45	A8PBG2	IYTPPLDDDENAEHAR	67
		LTVIDTPGFGDYVNNR	84
46	A8P332	TPDYISVVGFIDQTK	107
		LIPEGALTGVQFIR	92
47	A8N4X3	ASMENGVLTVTFPK	87

Table A.3: continued

ID	UniProtKB	Peptide sequence	Ion score
		VSSDYEESGYAVR	119
49	A8NDU3	APGVGLISIVNR	66
		FVQTESTGIYTIVNR	50
50	A8NCK1	AIEEAFGSLDNLK	99
		HHQTYVNALNAAEAAYAK	84
52	A8P981	IIPNFMLQGGDFTR	79
		KIEALGTASGNPK	73
53	A8N988	SIGTIASETGLVSLATK	87
		IQTSPNGTFPTAFGAALR	82
54	A8N6K7	LLGLAGGLVGGAALVGGAVAAYK	83
		SGQSSDPVNWVLGDGK	76
55	A8NVK7	NPNQVWSTGYLPTDLPNK	93
		TPEYVQITGVGDFTK	82
56	A8N3J7	ELADLYLSYVEK	51
		SGETENTIIADLAVALGVGQIK	68
57	A8NJ67	ISPLGTLTDAEK	71
		VEGYLPADDGLTK	55
58	A8N291	DLPLLTDAGVNTLR	98
		LAPSWSTNLLNLYIR	85
59	A8NWJ7	GPYTFTIGDVSNFGDYK	52
		LPAPSSLAGYR	50
60	A8NXB9	LITGQNPASASGVGEAILK	93
		PSVLFVFTSTNK	79
61	A8NN17	FSTVGGESGSADTAR	105
		IAAAIGHSPVQPLAVKPAAEAVR	73
63	A8P7W1	GLLDIVPAGVLTGDNVR	74
		SPIILQVSQGGSAFFAGK	87
64	A8NMI3	ITNNVITSQVASGLLR	74
		TPYGSNIFPR	105
65	A8NVM1	ADAGPTFDLSEGR	98
		TVLNTAYAVDSK	69
66	A8NGZ5	AMLGATNPLASPIGSIR	95
		NIIHGSDSVENAEK	70
67	A8N783	AQDPQNIIVGLGR	79
		GAICDKDAVAEALK	55
68	A8NNP4	IVIDDQFTTSAK	78

Table A.3: continued

ID	UniProtKB	Peptide sequence	Ion score
		GIDVENVSLNLPK	66
69	A8NI11	AIELAHAVAFR	64
		GDEAFFLDVLTSYITSAK	80
70	A8NZ12	ASPENFWTYSLALFK	61
		NQSDYFDIPTANLTPLQIR	64
71	A8N5I4	VLTDGGSGSIAGIVSGLDWIR	119
		GAVTGLPSTTANNLAQIGPL	95
72	A8P8P5	AGFYGEQFYYYMVK	60
		ITFCPGGQWPGPETWR	57
73	A8PC10	ITFKEDGTFK	54
		SPIDTWIR	53
74	A8N212	NPELESLLFDDFFNK	71
		SVFLGDITAAYTK	93
75	A8N1C8	SMFDAGSQIIAK	69
		YFPTQALNFAFK	69
78	A8NJH5	FANTYYDVNGK	61
		LFCLVGLVCR	71
79	A8PEQ3	TASSVVGHPPR	50
		VAEEELPR	66
81	A8PB28	SIGISNFQGALIIDLLR	66
		TPAQVLLR	60
83	Q12574	TAAVCEEQLSAVVK	85
		VIYGGSVNAANSK	80
84	A8N7G9	VGNAAFASLVESR	87
		VGVNNIFR	64
85	A8P5L0	AAAEQFLTDHVDGPLR	54
		STPGTEDWEVLDYGR	54
86	A8P7W6	DDLFNTNASIVR	71
		VFGVTTLDVVR	62
87	A8PA35	LGANSLLDIVVFGR	65
		NLLQCAIQTIVSAAAR	69
88	A8P7X6	GPILLNPGGPGGSGVDFLR	65
		LPEPGTVCKPVGTPFPSR	47
89	A8NFE7	ITEYQAQPYAQSR	63
		KLEVDHIYDR	66
90	A8P4Q2	AAPFTVQQNSMK	50

Table A.3: continued

ID	UniProtKB	Peptide sequence	Ion score
		TVNSDWFLR	55
91	A8NYS1	ICEDILDVLDK	83
		VASSDQELTVEER	80
92	A8P8M5	APSAITVAASTIADAK	108
		VTHSQFGGR	58
93	A8N2B0	DPVPYFMDGDSGILDVAR	83
		ELQNAASDEFGPAEDK	75
94	A8P1H4	AGSGVTFAAK	53
		LVGANFFITQRD	53
95	A8PBK9	GVENIYVIAVNDAYVTK	60
		ITSVSVEEEVPK	84
96	A8NFV0	LSKEEIDR	74
		NGLESYAYNLR	63
97	A8NJP4	LAEVQGLINPITSK	75
		LSQEEIDR	74
98	A8NLD8	GADGNGGACQYFNIWR	58
		GIEDYEMVFDGTGTSAEAR	91
100	A8NXT9	EFTTAYTELAPVAPK	52
		LGQQAIDAVGADR	76
101	A8NWI0	FANSDNANQFK	66
		VAADYAESPPTSETLAIR	83
102	A8NI41	TDNPLPSTFTWR	50
		YLLLIEAIGTDGR	59
103	A8NHD1	SVDDWLTTTLGLLR	53
		TFAIFNTDGSIADYSIAYPLR	49
104	A8NAG6	IPGQNPDAPIVVLGGHIDSLNSR	76
		NPTGPAPGADDDGSGSVNLLEALR	76
105	A8N076	LISINTVYWYK	56
		VYEVDPDTYEVMDYK	59
106	A8NBX1	ATLDASIFFTVFK	53
		IIGSYVGNR	47
108	-	DTNGSQFFITTVK	49
		HGDKDLGR	67
110	A8N5N7	LETGNFAWGSESITR	51
		LIGVVYNSSNNELVR	61
111	A8NRX3	FTNSIYVLNADANDR	78

Table A.3: continued

ID	UniProtKB	Peptide sequence	Ion score
		SSVYIYDATAQSWSK	87
112	A8P2Z0	APAQVFR	56
		GQSIVIWDFDSEDGR	57
113	A8N1H8	LPLQDVYK	65
		YLVTVIDAPGHR	55
114	A8N3F6	IEDFLER	47
		IGVLDESR	62
115	A8N208	SLTHDVLYETYR	56
		VADNATTAHIEEGIR	59
116	A8N7T4	ASMQDGVLTVTFPK	71
		NLVTATFELPGLK	67
117	A8NLJ6	VGYGGDFVYDR	80
		VSAEDIEEYEPLPK	50
118	A8N7K3	IDGVASQATPDGR	80
		MGFNDQEIVALSGAHALGR	44
119	-	AISSSSDVYYPGHYLYVK	52
		RIDPDNVMGLAGGFK	53
122	A8N7C4	TPQQWGDLVR	55
		YAIQNYGVDR	59
123	A8NEG5	LVLEVASHLGENSVR	46
		TVLIQELINNVAK	75
124	A8N3X1	GVIYAESQNLAR	52
		SVEVDNTDAEGR	62
125	A8NXM3	HGLTNWAAAYATGLLAAR	53
		IQGDFILTQAHSR	53
126	A8NCR3	LEADVEEFASR	82
		TYGPFSPDDVILLAAYASR	57
129	A8P9Q5	AMCNSVEGCVFANTYR	52
		LPCLTGPICK	63
130	A8NE72	GEPLPSADAEQNQDETR	59
		VLLTTSDNEQFNADR	50
131	A8NRB5	AVVLHAGTDDLGR	52
		VISLNGPFSIVGR	52
132	A8PG31	GLATNVSNYNPYK	70
		YFEEQGDELNAAR	50
133	A8N1J7	KADISVAVATPTGLITPIVK	51

Table A.3: continued

ID	UniProtKB	Peptide sequence	Ion score
		LSVNDFIVK	53
135	A8N592	AVLSGLHFAVAAGNDNR	64
		VLGTNGSGTMSDVIDGVLWAAQQAAK	51
137	A8P9R6	GGFNLAHAALNDLTNGQATQLGR	74
		GYSTFGSLPGFPR	54
138	A8N472	TQICGGPFR	47
		VIELSGPATIETCTAGCK	113
139	A8N0P0	AVLTASPDYAYGSR	46
		VILGWDEGVPQLSLGQK	67
140	A8PCC0	EYSDEWDGYIFTDLNR	83
		FDFYWISYDQK	57

Table A.4: Two peptide hits with the highest peptide scores for the proteins listed in Table A.1 obtained by the APS approach (Chepanoske et al., 2005; Shadforth et al., 2005); for further experimental details compare the legends of Figure A.1 and A.2.

ID	UniProtKB	Peptide sequence	Ion score
APS 1	A8PFE6	STGYGSLAINTHSFK	41
		TTPGTEQWEVIDWGR	39
APS 2	A8PEP4	KAEVTLFK	43
		FPLTEAQATSLLK	40
APS 3	A8P9E0	IGTLNGLFVLGR	56
		KDTLVDFLIR	44
APS 6	-	FENIGEPTFNWGEFK	46
		LTSPNSVEVTRPDGTK	43
APS 7	-	ASQASQSANTYAAIAER	57
		EADNVLTGWDNLVSASTR	38
APS 8	A8N9Y3	VPISDSVSSLK	49
		ELGTWLATPGVAEK	33
APS 9	A8N9T7	AAFGDGTVFIER	68
		GLTPVGAYLAQDEIIK	34
APS 11	A8PH25	IVVQLNGR	54
		HGYIGEFEIIDDHR	37

Table A.4: continued

ID	UniProtKB	Peptide sequence	Ion score
APS 12	A8PCJ0	GTAGEGSNVVVWK	45
		NVQTGGYLAVER	40
APS 13	A8NQJ9	FAVYDFEFEK	47
		ITFVSWSPDDAK	37
APS 14	A8NRF9	TEYSINHDIPADQGVAVGR	44
		TGVSANVGPDGAR	40
APS 15	A8N8Y4	AGGPSADGGGIGANAPK	48
		TEHAAAALSR	42
APS 16	A8PF48	ADGEEQFVK	52
		TVFPLNGGQFR	46
APS 17	A8NX92	IIEVSLADLNKDEEQSFR	48
		TTDGYLLR	43
APS 19	A8NEL1	LGIDYVDLFLVHSPSPANK	49
		IPDLTQEEIDAIQR	43
APS 20	A8NKR9	LRPTFNVVWHVISR	55
		DDGVVVFHESLPGGR	41
APS 21	A8NXY8	VNHSIISGVR	51
		EGVEYNVR	39
APS 22	A8NAL2	TPTAGNLGIMPTR	59
		TAVVTAIDPSAEYVK	31
APS 24	A8NQS8	GVQDFLDDLESK	68
		TPDIFPIFK	32
APS 25	A8N7U4	VLTIPAHLAYGER	57
		ELQIETTFTPEDCSVK	32
APS 26	A8NA16	NLVGVSHTVDEAR	41
		LADYMPAPYPNEEAAR	40
APS 27	A8N780	HGDKDLGR	67
		ITIGLFGGTVPK	45
APS 28	A8P338	LGLGPEYVQSSR	45
		IVGGPELLTYDEAAK	39
APS 29	A8N513	GVGVGLLNPEDSAK	49
		SGTDYSVYTGIDR	43
APS 30	A8N3K2	AIGSGSEAAQSELQDK	49
		TPEGVVLAVEK	35
APS 31	A8P4R3	VVLLNEGAFSGK	39
		VIIDGPTTDVPR	39

Table A.4: continued

ID	UniProtKB	Peptide sequence	Ion score
APS 32	A8NCX0	AGFFLEPTYWYYYIVR	71
		VADTNFCLDAGSTPGNGVPMK	38
APS 33	A8N1Q5	ATHTQEVLGEK	68
		GLSAIAQCESLR	37
APS 34	A8N1J1	AGLQAQISK	50
		KAELEDAQAEK	41
APS 35	A8N0W9	SSLVGPVDGKDYTYR	42
		GVTAQQQSLYYFQGQLQQATAR	38
APS 36	A8NU53	NTGHDFLGR	53
		STGYGSLAINTHSFK	41
APS 37	A8PJ25	GLPALIWDGSVLDAEEGIR	52
		LAELIPAEIENVK	37
APS 39	A8P6Q4	GRQEGLVVGSHYDYAGR	53
		QEGLVVGSHYDYAGR	36
APS 40	A8P3K1	NVPFEFHVVDYSK	51
		GPALIPTDLK	36
APS 41	A8NUE0	ATFFVVGSR	54
		NSGIEIPGYEPTAK	42
APS 43	A8NMJ8	WVVIGDHNYGEGSSR	50
		EHAALEPR	42
APS 44	A8NME0	FETTIAHLER	50
		YTGPIPPANVYR	32
APS 45	A8NIX0	HLVENEQEYKR	49
		VDDMAIR	26
APS 48	A8NFE2	EIVKEDSLTPPGWQAPR	50
		RNQALIAR	36
APS 49	A8NE22	LLTIFDVDVR	47
		TASSPEVDAVFK	35
APS 50	A8P4K9	HLPDDK	41
		LVGQIYNLVPDILLEAGK	39
APS 51	A8NH85	ELISNASDALDK	56
		YASLTDPSQLDSGK	31
APS 52	-	SVIGWFTR	53
		ATYHLQQFSGVQHGFATR	38
APS 54	-	SVYKPIVQHLTDIDEAAR	44
		TAFMFKPEVLK	42

Table A.4: continued

ID	UniProtKB	Peptide sequence	Ion score
APS 57	A8N2W8	AANSSLEHVVK	64
		DRFPAR	31

Chapter 4 - Post-translational Modifications in the Freely Secreted and the Cell Wall Proteome of *Coprinopsis cinerea*

Table A.5: Two peptides with the highest score used for the identification of the proteins of *C. cinerea* grown in liquid YMG medium at 37 °C listed in Table 4.1. The ID-numbers are referring to the own Access database generated in course of the shotgun experiment. For further experimental details see Material and Methods of Chapter 4 and Table 4.1.

ID	UniProtKB	2 representative peptides (ionscore)
4	A8P297	GAPEAENMEWVIER (80)
		FSILGSTDIPR (53)
4		GAPEAENMEWVIER (93)
		MYHSEVSLLADGR (66)
4		MYHSEVSLLADGR (61)
		IGGDPAGLGNWPDLPGFTLPGVR (58)
31	A8PFJ9	TPSDIAAGIHFANEHHLR (45)
		THPSAPLTVVELK (42)
31		SLSEANETFNSILSLAQER (83)
		THPSAPLTVVELK (53)
36	A8P8V7	SVDDGNAAFNTFFELAR (74)
		GGGGGTYGIVTSATYK (71)
36		SVDDGNAAFNTFFELAR (77)
		EAVENDPEMVAR (70)
36		SVDDGNAAFNTFFELAR (43)
		EAVENDPEMVAR (38)
36		SVDDGNAAFNTFFELAR (68)
		GGGGGTYGIVTSATYK (47)
36		GGGGGTYGIVTSATYK (55)
		SVDDGNAAFNTFFELAR (54)
36		SVDDGNAAFNTFFELAR (75)
		EAVENDPEMVAR (69)
36		GGGGGTYGIVTSATYK (47)
		EAVENDPEMVAR (45)
36		GGGGGTYGIVTSATYK (36)
36		VEYDPDSLFLVPLGVGSEDWDK (50)
		SVDDGNAAFNTFFELAR (50)

Table A.5: continued

ID	UniProtKB	2 representative peptides (ionscore)
43	A8NA51	IAPDWSTNLLDAYTR (70)
		LVGYAAINGAPDFR (67)
43		LGEHNIDVLEGNEQFINAAK (63)
		LSSPATLNSR (51)
46	A8P332	TFTHDNIPYKVDTDEGLIR (71)
		TPDYISVVGFIDQTK (70)
46		LIPEGALTGVQFIR (62)
		TPDYISVVGFIDQTK (59)
46		TPDYISVVGFIDQTK (41)
		LIPEGALTGVQFIR (35)
54	A8N6K7	LLGLAGGLVGGAALVGGAVAAYK (59)
		SGQSSDPVNWVLGDGK (57)
54		LLGLAGGLVGGAALVGGAVAAYK (77)
		SGQSSDPVNWVLGDGK (57)
62	A8NQL8	GTNQETVCGNTGR (48)
		YVQVTGDTDILAR (45)

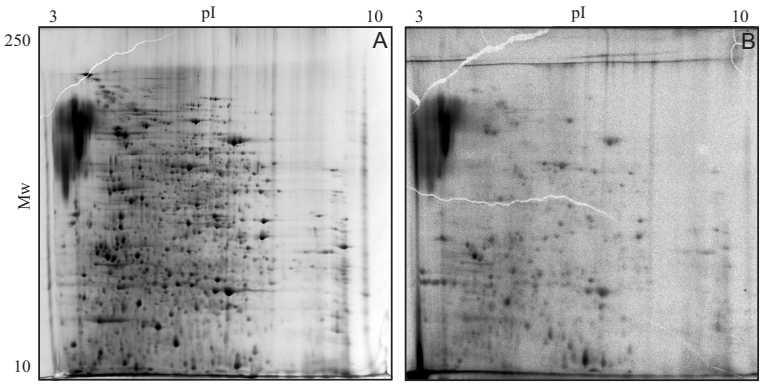

Figure A.1: 2-DE of the intracellular proteins used as a control from *Coprinopsis cinerea* grown at 37 °C in standard YMG medium (supplemented with 1 mCi ^{33}P-containing phosphate) on day 3 (A) of cultivation and corresponding autoradiography (B), respectively. Proteins were separated on 18 cm Immobiline DryStrip pH 3-10 (Amersham Biosciences) in the first dimension and on a 12% SDS-PAGE in the second dimension. Gels were stained with RuBP. The imager plate for autoradiography was exposed to the gel for 3 days and subsequently scanned using a fluorescence reader FLA-5100 (Fujifilm, Düsseldorf, Germany) with 50 μm resolution.

Table A.6: Elemental analysis of the dry mass from YMG medium (with and without reduction of the phosphate content by precipitation with 200 mM $CaCl_2$ used for the cultivation of *C. cinerea*; the analysis was done by the KOSI (Center for Stable Isotope Research and Analysis) at the Faculty of Forest Sciences and Forest Ecology, University of Göttingen.

Element	Ca	Mg	Fe	Mn	K	Na	S	P	Al(I)
Standard YMG medium									
Concentration (mg/g)	5.31	0.43	0.02	0.01	13.08	0.61	1.60	3.74	0.02
Phosphate reduced YMG medium*									
Concentration (mg/g)	129.39	0.14	0.01	0.01	5.10	0.25	1.71	0.77	0.01

*Anorganic phosphate was precipitated with 200 mM $CaCl_2$

Table A.7: Spots picked and identified from the gels of day 3 (early exponential phase), day 6 (late exponential phase), day 9 (stationary phase), and day 12 (cell lysis) of cultivation from *Coprinopsis cinerea* grown in liquid YMG medium at 37 °C in shaking cultures. Only proteins with at least 2 peptides higher than the homology score given by Mascot were regarded as confident and listed here. Molecular weight and pI value are predicted by EMBOSS (http://emboss.sourceforge.net/). The ID-numbers are referring to the own Access database generated in course of the shotgun experiment (see Table A.1).

Spot	ID	Gene code	Theoretical MW (kDa)	pI value	Peptides (ionscore)	Total score*
64	1	EAU87688	96,699	4.47	SLGGGPGDIIAIPYDEGLEIDYR (51)	97
					TSVSWLLDDFDLPR (51)	
186		EAU87688			NIVLIGSGAGGGR (47)	96
					SLGGGPGDIIAIPYDEGLEIDYR (47)	
120	2	EAU83081	72,212	6.31	YTFAAGAAALPDHR (58)	123
					NSPTGDESDALDQPGLK (53)	
218		EAU83081			YTFAAGAAALPDHR (40)	40
					ALGELLELDDTIR (20)	
2	4	EAU83456	104,983	4.24	GAPEAENMEWVIER (59)	130
					CTDACFNAGYR (51)	
3		EAU83456			QLNVGGWSLDSTFGVR (68)	322
					CTDACFNAGYR (59)	
6		EAU83456			MYHSEVSLLADGR (64)	387
					ILDPVTFDTIK (60)	
8		EAU83456			FSILGSTDIPR (74)	301
					GAPEAENMEWVIER (72)	
10		EAU83456			QLNVGGWSLDSTFGVR (64)	207
					ILDPVTFDTIK (55)	
11		EAU83456			MYHSEVSLLADGR (64)	203
					VLPNMPGAVNDFLGGR (50)	
4	6	EAU90899	94,639	4.73	IFGCEGFTQDDVIIAALFR (101)	893
					YPVYSGFITVSAPGENYR (89)	

Table A.7: continued

Spot	ID	Gene code	Theoretical MW (kDa)	pI value	Peptides (ionscore)	Total score*
5		EAU90899			IFGCEGFTQDDVIIAALFR (105) ISIANNGDAPK (67)	397
122	8	EAU93605	63,456	5.39	GLTSFDDDVCEGAVR (88) GPGVETASCDAQCR (48)	142
17	11	EAU82511	63,326	4.52	DGSLQNDIIVHEVAHGISNR (72) YGFTEAAFNFQQDNFGK (65)	520
89		EAU82511			YGFTEAAFNFQQDNFGK (55) LTGGGTASCLQTTESR (46)	109
234		EAU82511			LTGGGTASCLQTTESR (85) YGFTEAAFNFQQDNFGK (54)	251
13	15	EAU86023	61,178	6.38	LQQNSAPYAEQVER (78) VHCFLQSYWNPQQGYITANVGHR (77)	357
59		EAU86023			GSATYTALTNAVR (53) LQQNSAPYAEQVER (51)	124
40	17	EAU92553	45,249	6.51	FEGTYPGSSHIGSCR (62) VIDEWTFGQYLDR (61)	209
226		EAU92553			VIDEWTFGQYLDR (56) SFTGPASGYSQSYK (35)	68
255		EAU92553			SFTGPASGYSQSYK (73) FEGTYPGSSHIGSCR (76)	215
1	21	EAU90085	29,331	4.50	TTGLLGYATFPGDYER (52) EDEGNIPDSQLDSQLDVLNK (39)	115
16		EAU90085			TTGLLGYATFPGDYER (54) AGEKEDEGNIPDSQLDSQLDVLNK (51)	248
18		EAU90085			GAGPNNIQQADMK (72) TTGLLGYATFPGDYER (29)	91
44		EAU90085			EDEGNIPDSQLDSQLDVLNK (58) TTGLLGYATFPGDYER (56)	237
63		EAU90085			GAGPNNIQQADMK (64) EDEGNIPDSQLDSQLDVLNK (46)	118
73		EAU90085			GAGPNNIQQADMK (54) EDEGNIPDSQLDSQLDVLNK (49)	158
223	23	EAU84813	43,159	4.88	IGSSEEDGGEATFGGIDHEAYTGK (85)	85

Table A.7: continued

Spot	ID	Gene code	Theoretical MW (kDa)	pI value	Peptides (ionscore)	Total score*
14	26	EAU87896	52,468	4.68	LPSAPTEFSGR (59)	190
					TGYYDVELQTFPYLYSEGSAK (55)	
34		EAU87896			LPSAPTEFSGR (48)	59
					AAAHSVATYAR (39)	
219		EAU87896			LPSAPTEFSGR (53)	58
					RGECEFGLK (42)	
15	31	EAU80813	61,287	5.30	GGGGGTFGILTSVTYR (63)	252
					SLSEANETFNSILSLAQER (52)	
12		EAU80813			SLSEANETFNSILSLAQER (92)	266
					GGGGGTFGILTSVTYR (46)	
7	36	EAU82165	52,522	4.36	NPEDIQAAIEFANEHYLR (101)	433
					SVDDGNAAFNTFFELAR (67)	
27	39	EAU86463	65,360	6.03	MTGGGSGSCLQTTESR (63)	193
					DYVLGDYVYNNPGGIR (59)	
28		EAU86463			DYVLGDYVYNNPGGIR (85)	237
					VTVSVQDSSGTNNANFATPPDGQSGR (59)	
257		EAU86463			MTGGGSGSCLQTTESR (71)	151
					YGFTEAAFNFQNDNFGK (58)	
142	52	EAU82113	17,520	9.85	KIEALGTASGNPK (87)	125
					IIPNFMLQGGDFTR (45)	
220	55	EAU85085	42,690	6.81	TIPDGTLQGVHFVR (44)	66
					MNIPFGDDGGEMDNR (40)	
26	60	EAU84741	23,941	6.02	LITGQNPASASGVGEAILK (96)	184
					PSVLFVFTSTNK (83)	
205	68	EAU86652	54,017	7.66	IVIDDQFTTSAK (61)	101
					GALGGTCLNVGCIPSK (60)	
24	71	EAU91794	35,665	7.79	VLTDGGSGSIAGIVSGLDWIR (63)	176
					TPSAITVGATDIQDGK (61)	
25		EAU91794			GAVTGLPSTTANNLAQIGPL (82)	279
					TPSAITVGATDIQDGK (81)	

Table A.7: continued

Spot	ID	Gene code	Theoretical MW (kDa)	pI value	Peptides (ionscore)	Total score*
132	77	EAU92866	41,349	4.80	APGADDDGSGCVNLLEAFR (87) DTIAAIASQNPASR (77)	261
21	90	EAU83013	29,926	4.38	SPAFGCPVGR AAPFTVQQNSMK (41)	80
225	123	EAU88709	53,885	4.86	FTQAGSEVSALLGR (88) DEEGQDVLLFIDNIFR (69)	253
230		EAU88709			SIAELGIYPAVDPLDSK (45) FMSQPFQVAQVFTGYEGK (41)	72
100	136	EAU88001	34262	4.70	LPEDGFCTTAACR (61) DGNSDNFNTGISITPSR (57)	195
247		EAU88001			LPEDGFCTTAACR (81) DGNSDNFNTGISITPSR (68)	207
141	137	EAU81969	16,618	4,64	GYSTFGSLPGFPR (61) GGFNLAHAALNDLTNGQATQLGR (45)	107
126	140	EAU81416	29,593	4.55	EYSDEWDGYIFTDLNR (94) FPLPYDPTNGPPPTPIYGCPATTYR (59)	273
36	141	EAU86796	56,435	8.60	VGSESLVAAAASR (92) ELVSYDTPNIVR (43)	121
148		EAU86796			VGSESLVAAAASR (100) NGLGGTMFWELSTDK (68)	358
235		EAU86796			VGSESLVAAAASR (68) ELVSYDTPNIVR (37)	76
37	142	EAU85436	50,896	5.70	YEGLCDPDGCDYNPFR (57) VYLMENENR (45)	119
49	143	EAU88679	33,344	8.75	GLTVGNEVNDSPANIINK (77) SNGVNTPVSTVHTWVHIR (34)	161
139		EAU88679			GLTVGNEVNDSPANIINK (114) VLSVEPGCDALNR (19)	114
238		EAU88679			GLTVGNEVNDSPANIINK (51) VLSVEPGCDALNR (23)	78
9	144	EAU87901	61,646	4.76	VLGGSSSTNGMVYNR (58)	152

Table A.7: continued

Spot	ID	Gene code	Theoretical MW (kDa)	pI value	Peptides (ionscore)	Total score*
					EVVLSAGAFGSPQILLNSGIGPR (46)	
22	146	EAU87171	22,696	3.84	NIYIDEVLCIAK (61)	118
					CDNLVPGDELCLGTDEVDCR (53)	
20	147	EAU92752	21,900	4.69	FNVVDQSNFDCQVDR (60)	152
					LYDGGTLENYLR (54)	
51		EAU92576			SPPDLNPLNTTQAPPSTFPFTFR (74)	99
					FGVEFYEEQLR (48)	
189		EAU92576			SPPDLNPLNTTQAPPSTFPFTFR (78)	222
					FGVEFYEEQLR (67)	
244	148	EAU89154	34,708	5.18	GVGVAVGGGQSFER (61)	104
					SGSGSAEALYSIPSR (49)	
245		EAU89154			SGSGSAEALYSIPSR (44)	75
					FDVPEGNTAER (43)	
19	APS32	EAU89191	33,822	4.58	AGFFLEPTYWYYYIVR (76)	229
					VADTNFCLDAGSTPGNGVPMK (59)	
66		EAU89191			AGFFLEPTYWYYYIVR (36)	72
					VADTNFCLDAGSTPGNGVPMK (34)	
221		EAU89191			VADTNFCLDAGSTPGNGVPMK (51)	69
					AGFFLEPTYWYYYIVR (21)	
222		EAU89191			VADTNFCLDAGSTPGNGVPMK (50)	99
					AGFFLEPTYWYYYIVR (40)	
270		EAU89191			VADTNFCLDAGSTPGNGVPMK (40)	117
					AGFFLEPTYWYYYIVR (33)	
271		EAU89191			VADTNFCLDAGSTPGNGVPMK (57)	111
					AGFFLEPTYWYYYIVR (45)	

* Total protein score obtained from Mascot software (local server V. 2.0.04, Matrix Science).

Table A.8: Changes of the picked spots during the time of growth of the freely secreted proteins and the hyphal sheath proteins from *C. cinerea* grown in liquid YMG cultures at 37 °C obtained from Proteomweaver (Version 3.1.0.7 Defniens AG München, Germany). The numbers presented are corresponding to the change of the spot intensities in comparison to the gel of the supernatant from day 3. Changes higher than 1.5 can be regarded as significant; three replicates of each gel were analyzed.

ID	S3	HS3	S6	HS6	S9	HS9	S12	HS12
1	N.A.	0	0	0	0	N.A.	0	0
	N.A.	0	N.A.	N.A.	N.A.	0	N.A.	N.A.
2	N.A.	9.3452	0	0	0	0	0	0
4	N.A.	1.6738	0	0.4006	0	0	0	0
	N.A.	0.4943	0.2078	0	0.4198	0	0	0.379
	N.A.	0.4515	0	0	0.1724	0	0.0676	0
	N.A.	0	0.107	0	0.1357	0.19	0.177	0.147
	N.A.	1.0251	0	0.0698	0.1853	0.1829	0.2528	0.3494
	N.A.	1.3019	0	0.2523	0.1007	0.3056	0.2296	0.5939
6	N.A.	1.5355	2.7841	1.5023	1.6891	1.4649	1.7822	3.689
	N.A.	1.059	1.2422	1.5852	1.8538	1.9	1.4969	3.2797
8	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0	0
11	N.A.	1.3143	0.6066	0.9164	0.623	0.3901	0.9959	1.0558
	N.A.	0	N.A.	0	N.A.	0	N.A.	0
15	N.A.	0.5822	1.0616	0.8583	1.4356	0.691	0.794	0
	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
17	N.A.	0.4068	2.6227	0.5008	4.175	0.7058	2.0464	0.559
21	N.A.	1.6201	0.575	0.535	0.096	0.7122	0.1472	0.37
	N.A.	0	0.2413	0.2268	0.2909	0.3555	0.3759	0.3443
	N.A.	0	N.A.	0	N.A.	0	N.A.	0
	N.A.	0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	N.A.	0	N.A.	0	N.A.	N.A.	N.A.	N.A.
	N.A.	0.3125	0	0	0	0	0	0
26	N.A.	0	0	0	0	0	0	0
	N.A.	7.873	0.4777	24.327	0.3607	1.9745	0.3664	1.0087
31	N.A.	0	0.4609	0	0.7039	0	1.4226	4.7539
	N.A.	0.515	2.3483	0.5575	3.8137	1.9542	2.2661	1.8536
36	N.A.	0.9899	1.1927	1.0736	0.9098	1.0504	1.0356	1.2379
39	N.A.	2.188	0	0.5817	0	0.3374	0	0.1376
	N.A.	2.2906	0.8626	2.3939	0.6146	1.8249	0.4856	1.3924

Table A.8: continued

Spot number	S3	HS3	S6	HS6	S9	HS9	S12	HS12
52	N.A.	6.235	0	19.2811	0	29.8292	0	25.8848
60	N.A.	0.897	0.1933	0.3524	0	0.1151	0	0
68	N.A.	0	0	0	0	0	0	N.A.
71	N.A.	0.7313	0.4482	0.4591	0.3155	0.4198	0.0879	0.1153
	N.A.	2.037	2.0582	2.1794	2.5997	2.7281	2.2001	2.1579
77	N.A.	7.3374	3.0125	8.2615	4.3881	8.4441	4.7185	3.8808
90	N.A.	0.7295	1.4098	0.9161	1.0161	0.8097	0.5585	0.5413
136	N.A.	0	N.A.	N.A.	N.A.	0	N.A.	0
137	N.A.	8.8681	0.7069	3.8548	2.0502	0.9585	1.6541	2.4436
140	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
141	N.A.	0	N.A.	N.A.	N.A.	0	0	N.A.
	N.A.	0	N.A.	N.A.	N.A.	N.A.	0	0
142	N.A.	0.8072	5.4847	5.1835	7.0429	6.6306	7.6629	5.488
143	N.A.	9.4447	0	35.9018	23.0385	58.2389	9.45	57.4131
	N.A.	N.A.	N.A.	0	N.A.	N.A.	N.A.	N.A.
144	N.A.	1.6294	4.6007	3.3511	4.6769	5.6449	7.4121	11.3192
146	N.A.	0.5836	0	0	0	0	0	0
147	N.A.	0	0	0	0	N.A.	0	0
	N.A.	0.7314	0.6442	0	0.5147	0.0531	0.5862	0
	N.A.	1.0792	2.2777	2.3839	3.1872	0	4.9986	14.1403
APS32	N.A.	0.133	1.0175	0.0218	1.2609	0.0462	0.8167	0.0445
	N.A.	0.393	0.6155	0.243	1.6906	0.2501	2.7722	0

N.A.: Not applicable

Table A.9: Changes of the picked spots during the time of growth of the extractable cell wall proteins from *C. cinerea* grown in liquid YMG cultures at 37 °C obtained from Proteomweaver (Version 3.1.0.7 Defniens AG München, Germany). The numbers presented are corresponding to the change of the spot intensities in comparison to the gel of the supernatant from day 3. Changes higher than 1.5 can be regarded as significant; three replicates of each gel were analyzed.

ID	CW3	CW6	CW9	CW12
2	N.A.	0.1283	0	0
11	N.A.	0.5779	0.2636	0.3336
17	N.A.	23.807	20.348	15.495

Table A.9: continued

Spot number	CW3	CW6	CW9	CW12
	N.A.	0	47.865	3.178
23	N.A.	0	15.934	22.873
26	N.A.	121.988	4.824	23.441
39	N.A.	12.477	3.831	32.862
55	N.A.	11.354	0.4425	0.6776
123	N.A.	16.507	25.013	24.839
	N.A.	13.885	12.098	13.716
136	N.A.	14.466	26.216	23.592
141	N.A.	90.928	0.5757	0.8149
143	N.A.	N.A.	N.A.	0
148	N.A.	N.A.	N.A.	N.A.
	N.A.	77.335	91.704	49.383
APS32	N.A.	0.804	0.7621	0.4822
	N.A.	17.655	24.877	1.381
	N.A.	N.A.	N.A.	N.A.
	N.A.	N.A.	N.A.	N.A.

N.A.: Not applicable

Chapter 6 - General Discussion

Material and methods

Chemicals

Yeast extract and malt extract were obtained from Oxoid (Hampshire, UK) and agar was supplied by Serva Electrophoresis (Heidelberg, Germany). All other chemicals were of p.A. quality unless otherwise noted.

Culture conditions

Pleurotus ostreatus var. *florida* PC9 was obtained from Antonio G. Pisabarro (Universidad Pública de Navarra, Pamplona, Spain). The fungus was preserved on BSM (basal medium, (Braun-Lüllemann et al., 1997)) with 1.5% agar at 25 °C. For the main experiment the fungus was grown in plastic bags (1 l; 16.5 x 25 cm; Toppits®) suitable for autoclaving, containing 10 g wheat straw and 30 ml water. Two plaques of 5 mm diameter were used for the inoculation of the straw cultures. The cultures were grown at 25 °C in the dark. Every second day, starting at day 3, three inoculated bags plus one uninoculated control bag were harvested for the determination of enzyme activities.

Harvest and protein isolation

The straw cultures were washed with 50 ml Tris-buffer (0.5 M, pH 7.5) containing 2% (v/v) Tween 80 for 30 min under gentle shaking. Subsequently, the bags with the straw cultures were pressed between two wooden plates to obtain as much of the culture supernatant as possible. The culture supernatant was kept on ice for the measurement of the enzyme activities. After determination of the enzyme activities, the culture supernatants of the 3 replicates were pooled to obtain enough protein for 2-DE.

Protein samples isolated from the straw cultures were centrifuged at $48400g$ and 4 °C for 30 min to separated precipitated polysaccharides. Prior to protein precipitation soluble substances, hampering the IEF, were removed by hot SDS extraction under reducing conditions. 2% SDS and 5% β-mercaptoethanol were added to the centrifuged samples and incubated in boiling water for 20 min. After cooling the samples to room temperature, concentrated H_2SO_4 (4 μl/ml) was added to the samples to precipitate

phenolic compounds. The samples were incubated over night at room temperature and on the next day centrifuged at 1700g, transferred into a new tube and protein precipitation was performed by addition of 10% (v/v) from a 100% TCA stock solution (100 g TCA in 45.4 ml water) (Sivaraman et al., 1997). After mixing, samples were incubated overnight on ice and centrifuged at 1700g and 4 °C for 15 min. Excess of TCA was removed from protein precipitates by subsequent washings with few milliliters of ice cold 20% Tris-buffer (50 mM, pH 7.5) in acetone (v/v) (Fragner et al., 2009). Usually, three washing steps were required to remove TCA from the protein pellets. Finally, protein samples were washed with pure, ice-cold acetone, air dried, and stored at −20 °C until further processed.

The fungal biomass was determined as described in Chapter 5 (Section 5.3.3) and the protein concentration was determined as described in Chapter 2 (Section 2.5).

Enzyme assays

The activities of the following enzymes were determined on each day of harvest: manganese-dependent peroxidase (MnP), laccase with ABTS (2,2-azino-bis [3-ethylbenzthiazoline-6- sulfonate]) as substrate in spectrophotometric assays, phenoloxidases with MBTH (3-methyl-2-benzothiazolinon-hydrazone-hydrochloride) and DMAB (3-(dimethylamino)-benzoic acid) as substrate, cellobiose dehydrogenase, cellulase and xylanase. MnP was determined using phenol red for oxidation, according to the protocol of Kuwahara et al. (1984). The MnP activity was determined using the extinction coefficient $\varepsilon 610 = 4460\ \mathrm{M^{-1}cm^{-1}}$ (Michel et al., 1991). Laccase activity was determined in 120 mM sodium acetate buffer (pH 5.6) with ABTS as substrate. Substrate oxidation was determined at 420 nm according to Matsumura et al. (1986) and the laccase activity was calculated using an extinction coefficient of $\varepsilon 420 = 36000\ \mathrm{M^{-1}cm^{-1}}$ (Childs and Bardsley, 1975). Further phenoloxidases were assayed in succinate-lactate buffer (100 mM, pH 4.5) with MBTH and DMAB forming of a purple indamine dye product upon oxidation which was spectrophotometrically followed at 590 nm (Ngo and Lenhoff, 1980). Cellobiose dehydrogenase was measured with DCPIP (dichlorophenolindophenol) as electron acceptor as previously described by Rotsaert et al. (2003). The activities of cellulase and xylanase were determined according to Nair et al. (2008) using a dinitrosalicylic acid (DNS) reagent for the determination of reducing sugars produced by cellulase respectively xylanase (Miller, 1959). Enzyme activities are given in units

(U) defined as the amount of substrate in μmol transformed per min.

2-DE and Protein Identification

2-DE and masspectrometry were performed as described in the previous chapters. The data obtained by LC-MS^2 was searched against the NCBI database using the local Mascot software for the identification of the proteins.

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Abbreviations

1-DE	One dimensional gel-electrophoresis
2-DE	Two dimensional gel-electrophoresis
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACN	Acetonitril
APS	Average peptide scoring
BHT	2,6-di-tetra-butyl-4-methyl-phenol
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CAZy	Carbohydrate active enzyme database
CBM	Carbohydrate binding module
CDD	Conserved domain database
CDH	Cellobiose dehydrogenase
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMC	Carboxymethyl cellulose
CNBr	Cyanogene bromide
CRO	Copper radical oxidase
CW	Cell wall
CWP	Cell wall protein

DCPIP	Dichlorophenolindophenol
DMAB	3-(dimethylamino)-benzoic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
FAD	Flavine adenine dinucleotide
FGI	Fungal genome initiative
GC-MS	Gas chromatography coupled masspectrometry
GH	Glycoside hydrolase
GPI	Glycosylphosphatidylinositol
HF	Hydrogen fluoride
HS	Hyphal sheath
Hsp	Heat shock protein
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IUPAC	International Union of Pure and Applied Chemistry
JGI	Joint Genome Institute
LC-MS^2	Liquid chromatography coupled mass spectrometry
MBTH	3-methyl-2-benzothiazolinon-hydrazon-hydrochlorid, monohydrate
MnP	Manganese dependent peroxidase
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
PDH	Pyranose dehydrogenase

PFAM	Protein family
Pir	Proteins with internal repeats
PMSF	Phenylmethylsulfonyl fluoride
PTM	Posttranslational modification
RuBP	Ruthenium(II) tris(bathophenanthroline disulfonate)
S	Supernatant
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SMART	Simple Modular Architecture Research Tool
SP	Signal peptide
SQL	Structured query language
TFMS	Trifluoromethanesulfonic acid
Tris	Tris-(dimethylamino)-methane
VB	Visual basic
WGS	Whole genome shotgun
YMG	Yeast extract, malt, glucose medium

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