Heterobasidion annosum and wood decay: Enzymology of cellulose, hemicellulose, and lignin degradation

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Academic Dissertation

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Front cover: Different stages in the decay of spruce wood tracheids infected by *Heterobasidion annosum* are presented in the drawing above taken from R. Hartig (1878). The yellow-coloured parts contain lignin, the colourless parts contain mainly cellulose. The amino acid sequences below are segments of six aligned manganese peroxidases, which were translated from the manganese peroxidase encoding gene fragments identified in *Heterobasidion* and other related taxa.

ABSTRACT

Many fungi secrete enzymes that facilitate wood decay. Depending on the ecology of a particular fungus, the production of wood-decaying enzymes has evolved to sustain the niche the fungus is occupying. In some plant-fungus associations this has led to an effective wood decay; in other cases to the loss of efficient enzyme secretion.

Cellulolytic and hemicellulolytic enzyme production of a pathogenic white-rot fungus *Heterobasidion annosum* was studied and the activities were compared with cellulolytic activities of root symbiotic ectomycorrhizal fungi. Ligninolytic activity of *H. annosum* was studied in more detail by three different means: (1) enzyme activities were analysed by biochemical assays, (2) gene fragments encoding ligninolytic enzymes were characterised and (3) lignin mineralisation under solid state conditions was measured.

The cellulolytic activities of *H. annosum* were much stronger in comparison to the corresponding activities detected in the ectomycorrhizal fungi *Suillus bovinus*, *Paxillus involutus* and *Amanita regalis*. Both cellulolytic and hemicellulolytic activities of *H. annosum* were repressed by glucose while in *S. bovinus* clear cellulolytic activity was observed in the presence of glucose. When the enzymes were further characterised by native gel electrophoresis, some of the putative endoglucanases appeared to have similar mobilities in both the ectomycorrhizal fungi and in *H. annosum*. A number of cellulose-binding proteins were detected in *H. annosum* that were absent in ectomycorrhizal fungi. Several endoglucanase isozymes with different isoelectric points were identified in the P and S intersterility groups of *H. annosum*. The recognition of endoglucanases and cellobiohydrolases of *H. annosum* by polyclonal antibodies raised against cellulolytic enzymes of *Trichoderma reesei* indicated serological similarities between the enzymes of these two phylogenetically distant fungal species.

The activities of a range of hemicellulolytic enzymes, xylanase, ß-xylosidase, acetyl esterase, acetylxylan esterase, mannanase and α-galactosidase, were investigated in *H. annosum*. The P- and S-intersterility groups of *H. annosum* each had a characteristic xylanase and mannanase isoenzyme pattern. A xylanase with a p*I* of 8.4 was only detected in the P-strain in a medium supplemented with pure xylan. The p*I*-values of the acidic isoenzymes in both strains overlapped with those having mannanase and endoglucanase activities. Young mycelium of the P-strain produced mannanases with p*I*-values which differed from those expressed during later stages of growth. Xylanases and mannanases of *H. annosum* also cross-reacted with polyclonal antibodies to xylanase and mannanase from *T. reesei*.

H. annosum was shown to be a moderately ligninolytic fungus. Manganese peroxidases from a homokaryotic P-strain were partially purified by ion exchange chromatography and characterised using isoelectric focusing. Three isoenzymes with p*I*s of 3.3, 3.5, and 3.7 were found in spruce wood chips cultures. Interestingly, three manganese peroxidase encoding gene sequence fragments were identified in European *H. annosum* P-type, and four distinct fragments in the S-type of *H. annosum* using PCR with degenerate primers. Similar gene fragments were also detected in several other closely related wood-rotting basidiomycetes. The gene sequences among different species of *Heterobasidion* genus share about 80 to 92% identity at the

tentative amino acid level, and are similar with other known fungal lignin peroxidase and manganese peroxidase genes. The intron positions were identical in all the analysed genes and species.

The characterised fragments share several conserved residues which are important for the structure and function of a manganese peroxidase. Residues involved in Ca^{2+} and Mn^{2+}-ion binding, and the proximal histidine-residue involved in the catalysis were highly conserved in all the investigated fragments. The sequence data of these fragments was used for the construction of phylogenetic trees and analysis of relationships among the different intersterility groups. Variation within the sequences was also noted between the different intersterility groups of *H. annosum*.

PREFACE

Most of this work was carried out at the then Department of Botany, Division of Plant Physiology, Helsinki University, and during the last three years at the Division of Plant Physiology of the new Department of Biosciences. I am very grateful to Professor Liisa Simola for her support and encouraging attitude towards my thesis, and for providing good working facilities at her Department. I thank Docents Marja-Leena Niku-Paavola and Robin Sen for their critical reading of the manuscript and for their valuable comments.

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My thanks are due to my dearest ones, family and friends for giving me so much joy and happiness outside the lab. My wife Hanna has given me enormous support, love and understanding throughout the years. Without her patience this work would have never been realised. Lastly, my dearest thanks are due to my parents Mielikki and Mikael for their love and support.

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Helsinki, March 2000

Pekka Maijala

Dear flat rock
facing the stream

Where the willows are sweeping
over my wine cup again

If you say that the spring wind
has no understanding

Why should it come blowing me
these falling flowers?

Wang Wei

To Hanna

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LIST OF ORIGINAL PUBLICATIONS

I. Maijala, P., Fagerstedt, K.V. & Raudaskoski, M. 1991: Detection of extracellular cellulolytic and proteolytic activity in ectomycorrhizal fungi and *Heterobasidion annosum* (Fr.) Bref. New Phytologist 117: 643-648.

II. Maijala, P, Raudaskoski, M. & Viikari, L. 1995: Hemicellulolytic enzymes in P- and S-strains of *Heterobasidion annosum*. Microbiology 141: 743-750.

III. Maijala, P., Kulju, K. & Raudaskoski, M. 1998: Ligninolytic activity in *Heterobasidion annosum*: peroxidase involvement in pure cultures and in pathogenesis. In: Delatour, C., Guillaumin, J.J., Lung-Escarmant, B. & Marçais, B.(eds.): Root and Butt Rots of Forest Trees (9th International Conference on Root and Butt Rots), INRA Editions (France), Les Colloques n° 89, pp. 325-340.

IV. Maijala, P., Harrington, T.C. & Raudaskoski, M. Peroxidase gene structure and gene trees in *Heterobasidion* and related taxa. Submitted manuscript.

ABBREVIATIONS

2D NMR	Two-dimensional nuclear magnetic resonance
3-HAA	3-hydroxyanthranilate
AAO	Aryl alcohol oxidase
ABTS	2,2'-Azino-di-(3-ethylbenzthiazoline-6-sulphonic acid)
AXE	Acetylxylan esterase
bp	Base pair(s)
CBD	Cellulose binding domain
CBH	Cellobiohydrolase
CBP	Cellulose binding protein
CDH	Cellobiose dehydrogenase
cDNA	Complementary deoxyribonucleic acid
CMC	Carboxymethylcellulose
CREA	Catabolite repression effector of *Aspergillus*
DHP	Dehydrogenation polymerisate, synthetic lignin
DNS	Dinitrosalisylic acid
DP	Degree of polymerization
dw	Dry weight
E.C.	Enzyme Commission
EG	Endoglucanase
FAD	Flavin adenine dinucleotide
GC	Guanine-cytosine
GLOX	Glyoxal oxidase
HEC	Hydroxyethylcellulose
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IG	Immunoglobulin
IGS	Intergenic spacer region
IS	Intersterility group
ITS	Internal transcribed spacer region of the rDNA
kb	Kilo base pair(s)
K_{cat}	Catalytic efficiency, turn over
kDa	Kilodalton
K_m	Michaelis constant
LCC	Lignin-carbohydrate complex
LiP	Lignin peroxidase
MnP	Manganese peroxidase
NADH	Nicotinamide adenine dinucleotide, reduced form
NAD(P)H	Nicotinamide adenine dinucleotide(phosphate), reduced form
p*I*	Isoelectric point
RAPD	Randomly amplified polymorphic DNA
rDNA	Recombinant DNA
RFLP	Restriction fragment length polymorphism
rMnP	Recombinant manganese peroxidase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
VA	Veratryl alcohol

1 INTRODUCTION

1.1 THE IMPORTANCE OF *HETEROBASIDION ANNOSUM*

Robert Hartig stated in 1882: "Der gefährlichste Parasit der Nadelholzbestände is zweifellos die *Trametes radiciperda*" (the most dangerous parasite of conifers is without doubt *Trametes radiciperda*) (Hartig, 1882), and even today, renamed as *Heterobasidion annosum*, this fungus is the most harmful fungus to the modern forest industry in causing root and butt rot disease. Economic losses have been estimated to exceed 790 million ECU *per annum* in Europe (Woodward *et al*., 1998).

This thesis presents investigations on cellulolytic, hemicellulolytic, and ligninolytic activities that are required for lignocellulose degradation in *H. annosum*. A comparison of activities with those in ectomycorrhizal fungi is also presented. *H. annosum* is a pathogen that mainly attacks conifers causing heart rot on spruce and butt-rot on pines. The pathogen has spread widely through the Northern hemisphere, and during the course of evolution, has separated into different subtypes or intersterility groups that are classified as P, S, and F, based on the host preference of each subtype. Recently, these three subtypes have been described as three separate species (Niemelä & Korhonen, 1998), but the still commonly used nominations P, S, and F, are retained in this thesis. As wood-rotting fungi require extracellular enzymes for lignocellulose degradation, the application of these decay fungi, and the enzymes they secrete, in the production of food, industrial compounds and environmentally friendlier technologies, has been recognised. The great versatility that fungi have in both quantitative and qualitative terms in the production of hydrolytic and oxidative enzymes, offers an interesting field for a researcher, as the regulation of enzyme expression, structural modifications, or implications of these enzymes for both wood decay and fungal taxonomy, are all open for investigation.

1.2 WOOD, FUNGI AND NUTRIENT SUPPLY

Fungi are the most important group of organisms contributing to wood decay. On the other hand, fungi living in symbiosis with living trees and forming mycorrhiza play a critical role in the enhancement of tree growth and vigor. Concerted evolution of woody plant-interacting fungi and their plant hosts is clearly apparent, and can be traced back to the Silurian period around 400 million years ago, when the first vascular plants emerged (Simon *et al*., 1993; Remy *et al*., 1994). As the first vascular plants lacked highly developed root systems, symbiotic associations with fungal endophytes might have been crucial for plant survival. Basidiomycetes, including wood-rotting fungi, diverged probably during the Jurassic period around 300 million years ago, at the time of emergence of the early gymnosperms (Berbee & Taylor, 1993). Fungi took advantage of increasing availability of plant materials. As all fungi are heterotrophic, they are dependent on extracellular nutritional sources, e.g. carbon and nitrogen. For this purpose they can either form symbiotic associations with plants or they secrete

extracellular enzymes to degrade complex carbon and nitrogen sources, such as starch, pectin, cellulose, lignin and proteins (Carlile & Watkinson, 1994).

The fungal kingdom consists of a wide variety of organisms including yeasts, puffballs, moulds, morels, toadstools and polypore fungi which form large bracket-shaped fruiting bodies on wood. Polypore fungi belong to the basidiomycetes, a class that consists of highly advanced forms of fungi which have a special sexual life cycle. Basidiomycetes in particular are key players in wood degradation processes. Ascomycetes, such as morels, also produce typical structures for sexual spore formation and reproduction. By contrast, in many molds only a simple filamentous form is known. These fungi are often classified as deuteromycetes. These "lower fungi" can also potentially degrade complex plant polysaccharides and even lignin.

Based on their mode of plant interaction, fungi are presently divided into biotrophic, saprotrophic, and necrotrophic species. Typically, wood-colonising micro-organisms, such as basidiomycete fungi, are saprotrophic and either pathogenic or harmless to the host plant. Necrotrophy describes a process, in which host tissues are first killed by the fungus and then utilised saprotrophically. Biotrophic fungi utilise living plant materials, whereas saprotrophic fungi gain essential nutrients from dead cell material. A special group of biotrophic fungi are those that form mycorrhiza. These nutritional traits do not represent unvarying trends. In fact, many fungi are not restricted to a single mode but show various degrees of flexibility during their life cycle (Cooke & Whipps, 1993).

Mycorrhizal fungi, which normally show strict biotrophic behaviour, may sometimes act as saprophytes. Under tree-less conditions e.g. after clear-cutting, some ectomycorrhizal fungi may produce extracellular enzymes for acquisition of nutrients and thus survive as saprophytes. There are indications that these fungi possess these traits (Trojanowski *et al*., 1984; Cairney & Burke, 1994, 1998), although whether the low observed activity of the respective enzymes would be enough for survival in forest soil is questionable. Hydrolytic enzyme activities could also be necessary for the development of functional structures of the symbiosis, as plant cell wall modifications to accommodate the Hartig net structure in ectomycorrhizal, or arbuscule structure in endomycorrhizal associations, is required. In contrast, pathogenic fungi, such as *Heterobasidion annosum*, attack living cells and in a step-wise fashion kill the cells and finally the whole infected plant. The infection and penetration by *H. annosum* often starts via entry through roots as in the mycorrhizal colonisation process. Plant root tips are the target for both classes of fungi. In the present work the different infection modes are investigated with special emphasis on the hydrolytic enzymes produced by the saprophytic and ectomycorrhizal fungi.

1.3 COLONISATION OF LIVING PLANTS

Fungal attack of woody plants is an everpresent stress factor. The subsequent plant response to fungal attack will determine the consequences of the interaction; whether it is localised to the invaded cells, or whether the fungus is able to invasively colonise the tissue(s) and cause disease or even death of the plant. A notable active resistance mechanism in woody plants is mediated by a non-specific response that appears to

involve the formation of tissues resistant to enzymatic attack by the fungus, but also the production of toxins and phytoalexins. This occurs in the cortex-phloem and xylem of roots, stems, and the foliage (Merrill, 1992). Such walling-out reactions in xylem tissues had been clearly illustrated over a 100 years ago by Robert Hartig (Hartig, 1882), and much later termed "compartments" by Shigo (1979, 1984), in descriptions of *H. annosum* infection of *Pinus resinosa*. Further colonisation by the fungus and decay distribution in wood is dependent on anatomical characteristics of xylem vessels or chemical composition of the wood. High content of secondary metabolites and phenolics in the heartwood of many species or physical factors, such as gaseous regime, pH of the environment, and moisture content, may affect fungal growth. In particular, fungi growing in the heartwood must be adapted to low oxygen tensions inside the trunk (Rayner & Boddy, 1988).

The outermost plant tissues covering the xylem form the protective layer consisting of alternating layers of periderms and associated tissues, called rhytidome (Esau, 1977). These tissues are corky, suberized, and often contain antimicrobial compounds, which together form the constitutive defence or preformed anatomical barrier to fungal attack. Most pathogens are unable to directly invade these tissues. However, *H. annosum* has been reported to be able to penetrate, to a certain extent, the intact bark (Peek *et al*., 1972). Fungal ingress may be mediated by the toxin production (Donnelly *et al*., 1988; Sonnenbichler *et al*., 1989) and killed cells may open up a route for further penetration. Young, meristematic cells, e.g. root tips in short roots of *Pinus*-species, do not contain such barriers, and successful invasion is instead more dependent on an active response of the host to a fungus. Once a saprotrophic/pathogenic fungus has succeeded in pre-colonising host tissues, it rapidly enters xylem vessels growing axially within the tracheid lumen and enters adjacent cells through pits or directly through the secondary wall. Ray cells provide a route for horizontal spread, and when killed, the readily assimilable nutrients provide resources for further colonisation.

The course of infection development is determined by the molecular signals released at the plant-fungus interaction and is further mediated by the signal transduction pathway of the host cell. Rapid responses in host cell wall, membranes, and cytosol will generate local and systemic wound signals. These enhance further transcriptional and translational activities leading to the activation of secondary responses, e.g. induction of pathogen cell wall degrading enzyme production in plant cells, and enzymes required for suberin and lignin biosynthesis. In symbiotic associations, a delicate control of these responses is required, and a successful interplay may include other factors (Harrison, 1999; Martin & Tagu, 1998). The growth rate of the *Pinus* short roots is suggested to help slow-growing ectomycorrhizal species to establish the symbiosis in the root cortex tissue (Niini & Raudaskoski, 1998).

1.4 CONSEQUENCES OF FUNGAL INFECTION - DECAY TYPES

Most wood-inhabiting fungi are strictly saprotrophic and utilise dead wood as a food base. The saprophytic fungi, especially polypores, are extremely important in their role

as decomposers in global ecosystem carbon recycling. The main energy sources for fungal growth are wood polysaccharides, although many wood-inhabiting fungi and bacteria also are able to modify lignin (Kirk & Farrell, 1987). Only a specialised group of fungi, either pathogenic and/or saprophytic, are able to efficiently degrade lignin. They are called white rot fungi. In order to break down lignin, white rot fungi require a co-metabolisable carbon source, which within wood are the breakdown products of cellulose and hemicellulose. Typically white rot appears as a spongy, stringy, or laminated structure in affected wood, where lignin and polysaccharides present in sound wood are removed in equal proportions, although lignin removal may be preferred. The pathogen *H. annosum* can preferentially degrade lignin, which is seen in Fig. 1 as loss of the middle lamella between the tracheids. A similar decay pattern in spruce has been observed in later investigations (Blanchette, 1984b, Schwarze, 1995). *H. annosum* caused simultaneous lignin and cellulose degradation in laboratory conditions in pine (Schwarze, 1995) and in spruce (Meier, 1955). Selective lignin removal of birch wood degraded by *H. annosum* has been reported (Meier, 1955). It is not known, if the likelihood for a certain decay pattern differs in different intersterility groups of *H. annosum*. The substrate cell types and other micro-environmental conditions affect degradation patterns (Blanchette, 1991, 1995), but the exact mechanism, by which this selectivity for lignin removal is achieved, is not well known.

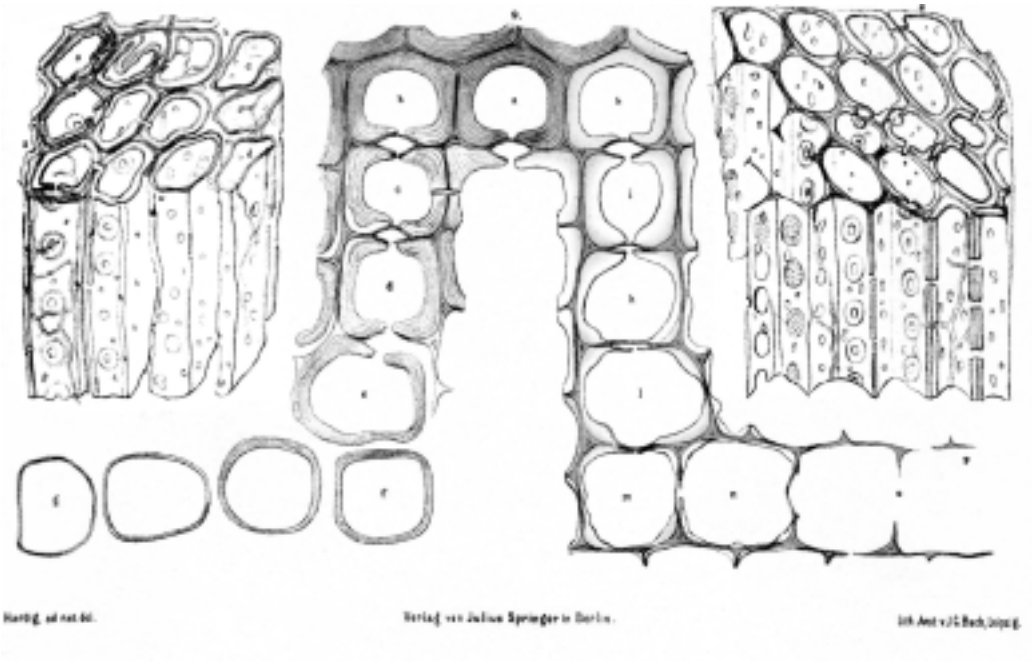

Fig. 1. Decay patterns of spruce wood infected by *H. annosum*. Selective decay development, where lignin is preferentially removed, is visualised in tracheids b-g on the left, and simultaneous wood deterioration in tracheids h-p on the right. Drawing by R. Hartig (1878). Tracheid a denotes a healthy cell.

Brown rot fungi include both pathogenic and saprophytic species, which selectively remove cellulose and hemicellulose from wood, especially conifers. Infected wood rapidly loses its inherent strength and undergoes drastic shrinkage and cracking across the decayed wood fibres. In the advanced stages the wood remains as cubic, dark brown crumbly chunks composed mainly of modified structures of lignin. Brown rot fungi are basidiomycetes, and comprise the clear minority (10-20%) of all wood-degrading basidiomycetes. They do not possess oxidative phenoloxidase or peroxidase activities required for efficient lignin degradation. However, they have developed an efficient mechanism for cellulose hydrolysis, which probably involves low molecular weight chelators, oxalate, and hydroxyl radicals produced by Fenton reaction (Goodell *et al*., 1997). The hydroxyl radical is a strong oxidant being able to degrade cellulose, and may affect lignin substructures (Backa *et al*., 1992; Gierer *et al*., 1992). Recent results indicate that brown rot fungi will efficiently utilise the extracellular one-electron oxidation system to cleave carbon-carbon bonds in a poly(ethylene oxide) molecule (Kerem *et al*., 1998), and possibly the cellulose structure as a natural substrate.

Fungi that cause soft rot may attack wood exposed to moisture and water. These species belong to Ascomycetes (or "Deuteromycetes"), including the genera *Penicillium*, *Thielavia* and *Dactylomyces*. On contact, soft rot fungi may cause erosion of the secondary wall forming cavities within the S_2-layer of the plant cell wall. Morphology of these cavities varies depending on the fungus and the substrate (Nilsson, 1985). They can remove significant amounts of cellulose and hemicellulose from cell walls of wood. Several *Aspergillus* spp. forming soft rot are strong producers of xylanases and they are able to degrade lignin model compounds, but high molecular weight lignin or ^{14}C-labelled synthetic dehydrogenation polymerisate (DHP) -lignin is very poorly degraded (Duarte & Costa-Ferreira, 1994). Similar degradation patterns have been observed in soil-inhabiting *Fusarium* spp. (Regalado *et al*., 1997).

Bacteria attack cell walls of woody plants through different mechanisms and may adversely or synergistically affect the growth of various fungi in wood. Some bacteria utilise the cell content of rays and affect the wood permeability to liquids by dissolving pit membranes, resin etc., whilst others attack the cell walls directly and affect the tensile strength properties of wood. Bacteria may be associated with other micro-organisms and contribute to the cell wall degradation process, or they can be antagonistic and inhibit the growth of other microbes. Deterioration of cell walls may resemble similar degradation by brown rot fungi, including the destructed crystalline cellulose fraction (Greaves, 1969). Lignin model compounds have been shown to be degraded by bacteria (Pellinen *et al*., 1984, 1987; Daniel *et al*., 1987).

Among bacteria, actinomycetes, particularly *Streptomyces*, have been studied (Crawford *et al*., 1977, 1983). The preliminary studies suggest that they may play an important role in wood cell wall modifications. Lignin peroxidase activity has been described in *Streptomyces viridosporus* (Ramachandra *et al*., 1988). Wood degrading *Streptomyces*-species form an important group with antagonistic properties against other wood-invading micro-organisms. On the other hand, many bacteria has been detected in connection with white rot fungi. They may provide substances that promote hyphal growth and they may also diminish catabolic inhibition of cellulose degradation caused by cellobiose and glucose by utilising these sugars (Eriksson *et al*., 1990). In ectomycorrhizal associations, the intimate association of fungi with bacteria,

has been reported (Nurmiaho-Lassila *et al.*, 1997). So called mycorrhization helper bacteria, has been proposed to improve fungal growth and include species that facilitate degradation of organic polymers around hyphae (Garbaye, 1994; Sarand *et al.*, 1998).

1.5 CHEMICAL STRUCTURE OF WOOD COMPONENTS

1.5.1 Cellulose

Cellulose is the main polymeric component of the plant cell wall and is the most abundant polysaccharide on earth. The physical structure and morphology of native cellulose are complex, and fine structural details are difficult to determine experimentally (O'Sullivan, 1997). The chemical composition of cellulose is simple: the polysaccharide consists of D-glucose residues linked by ß-1,4-glycosidic bonds to form linear polymeric chains of over 10 000 glucose residues. The individual chains adhere to each other along their lengths by hydrogen bonding and van der Waals forces, and crystallise shortly after biosynthesis. Although highly crystalline, the structure of cellulose is not uniform. Physical and chemical evidence indicates that cellulose contains both highly crystalline and less-ordered amorphous regions (Atalla, 1993; Hon, 1994) (Figs. 3, 5). Although chemically simple, the extensive intermolecular bonding pattern of cellulose generates a crystalline structure that, together with other cell wall components such as hemicellulose and lignin, results in very complex morphologies. Multiple enzyme systems are thus required to efficiently degrade cellulose.

In laboratory experiments, cellulolytic enzyme activities can be measured using artificial soluble cellulose derivatives. Hydroxyethyl (HEC) or carboxymethyl (CMC) substitution occurring at every two to five sugars unit positions can solubilise long cellulose polymers. Endoglucanases cleave bonds between the substituent groups, which results in a rapid decrease in the degree of polymerisation (DP) and viscosity of the substrate. Exoglucanases seem to be limited to the hydrolysis of a few, unsubstituted sugar residues at the chain ends of HEC and CMC and do not decrease their DP or viscosity (Wood and Bhat, 1988).

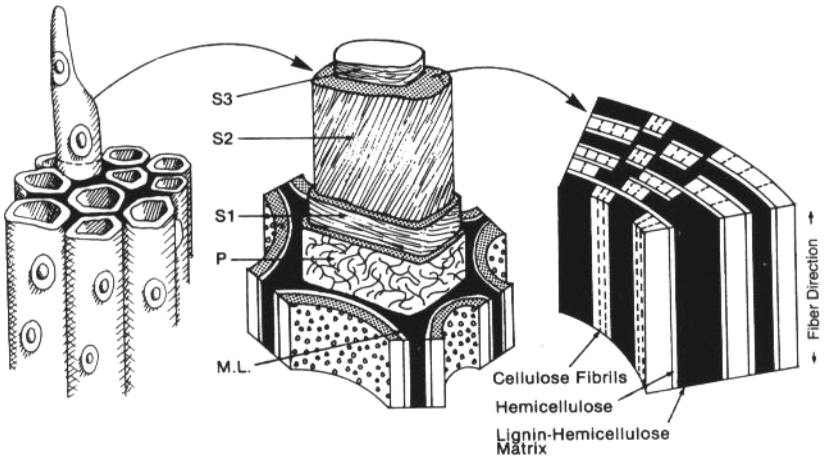

Fig. 2. Schematic illustration of the morphology of the tracheids, secondary wall layers and the relationship of the lignin, hemicelluloses, and cellulose in the secondary wall of a tracheid. Cell diameter is about 25µm. S1-S3, secondary cell wall layers; P, primary wall; M.L., middle lamella (Kirk & Cullen, 1998; partly from Goring, 1977).

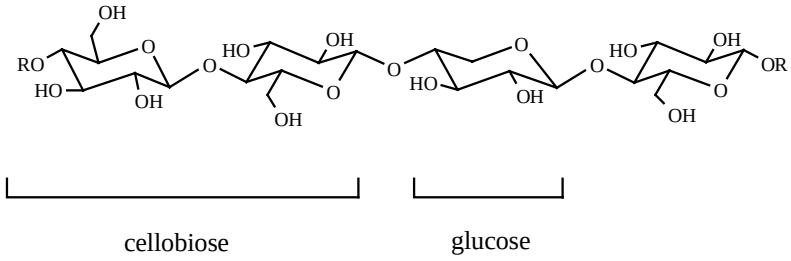

Fig. 3. A schematic structure of a cellulose chain.

1.5.2 Hemicellulose

Hemicellulose consists of several different sugar units and substituted side chains in the form of a low molecular weight linear or branched polymer. This polymer is more soluble than cellulose with a DP of less than 200. Hemicelluloses are named according to their main sugar residues in the backbone. Xylans, consisting of D-xylose units, and glucomannans, consisting of D-glucose and D-mannose units (Table I, Fig. 6), contribute to the main hemicelluloses in hardwoods and softwoods, respectively. Larch (*Larix* sp.) wood and compression wood of softwoods contain a large amount of arabinogalactans and galactans, respectively (Dekker, 1985). Branched polymers contain neutral and/or acidic side groups. These groups render hemicelluloses noncrystalline or poorly crystalline, so that they exist more like a gel than as oriented fibres. Hemicelluloses form a matrix together with pectins and proteins in primary plant cell walls and with lignin in secondary cell walls. Covalent hemicellulose-lignin bonds involving ester or ether linkages form lignin-carbohydrate-complexes (LCCs)

(Watanabe *et al*., 1989; Jeffries, 1990). The cleavage of these bonds in LCCs has been proposed to be important for facilitation of the biopulping-effect and preferential lignin degradation by white-rot fungi (Watanabe, 1995). Similar bonds between lignin and cellulose have not been documented. Hemicelluloses can be occluded within and between crystalline cellulose domains during the synthesis of cellulose microfibrils, as concluded from the studies with the bacterium *Acetobacter xylinum* (Atalla *et al*., 1993). The relationship between cellulose and hemicellulose in the cell walls of higher plants may be extremely intimate, so it is possible that molecules at the cellulose-hemicellulose boundaries and those within the domains of crystalline cellulose require different enzymes for efficient hydrolysis. This may help to explain why cellulolytic micro-organisms typically secrete a range of different cellulases with overlapping specificities and why some xylanases carry substrate-binding domains with affinity for cellulose (Tomme *et al*., 1995).

Table I. The main hemicelluloses in hardwoods and softwoods.

Wood species	***Polysaccharide***	***Amount (%)***	***Composition (molar ratios)***						
			Xyl	***GlcA***	***Ara***	***Man***	***Glc***	***Gal***	***Ac***
Hardwood	Glucuronoxylan	15-30	10	1					7
	Glucomannan	2-5				1-2	1		
Softwood	Arabinoglucuronoxylan	7-10	10	2	1.3				
	(Galacto)glucomannan	10-15				4	1	0.1	1
	Galactoglucomannan	5-8				3	1	1	1

Abbreviations: Xyl = xylose, GlcA = 4-O-methyl-α-D-glucuronic acid, Ara = arabinose, Man = mannose, Glc = glucose, Gal = galactose, Ac = acetyl group.

1.5.3 Lignin

Lignin is a branched polymer of substituted phenylpropane units joined by carbon-carbon and ether linkages. Biosynthesis of lignin formation proceeds *via* polymerisation of the free radical forms of precursors, i.e. the monolignols *para*-coumaryl, coniferyl and sinapyl alcohol. In the final polymer they form *p*-hydroxyphenyl-, guaiacyl-, and syringyl type units, respectively. Plant laccases and peroxidases catalyse the generation of radical formations. It seems plausible that lignin polymerisation pattern and assembly is guided by the orientation of cellulose and the structure of hemicelluloses (Atalla, 1995). The major linkage in lignin, the arylglycerol-ß-aryl ether substructure, comprises about half of the total interunit linkages. Lignin in gymnosperms (softwood lignin) and angiosperms (hardwood lignin) have different monolignol compositions: softwoods contain mainly guaiacyl type lignin with some *p*-coumaryl subunits, whereas hardwood lignin consists of both guaiacyl and syringyl type lignin with few *p*-coumaryl residues (Sjöström, 1993). Softwood lignin structure has been recently refined using 2D NMR methods (Brunow *et al*., 1998) (Fig. 4).

A general response to fungal infection of plants is a formation of lignin and lignin-related compounds, such as suberin, in the vicinity of the invading hyphae. It is

unclear, if the monomer composition of this newly formed defence lignin differs from the lignin in the native xylem tissue (Nicole *et al*., 1992).

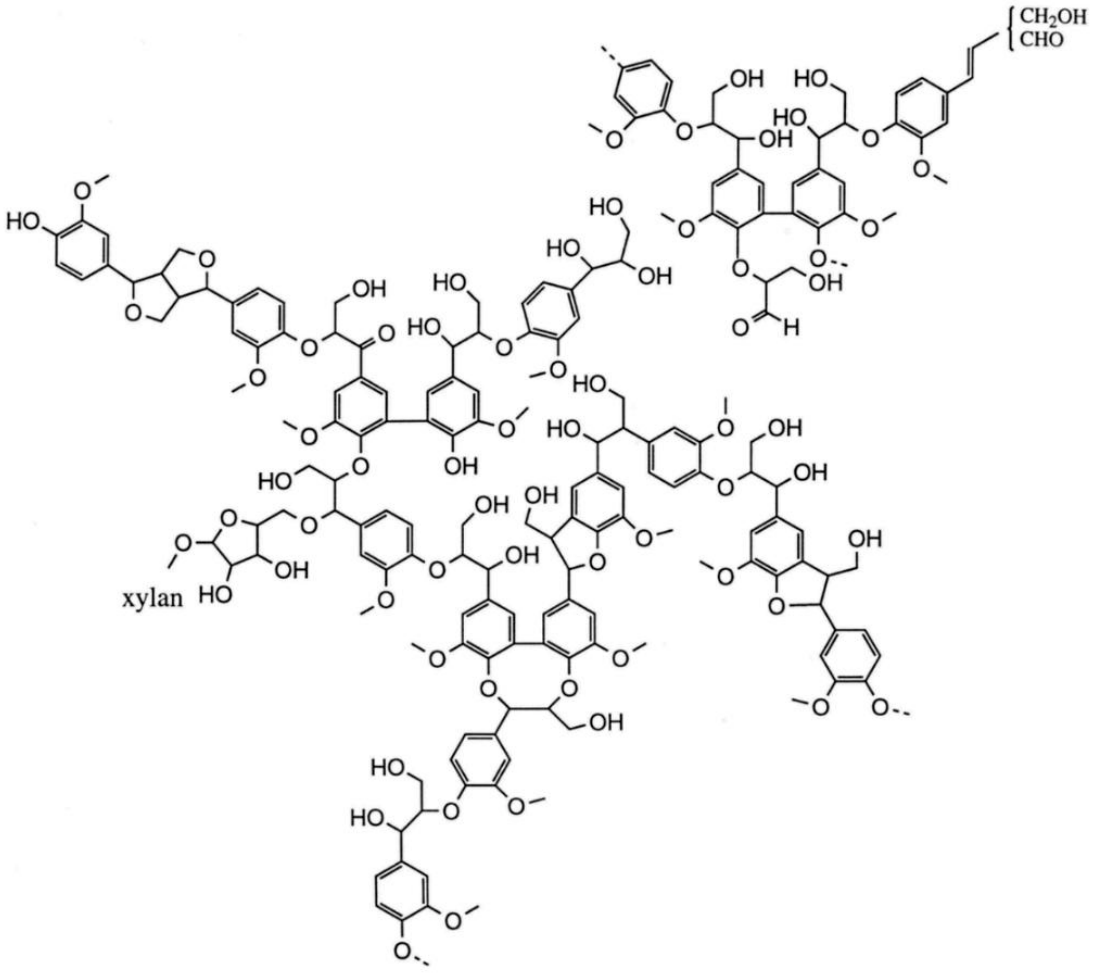

Fig. 4. A structural model of softwood lignin according to Brunow *et al*., 1998.

1.5.4 Other plant substances affecting fungal colonisation

Pectin is a common name for a group of amorphous polymers that exist in plant cell walls, especially in the middle lamella between xylem cells. Pectic polymers rich in galacturonic acid are structurally important especially during cell wall formation. They exist in nature both in a methylesterified and in a free acidic form. Polymers also contain neutral sugars, notably D-galactose, L-arabinose, L-rhamnose, D-xylose, L-fucose, and D-apiose (Aspinall, 1981). L-arabinose and D-galactose are also present in polymeric forms as 1,5-linked arabinan and as ß-1,4- or ß-1,3-linked galactans or as arabinogalactan I containing ß-1,4-galactan.

Pectin in the form of a calcium salt forms crosslinks that affect the structural properties of the amorphous matrix. During lignification of the xylem cells, pectin is partly removed (Westermark *et al*., 1986). The content of galacturonans is less than 1% in lignified soft- and hardwoods (Fengel & Wegener, 1989). Pectin has a high

charge density that may play a role for Ca^{2+} -ion binding and the localisation of metal ions in the cell wall, which may also affect the swelling of the cell walls in xylem (Westermark & Vennigerholz, 1995). Cambial cells, containing only the primary cell wall, may provide easy access for fungal infection. The fungi can utilise pectin as a carbon source by producing pectinolytic enzymes, such as pectin acetylesterases, pectinmethylesterases and polygalacturonases.

Pectinolytic activity has been found in several ectomycorrhizal fungi (Nylund & Unestam, 1982; Ramstedt & Söderhäll, 1983; Keon *et al*., 1987; Niini *et al*., 1988). Pectinases have also been detected during colonisation of the root in endomycorrhiza (Gianninazzi-Pearson & Gianninazzi 1995). In *H. annosum*, the P-group appears to produce more pectinolytic enzymes than the S-group (Johansson, 1988; Karlsson & Stenlid, 1991). Pectinases were not investigated in the present study.

Suberin contributes to both cell wall strength and resistance to water loss during plant growth and development. Plant root periderm and cork tissues are often suberized. Suberin and lignin biosyntheses require the same precursors, and suberinisation may precede lignification in fungal colonisation of plants, thus forming an important component of the plant defence responses. Suberin consists of spatially separate polyaromatic (polyphenolics) and polyaliphatic (lipids) domains, but its assembly has not been established (Bernards & Lewis, 1998). Suberised tissue contains phenolics and acids which can be toxic to invading micro-organisms. Terpenes, such as resin acids and phenolic extractives, such as lignans and stilbene derivatives, have fungicidal properties. These compounds are concentrated in heartwood and bark tissues of trees, and have been noted to affect host resistance against pathogens like *H. annosum* (Alcubilla *et al*., 1974), although the mode of action has not been conclusively clarified. Lignans, stilbenes and other phenolic compounds may inhibit extracellular enzyme activity and fungal growth (Woodward & Pierce, 1988; Lindberg *et al*., 1992). *H. annosum* appears to be able to detoxify and penetrate these barriers in a successful infection.

Close assembly with other cell wall polymers makes the extraction of cellulose, hemicellulose, or lignin as the pure, native product very difficult for structural analysis or as a substrate for enzymes. On the other hand, the complex polymer structure, with a variety of side chains attached to the polymer backbone, makes the plant cell wall particularly resistant to microbial degradation. In fungi a variety of mechanisms must have evolved to overcome these barriers in order to infect the plant and utilise the organic plant material for growth.

1.6 DEGRADATION OF WOOD COMPONENTS

1.6.1 Enzymes required for the hydrolysis of cellulose

An efficient cellulolytic enzyme system requires endo- and exo-type enzymes and ß-glucosidases. Microbial cellulases have been classified into more than 60 different families depending on their structure and substrate specificity, but also according to the mode of action: some microbial cellulases display both endo- and exo-type attack

features (Henrissat & Davies, 1997). The complementary activities of endo- and exo-type enzymes lead to synergy, an enhancement of activity, which is more than the added activities of individual enzymes. Exo-exo-synergy is also observed, and may indicate a low, inherent endoglucanase activity of the exoglucanases (Tomme *et al.*, 1995; Shen *et al.*, 1995). **Endoglucanases** (EGs, E.C. 3.2.1.4) belong to endo-type enzymes that hydrolyse cellulose microfibrils preferentially in the amorphic parts of the fibril (Fig. 5). The catalytic region of the enzyme is groove-shaped that enables the attachment of the enzyme and the hydrolysis in the middle part of the cellulose fibre (Divne *et al.*, 1994). Endoglucanase activity in other white rot fungi is a common feature, and it probably exists in all wood-degrading fungi including brown-rot fungi (Highley, 1988). **Cellobiohydrolases** (CBHs, E.C. 3.2.1.91) are exo-type enzymes that attack cellulose fibres from both reducing and non-reducing ends. In *Trichoderma reesei*, CBH I attacks reducing ends, and CBH II the non-reducing ends of the fibre (Teeri, 1997). No exo-type activity has been observed in brown-rot fungi, so they cannot degrade pure crystalline cellulose. Exceptions to this generalisation can be found in a few brown rot fungi belonging to the family *Coniophoraceae* (Nilsson & Ginns, 1979). In *Coniophora puteana* two CBHs have been isolated (Schmidhalter & Canevascini, 1993). The product of CBH action, cellobiose is hydrolysed by **ß-glucosidases** (E.C. 3.2.1.21) to two glucose units. Cellobiose and glucose can be taken up and assimilated by the hyphae. The cellobiose taken up is probably hydrolysed to glucose by cell wall bound or intracellular ß-glucosidases.

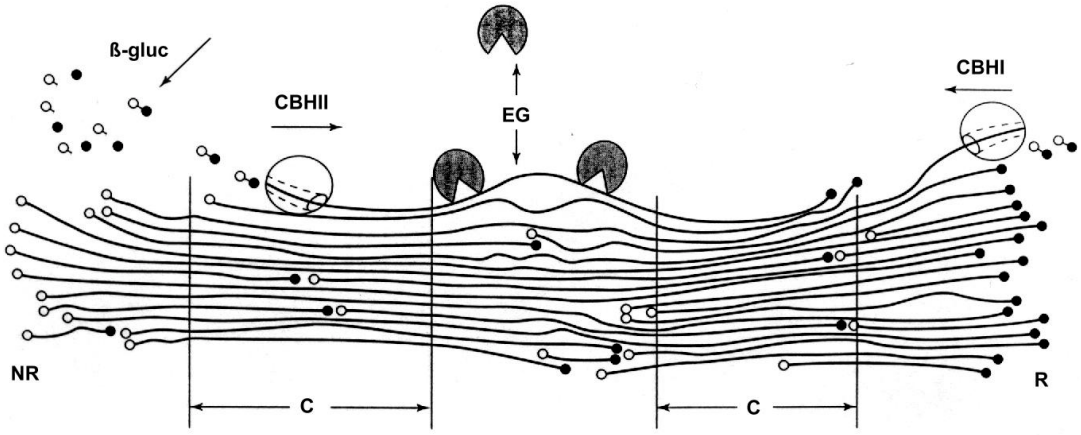

Fig. 5. A schematic view of the cellulose structure and action of the CBHs, EGs, and ß-glucosidases (ß-gluc) in *Trichoderma reesei*. C defines the highly ordered crystalline region, R the reducing ends (filled circles), and NR the nonreducing ends (open circles). EGs attack the more disordered structures of the cellulose. ß-glucosidase action produces glucose. Modified from Teeri (1997).

All cellulolytic enzymes share the same chemical specificity for ß-1,4-glycosidic bonds, which they cleave by a general acid-catalysed hydrolysis. A common feature of most cellulases in different fungal genera is a domain structure with a catalytic domain linked with an extended linker region to a cellulose-binding domain (CBD) (Gilkes *et al*., 1991). CBDs are obviously required for efficient hydrolysis of crystalline, but not for soluble, substrates (reviewed by Klyosov, 1990). Similar cellulase and xylanase structures in fungi and bacteria suggest that cellulolytic genes could have spread to a significant extent by horizontal transfer across a wide range of organisms during the course of evolution (Béguin & Aubert, 1994).

Oxidative enzymes participating in cellulose hydrolysis were detected in *Phanerochaete chrysosporium* in the seventies (Westermark & Eriksson, 1974). Originally this enzyme was proposed to be present only in basidiomycetes, but has been later found in several other fungal groups, including ascomycetes and deuteromycetes. Biochemically the enzyme is currently described as **cellobiose dehydrogenase** (CDH, E.C. 1.1.3.25) (Wenjun *et al*., 1993), which consists of two domains, a heme-containing domain and a flavin adenine dinucleotide (FAD) -domain. Proteolytic cleavage will separate the FAD-domain, which will produce cellobiose oxidase (E.C. 1.1.5.1) (Ander, 1994). CDH will oxidise cellobiose, lactose and mannobiose that all have ß-1,4-bonds, whereas monosaccharides and α-1,4-glycosidic bond containing maltose are not oxidised (Henriksson *et al*., 1998). As electron acceptors CDH reduces quinones, phenoxy radicals, cytochrome *c*, complexed Fe^{3+}, manganese and molecular oxygen, which leads to the production of hydrogen peroxide. Hydrogen peroxide production has been demonstrated in *P. chrysosporium* CDH (Kremer & Wood, 1992), but not in *T. versicolor* CDH (Roy *et al*., 1996). These properties make this enzyme an interesting component of fungal wood decay systems, as it combines cellulose and lignin degradation. Moreover, in a brown rot fungus *Coniophora puteana* CDH appears to be important in generating Fe(II) in acid conditions, and subsequent H_2O_2 production leads to hydroxyl radical formation and cellulose hydrolysis (Hyde & Wood, 1997). CDHs have been characterised from several white rot fungi, such as *Heterobasidion annosum* (Hüttermann & Noelle, 1982), *Phanerochaete chrysosporium* (Westermark & Eriksson, 1975; Bao *et al*., 1993), *Pycnoporus cinnabarinus* (Temp & Eggert, 1999), *Schizophyllum commune* (Fang *et al*., 1998), and *Trametes versicolor* (Roy *et al*., 1996). In addition, CDHs are known from the brown rot fungus *Coniophora puteana* (Schmidhalter & Canevascini, 1992), ascomycetes *Myceliophtora thermophila* (Canevascini, 1988), *Neurospora sitophila* (Dekker, 1980), *Chaetomium cellulolyticum* (Fähnrich & Irrgang, 1982) and *Humicola insolens*, a thermophilic fungus (Schou *et al*., 1998). CDH encoding cDNAs have been cloned and sequenced from *P. chrysosporium* (Raices *et al*., 1995; Li *et al*., 1996). No cellulase-like CBD was recognised. Northern blot studies identified CDH transcripts in the presence of cellulose but not in glucose or cellobiose containing cultures (Li *et al*., 1996).

Cellulolytic enzyme systems in white-rot fungi have gained less attention than in asco- and deuteromycetes. Few studies exist in basidiomycetes, such as *P. chrysosporium* or its anamorph, *Sporotrichum pulverulentum* (Eriksson & Hamp, 1978) and *Ischnoderma resinosum* (Sutherland, 1986). The work done with the white

rot fungus *P. chrysosporium* has shown that in this fungus the cellulase systems are analogous to systems well-known in the ascomycete/deuteromycete fungus *T. reesei*, which is illustrated in Fig. 5. The enzymes involved in cellulose degradation are inducible by the substrate and repressed by the products of their action. However, in most brown rot fungi, endoglucanases are constitutively expressed and are not catabolically repressed by glucose (Highley, 1973; Cotoras & Agosin, 1992).

Two acidic proteases has been observed to cause increased activities of endoglucanases but not proteolysis in the cultures of *P. chrysosporium* (Eriksson & Pettersson, 1982). Similar modulation of cellulase activities by specific proteolysis has been suggested in *T. reesei* (Teeri, 1987). A glucose-inhibited permease is known from *T. reesei* (Kubicek *et al*., 1993). The permease may facilitate the uptake of putative inducing diglucosides, such as cellobiose, and thus functions as a regulatory component of the system.

An interesting enzyme system for ß-glycosidic bond cleavage of cellobiose has been reported from *H. annosum* (Hüttermann & Volger, 1973b). The phophorolytic cleavage of this bond producing glucose-1-phosphate may increase the energy capacity for growth, and may explain the higher biomass production in cultures supplemented with cellobiose (Hüttermann & Volger, 1973a; Hüttermann & Noelle, 1982). The cellobiose phosphorylase activity has not been reported in other white-rot fungi.

1.6.2 Enzymes required for hemicellulose degradation

The enzymatic degradation of hemicelluloses requires a complex set of different enzymes reflecting the variability of the hemicellulose structure. The enzymology is reasonably well known in ascomycetes and deuteromycetes (Wong *et al*., 1988; Eriksson *et al*., 1990; Biely, 1993; Coughlan & Hazlewood, 1993), but only a few studies have been made on the hemicellulases of basidiomycetous white rot fungi. Although the systems of hemicellulose degradation is analogous to that of non-wood decaying ascomycetes, it seems possible that evolutionary pressures to function in the tight molecular architecture of lignified cell walls could have altered enzyme sizes, shapes, and specificities (Kirk & Cullen, 1998). Hemicellulose hydrolysis proceeds through the action of endo-type enzymes, that liberate shorter fragments of substituted oligosaccharides, which are further degraded by side-group cleaving enzymes and exo-type enzymes. Alternatively, side-branches may be cleaved first. As a result, acetic acid and monomeric sugars are liberated and can be used as carbon sources for the fungal growth. Similarly to cellulose hydrolysis, the hydrolases act synergistically to convert hemicellulose polymer into soluble units (Tenkanen *et al*., 1996).

Hemicellulose degrading enzymes are hydrolytic, and specifically degrade those glycans that make up the backbone of the hemicelluloses. Typical hemicellulases are therefore endo-1,4-ß-D-xylanases (E.C. 3.2.1.8), endo-1,4-ß-D-mannanases (E.C. 3.2.1.78), and endo-1,4-ß-D-galactanases (E.C. 3.2.1.90). Xylan-degrading enzyme system is well characterised in fungi. **Xylanases** are probably the most widely studied group of hemicellulases in bacteria and fungi due to their numerous biotechnological applications (Wong & Saddler, 1992; Viikari *et al*., 1994). Endo-ß-1,4-xylanases catalyse the random hydrolysis of ß-1,4-glycosidic bonds in xylans. A range of

different endoxylanases with different specificities has been proposed in fungi. They show the highest activity against polymeric xylan, and the rate of the hydrolysis decreases with decreasing chain length (Coughlan *et al*., 1993). Some xylanases prefer hydrolysis of the backbone xylan chain near the specific sugar substituent. The effective native xylan degradation seems to involve probably multiple isoenzymes of four enzyme types: endoxylanases, **α-glucuronidases** (E.C. 3.2.1.131), **α-arabinofuranosidases** (E.C. 3.2.1.55)/**α-arabinosidases** (E.C. 3.2.1.99), and **acetyl xylan esterases** (E.C. 3.2.1.72). They all differ in specificity in respect to the neighbouring substituents and chain length (Tenkanen *et al*., 1995). In softwoods, where the xylan has arabinose as a substituent, xylan degradation requires a α-arabinofuranosidase, but not the esterase. Synergy between endoxylanase, α-glucuronidase, **ß-xylosidase** (E.C. 3.2.1.37), and α-arabinofuranosidase was reported in *P. chrysosporium* in the hydrolysis of a birch wood xylan (Castanares *et al*., 1995).

α-Glucuronidases have been characterised from *P. chrysosporium* (Castanares *et al*., 1995), *S. commune* (Johnson *et al*., 1989) as well as from the ascomycete *Thermoascus aurantiacus* (Khandke *et al*., 1989) and *T. reesei* (Siika-aho *et al*., 1994). α-Arabinofuranosidases have been studied widely in filamentous ascomycetes, such as *Aspergillus terreus* (Luonteri *et al*., 1995), *A. awamori* (Wood & McCrae, 1996) and *Penicillium capsulatum* (Filho *et al*., 1996) due to their use in biotechnological applications in food industry. In white rot fungi, only *P. chrysosporium* α-arabinofuranosidase has been studied (Coughlan & Hazlewood, 1993). **Ferulic acid esterase** has been characterised from *S. commune* (Mackenzie & Bilous, 1988). This enzyme form functions only together with xylanase, thus forming a component of the xylanolytic enzyme system in *S. commune*. No ferulic acid esterase activity from other white rot fungi has yet been reported.

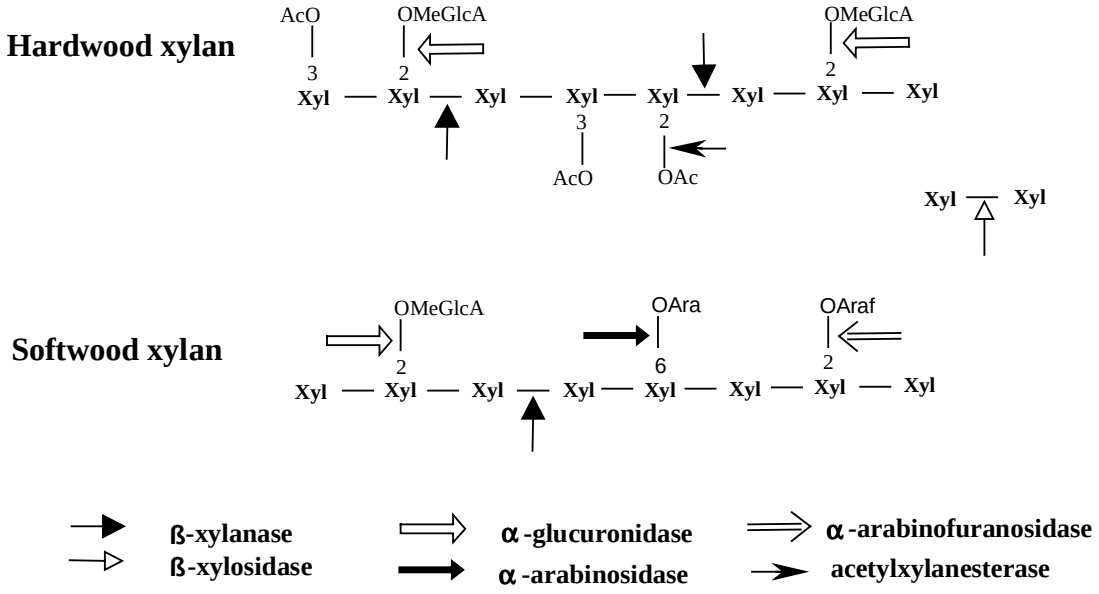

Fig. 6. Schematic structures of hardwood and softwood xylan and the action of different hemicellulolytic enzymes.

Complete glucomannan hydrolysis also requires five different enzyme types. **Endo-1,4-ß-D-mannanases** hydrolyse randomly the 1,4-ß-D-mannopyranosyl linkages of gluco- and galactoglucomannans, releasing oligomeric fragments. **Acetyl(gluco)mannan esterase** removes the acetyl groups and **α-galactosidase** (E.C. 3.2.1.22) removes galactose. **ß-mannosidase** (E.C. 3.2.1.25) and **ß-glucosidase** cleave the ß-1,4-linkages between oligomeric fragments. Only few studies have been conducted on these enzymes in white rot fungi, although white rot fungi obviously can effectively remove mannan from the cell walls of wood (Eriksson *et al*., 1990). Endo-1,4-ß-D-galactanase has been characterised in *Irpex lacteus* (Tsumuraya *et al*., 1990), and they are also present in bacteria (Dekker, 1985). There are no reports on acetylglucomannan esterase activity in white rot fungi. The enzyme is isolated and partially characterised in several *Aspergillus*-species (Puls *et al*., 1992; Tenkanen *et al*., 1995). ß-Mannosidases have not been characterised in white rot fungi. In a brown rot fungus *Polyporus* (*Laetiporus*) *sulfureus* ß-mannosidase was characterised as a 64 kDa protein (Wan *et al*., 1976).

Microbial hemicellulase production appears to be not as strictly controlled by induction through the growth substrate as the cellulase production. The regulation of hemicellulase genes appears to be more complex than that of the cellulase genes. Both constitutive and inductive production of hemicellulases have been reported (Dekker, 1985, Eriksson *et al*., 1990). No information on aspects of the genetic control of hemicellulases in white rot fungi exist, but, as with other fungi, hemicellulases appear to be regulated at the transcriptional level. A common transcriptional activator XlnR has been recently characterised in *A. niger* that regulates not only endoxylanase and ß-xylosidase expression, but also the expression of α-glucuronidase, acetylxylan esterase, arabinoxylan arabinofuranohydrolase, feruloyl esterase and two endoglucanase-encoding genes (van Peij *et al*., 1998) and even two *cbh*-genes (Gielkens *et al*., 1999). In the basidiomycete *S. commune* (Yaguchi *et al*., 1992; Oku *et al*., 1993) and in a basidiomycetous yeast, *Cryptococcus albidus* (Boucher *et al*., 1988) endoxylanase genes have been cloned.

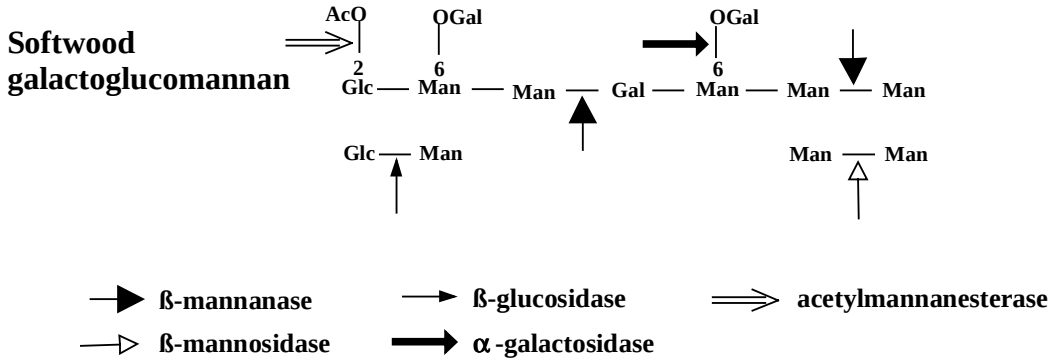

Fig. 7. Schematic structure of softwood galactoglucomannan and the action of different hemicellulases.

1.6.3 Enzyme systems required for lignin degradation

Since white-rot fungi are the only organisms capable of efficient lignin degradation, their ligninolytic enzyme system has been studied extensively. Lignin polymer structure is irregular, which means that the degradative enzymes must show lower substrate specificity compared to the hydrolytic enzymes in cellulose or hemicellulose degradation. Because lignin consists of interunit carbon-carbon and ether bonds, the enzymes must be oxidative rather than hydrolytic. White rot fungi produce extracellular peroxidases and phenoloxidases that act nonspecifically via generation of free radicals, which are unstable and undergo a variety of spontaneous cleavage reactions. The major consequence of enzymatic one-electron oxidation of lignins and lignin-related phenols is oxidative coupling/polymerisation (Lewis & Yamamoto, 1990). However, no lignin polymerisation has been observed during fungal delignification *in vivo* (Kirk & Farrell, 1987; Kurek *et al.*, 1998).

Three main fungal enzymes that affect lignin structures either directly or indirectly are lignin peroxidases (LiPs), manganese peroxidases (MnPs) and laccases. All three enzymes can act with low-molecular-weight mediator molecules to bring about lignin oxidation. Some white-rot fungi obviously do not produce all three, but only two or one of the enzymes (Hatakka, 1994). No ligninolytic peroxidase activities have been reported from soft rot fungi, although a gene with high similarity to *P. chrysosporium* lignin peroxidase has been found in *Fusarium oxysporum* (Mönkmann *et al.*, 1996). Peroxidase function requires extracellular hydrogen peroxide, which is supplied by enzymes such as glyoxal oxidase and glucose-2-oxidase or by non-enzymatic systems via Fenton reaction.

Lignin peroxidases (EC 1.11.1.14) were first characterised by Tien & Kirk (1983) as “ligninases”, but soon thereafter another ligninolytic peroxidase was found, that required manganese (Tien & Kirk, 1984; Kuwahara *et al.*, 1984). They resemble other peroxidases like horseradish peroxidase. LiP operates via a typical catalytic cycle. Enzyme is oxidised by H_2O_2 to a two-electron deficient intermediate termed Compound I, which returns into its resting state through two one-electron oxidations of donor substrates. The one-electron deficient intermediate is termed Compound II. The key characteristic of LiP is its capability to oxidise non-phenolic compounds or moieties. LiPs are powerful oxidants that can also oxidise several aromatic ethers and polycyclic aromatics (Kersten *et al.*, 1990; Field *et al.*, 1993). LiP-catalysed oxidation results in aryl cation radicals that can react as a radical and as a cation, forming a wide variety of degradation fragments (Higuchi, 1989). LiPs are produced by several white rot basidiomycetes, but species seem to exist that do not possess any LiP activity (Orth *et al.*, 1993; Hatakka, 1994). Although LiP has been regarded for a long time as the central enzyme influencing delignification, recent studies with other fungi and natural woody substrates have indicated that other enzyme systems may be equal effective and efficiently utilised by white rot fungi in lignin biodegradation.

Manganese peroxidase (E.C. 1.11.1.13) has a similar catalytic cycle as LiP, but it utilises Mn(II) as a substrate. Compound I can oxidise phenolic compounds or Mn(II), but compound II can apparently oxidise only Mn(II) (Wariishi *et al.*, 1988). The product of Mn(II) oxidation, Mn(III), must be chelated by organic acids, such as oxalate or glycolate, which stabilise the ion and promote its release from the enzyme

(Glenn & Gold, 1985; Glenn *et al*., 1986; Wariishi *et al*., 1988; Kishi *et al*., 1994). Mn(III)-chelate may diffuse from the enzyme and function as diffusible oxidant to promote lignin degradation. However, the Mn(III)-chelate is not a strong oxidant, and can oxidise only the phenolic moiety of the lignin (Wariishi *et al*., 1991; Tuor *et al*., 1992). The resulting phenoxy radicals can undergo a variety of reactions, which may result in polymer cleavage between the aromatic nuclei and Cα in a propane side chain (Wariishi *et al*., 1991; Tuor *et al*., 1992). Also LiPs have been shown to be able to oxidise chelated Mn(II) in the presence of H_2O_2 (Popp *et al*., 1990; Khindaria *et al*., 1995a). Recent studies indicate that MnP may oxidise Mn(II) without H_2O_2 with decomposition of malonic acid, and concomitant production of peroxyl radicals that may affect lignin structure (Hofrichter *et al*., 1998). Moreover, MnP is able to liberate CO_2 directly from lignin substructures (Hofrichter *et al*., 1999). It can also produce H_2O_2 in O_2-requiring oxidation of NAD(P)H (Glenn *et al*., 1986), glutathione and dithiotreitol (Glenn *et al*., 1986; Paszsyñzski *et al*., 1986).

MnP and LiP encoding gene sequences have been isolated from several basidiomycetes. The first LiP encoding gene was cloned and sequenced in 1987 from *P. chrysosporium* (Tien & Tu, 1987). Since then several peroxidase encoding genes have been reported from *P. chrysosporium*, as well as from other fungal species. MnP and LiP gene sequences are conserved among basidiomycetes, which has helped the identification of these genes in different white rot fungi. Heterologous hybridisation experiments have revealed tentative *P. chrysosporium* LiP-like sequences in *Fomes lignosus* (Huoponen *et al*., 1990), *Phlebia brevispora* and *C. subvermispora* (Rüttimann *et al*., 1992). *P. chrysosporium* LiPs are encoded by at least ten closely related genes, which have been designated *lip*A through *lip*J (Gaskell *et al*., 1994), although a wide range of designations have been used for these genes in literature. A transposon-like element *Pce*1 was found in one of the LiP genes of *P. chrysosporium* (Gaskell *et al*., 1995). MnP genes are not so well characterised in white rot fungi. In *P. chrysosporium* three MnP genes have been reported, but the total number of MnP genes in this or in any white rot fungus remains to be established (Datta *et al*., 1991; Pease & Tien, 1992).

Fungal **laccases** (E.C. 1.10.3.1) have been studied for over a hundred years. The enzyme is blue copper-containing polyphenol oxidase. It catalyses the reduction of molecular oxygen to water by four consecutive 1-electron oxidation steps of a phenolic substrate, or aromatic amines and other electron-rich substrates. Laccase is produced by most of the white rot fungi, but its significance as a delignifying enzyme seems to vary in different species. Laccase has also other roles, which are often related to fungal morphogenesis, such as fruiting body formation, conidiogenesis, colour and melanin biosynthesis, or pathogenesis, as in *Botrytis cinerea* or *Cryphonectria parasitica* (Thurston, 1994).

Laccase catalyses splitting the Cα-Cß-bonds in the ß-1- and ß-O-4-dimers by oxidising α-carbon and by cleavage of the aryl-alkyl bond (Higuchi, 1989; Youn *et al*., 1995). It can also modify the synthetic DHP-lignin forming water-soluble products (Iimura *et al*., 1995) and cause demethoxylation (Eriksson *et al*., 1990). Since *P. chrysosporium* evidently is able to mineralise lignin without producing laccase (Kirk & Farrell, 1987), the significance of laccase as a delignifying enzyme has gained little attention. Recently, as more fungi have been studied, laccase has also been shown to

act as an important part of the delignifying system of fungi. *Pycnoporus cinnabarinus* is reported to produce laccase but not peroxidases (Eggert *et al*., 1996). Also in *P. chrysosporium* laccase has been reported to be produced in the presence of cellulose (Snirivasan *et al*., 1995). However, the low oxidase activity has not been conclusively proved to originate from true laccase, and might be produced by LiP or MnP (Paszczyñski *et al*., 1986; Glenn *et al*., 1986; Ander & Pettersson, 1992).

Hydrogen peroxide-generating systems form an important part of the fungal delignification system, as peroxides are required for peroxidase function. Hydrogen peroxide may be also utilised by CDH together with Fe(III) and Mn(IV) reduction (Roy *et al*., 1994) for the generation of oxyradicals that are able to cause bond cleavages in lignin and cellulose. Several fungal enzymes have been characterised as H_2O_2 producers, but most of them are intracellular, and only few extracellular enzymes have gained more attention.

Extracellular enzymes which produce hydrogen peroxide include glyoxal oxidase (GLOX, E.C. number not yet assigned) (Kersten & Kirk, 1987) and glucose-2-oxidase (E.C. 1.1.3.10) (Daniel *et al*., 1994). Intracellular methanol oxidase catalyses the reaction from methanol to formaldehyde and H_2O_2. Extracellular formaldehyde and acetaldehyde, formed in ethanol oxidation, can then be oxidised by GLOX to produce more H_2O_2. GLOX has been found in several white rot fungi, although a few fungi seem to lack GLOX activity (Orth *et al*., 1993; Hatakka, 1994). GLOX appears to be transcriptionally regulated, and expression coincides with that of MnP and LiP genes in *P. chrysosporium* (Stewart *et al*., 1992). Some fungi also produce extracellular aryl alcohol oxidase (AAO, E.C. 1.1.3.7). This enzyme oxidises benzyl alcohols produced by the fungus to the aldehydes by transferring electrons to O_2 and producing H_2O_2. This is a typical extracellular enzyme in *Pleurotus*-species and *Bjerkandera adusta* (Waldner *et al*., 1988, Marzullo *et al*., 1995), but has been found also from *T. versicolor* (De Jong *et al*., 1994), and as an intracellular enzyme from *P. chrysosporium* (Asada *et al*., 1995b).

Low molecular weight compounds have gained increasing interest in lignin biodegradation studies as potential oxidants of lignin in locations distant from fungal hyphae and in substrates that enzymes are unable to penetrate. Oxalate has been proposed to be important in connection with MnP and manganese in the oxidation of aromatics (Perez & Jeffries, 1992; Kuan & Tien, 1993; Khindaria *et al*., 1994, Shimada *et al*., 1994; Lequart *et al*., 1998; Moreira *et al*., 1998), and it can also function with LiP-systems (Popp *et al*., 1990). Oxalate is produced by several white rot fungi, including *H. annosum* (Volger *et al*., 1982; Dutton *et al*., 1993; Galkin *et al*., 1998) and appears to have versatile roles in fungi. It may be an important factor in fungal pathogenesis, and may increase phosphate and sulphate availability in mycorrhizal systems (Kritzman *et al*., 1977; Dutton & Evans, 1996). Iron-binding chelators produced by fungi have also been proposed to be potential compounds in wood degradation and biological pulp bleaching (Durán & Machuca, 1995, Goodell *et al*., 1997), as well as compounds such as copper peroxydisulfate that have been shown to mimic LiP activity (Huynh, 1986).

1.6.4 Factors affecting lignin degradation

Lignin degradation is thought to generally occur at the onset of the secondary growth phase of the fungus, as early utilisable nutrients are depleted and primary fungal growth ceases. Nitrogen (Keyser *et al*., 1978), carbon or sulphur (Jeffries *et al*., 1981) depletion triggers the production of ligninolytic enzymes and lignin degradation in *P. chrysosporium*, and similar culture conditions for studies of fungal delignification in general has been adopted for other species. It seems however, that although nitrogen, carbon, and sulphur limitation is a common regulatory aspect in triggering fungal ligninolysis, it is not a rule. When greater number of species have been analysed, a more variable picture concerning the regulatory aspects of fungal lignin degradation have been observed (Leatham & Kirk, 1983; Hatakka & Uusi-Rauva, 1983; Eriksson *et al*., 1990; Hatakka, 1994).

The ligninolytic system in *P. chrysosporium* is expressed under secondary metabolic conditions, triggered by starvation for nutrient nitrogen or carbon (Gold & Alic, 1993). Transcription levels of LiP and MnP genes is strictly dependent on the culture conditions. In *P. chrysosporium*, different LiP transcripts are expressed depending on the limiting component, i.e. carbon or nitrogen, in the medium (Holzbaur & Tien, 1988; Stewart *et al*., 1992; Reiser *et al*., 1993). However, strain variation seems to also affect *P. chrysosporium* transcript profile: widely used strain BKM-F-1767 expresses different dominant LiP transcript than strains ME446 (James *et al*., 1992; Broda *et al*., 1995), and OGC101 (Ritch *et al*., 1991; Ritch & Gold, 1992).

MnP production in *P. chrysosporium* is transcriptionally regulated by a number of factors, and the induction of different MnP isoforms by their primary substrate Mn^{2+} (Brown *et al*., 1991) appears to be co-ordinately regulated (Pease & Tien, 1992; Gettemy *et al*., 1998). The expression analyses with MnP genes support the earlier observations that manganese-ion content affects ligninolytic efficiency in many fungi (Leatham, 1986; Boyle *et al*., 1992; Kerem & Hadar, 1995). The expression of a novel type peroxidase encoding gene *mnpl* from *Pleurotus eryngii* was not induced by Mn^{2+}-ions, but peptone as the nitrogen source and oxidative stress did elevate *mnpl* gene expression (Ruiz-Dueñas *et al*., 1999c). In *Pleurotus*-species lignin degradation appears not to be repressed by nitrogen (Leatham & Kirk, 1983).

Many other factors have been observed to affect fungal ligninolysis, including the presence of detergents, such as Tween 80 (Jäger *et al*., 1985), veratryl alcohol (VA) and other aromatic compounds (Faison & Kirk, 1985), as well as culture pH and availability of oxygen (Kirk *et al*., 1978; Hatakka & Uusi-Rauva, 1983; Boyle *et al*., 1992). Gene expression analyses of MnPs from *P. chrysosporium* have supported the observations of potential factors regulating ligninolysis in this fungus (Brown *et al*., 1993; Li *et al*., 1995).

2 AIMS OF THE STUDY

The initial aim of this study was to characterise the wood degrading enzymes in ectomycorrhizal fungi and to compare their activity with that in the wood-degrading pathogenic fungus *Heterobasidion annosum*. During the course of the work the emphasis moved away from the mycorrhizal fungi and on to the plant cell wall hydrolysing enzymes of the latter fungus. Although the ecology, infection biology, and disease development in *H. annosum* have been actively investigated, there is still a critical need for a more detailed characterisation of the wood degrading enzymology of this important forest pathogen. A thorough evaluation of the enzymes and enzyme activities is crucial for a deeper understanding of the infection process and wood decay development. *H. annosum* is divided into different intersterility groups with differing host preferences. By analysing wood degrading enzymes in these groups, a more functional basis for the development of intersterility groups may be identified.

Specific aims were:
1. To study the production of endoglucanase activities in *H. annosum* and to compare those with the activities produced by ectomycorrhizal fungi (I, II).

2. Characterise the hemicellulase components of *H. annosum* and identify differences relating to intersterility group membership (II).

3. Investigate enzymes involved in lignin degradation both by biochemical and molecular biological methods (III, IV).

4. Utilise peroxidase-encoding gene fragments for the clarification of the relationships among different intersterility groups of the genus *Heterobasidion* (IV).

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 The fungi

The origin of the strains of ectomycorrhizal fungi *Paxillus involutus, Suillus bovinus* and *Amanita regalis* and the isolates of *H. annosum* are described in Table II. The origins of other strains were as explained in Harrington *et al*. (1998) and in IV. Fungal culture media were prepared according to Brown & Wilkins (1985) or as described in II and III.

Table II.

Fungus	Used in	Isolate code and information	Collection
Amanita regalis	I	-	Dept. of Microbiology, University of Helsinki[*2]
Suillus bovinus	I	-	Veikko Hintikka
Paxillus involutus	I	-	Veikko Hintikka
H. annosum P[*1]	I	heterokaryon	Kari Korhonen
H. annosum S	I	heterokaryon	Kari Korhonen
H. annosum P	II, III, IV, DHP-exp.	910517 (HaE-EP0);Utti, on *Pinus sylvestris*; heterokaryon	Kari Korhonen
H. annosum S	II	900509; Tuusula, on *Picea abies*; heterokaryon	Kari Korhonen
H. annosum P	MnP purification	94177; Kärkölä, on *Pinus sylvestris*; homokaryon	Kari Korhonen

[*1]Indicates intersterility group P or S, [*2]now Dept. of Applied Chemistry and Microbiology

3.1.2 Other materials

Plant seed material used in study III was obtained from Haapastensyrjä tree breeding centre of the Foundation for Forest Tree Breeding. *Pinus sylvestris* seeds were imbibed overnight in distilled water at 4°C, surface-sterilised in 30% H_2O_2 for 20 min and rinsed thoroughly with sterile water. Seeds were germinated on water agar plates in darkness. Culture set-up was as described in III.

3.2 METHODS USED IN PUBLICATIONS

The experimental methods used in the present investigation and described in the included publications are listed in Table III.

Table III.

Method	Described and used in
Plate assays	I
Native protein gel electrophoresis	I
Diffusion blotting	I
Isoelectric focusing	II, III
DNA methods and Southern blotting	III, IV
Measurement of hemicellulolytic enzyme activities	II
Measurement of laccase, MnP, and LiP activities	III
Measurement of total proteins	I, II
Determination of fungal biomass	II
Analysis of enzymatic xylan hydrolysis products	II
Analysis of DNA sequences	IV

Methods not described in the publications but contributing to this research are presented in detail below.

3.3 OTHER METHODS

3.3.1 Western blotting

Polyclonal antibodies raised against cellulolytic and hemicellulolytic enzymes of *Trichoderma reesei* were obtained from VTT Biotechnology and Food Research Laboratory. Transfer of proteins from polyacrylamide gels onto nitrocellulose filters and immunodetection of cellulolytic and hemicellulolytic proteins were performed according to Towbin *et al.*, 1979. Membranes were blocked by incubation either in 0.05% (w/w) Tween 20 or 1% (w/v) bovine serum albumin. After the primary antibody treatment, secondary antibody, either alkaline phosphatase-conjugate of Goat anti-rabbit IgG [H+L] (Bio-Rad, PROMEGA) or rabbit anti-mouse IGs linked to horseradish peroxidase (Dako A/S, Glostrup, Denmark) was added under the same conditions.

3.3.2 Measurement of glucose and total reducing sugars in the medium

Amount of glucose was measured enzymatically using a glucose oxidase based method (Boehringer). Total reducing sugars were analysed by a dinitrosalisylic acid (DNS) -

method (Sumner & Somers, 1949), using glucose as a standard. Measured values were used to evaluate the germination efficiency of P- and S-strain of *H. annosum* conidia as the time required to reduce the day 0 sugar content (approximately 300mg/100ml medium) by 50%.

3.3.3 ^{14}C-DHP mineralisation experiments

^{14}C-(ring)-labelled synthetic lignins (dehydrogenation polymerizate, DHP) were originally obtained from Dr. K.E. Hammel (Forest Products Laboratory, Madison, Wisc., USA). Specific activities were: non-phenolic, methylated DHP-lignin $9x10^5$ dpm/mg, phenolic, non-methylated DHP-lignin $9x10^5$ dpm/mg. Solid state cultures for isotope trapping experiments were prepared as follows: 10ml of water agar was added to conical 100ml flasks to balance the moisture content inside the flasks. A nylon spacer was placed over agar. Bleached wood pulp sheets (Lenzing AG, Lenzing, Austria) (approximately 20mm x 20mm, 2 g) were labelled by pipetting ^{14}C-DHP-lignin in dimethylformamide with the total of 20.000 dpm per flask. Solvent was evaporated under vacuum, and the labelled sheets were placed on the nylon spacer. 5% mycelial suspension prepared in mineral salt medium (III) was added on top of the pulp adjusting the water content to 80%. Flasks were fitted with rubber stoppers with gasing manifolds. Evolved $^{14}CO_2$ were trapped in an ethanolamine-containing cocktail containing toluene, methanol, ethanolamine, 2,5-diphenyl-oxazole (PPO) (Sigma) and 1,4-bis(5-phenyl-2-oxazolyl-benzene; 2,2'-*p*-phenylene-bis(5-phenyloxazole) (POPOP) (Sigma) at three-day intervals by flushing the flasks with air. Trapped $^{14}CO_2$ were quantified by scintillation counting.

3.3.4 Partial MnP purification and characterisation

For the characterisation of MnP in *H. annosum*, a homokaryotic strain (IS-group P, strain 94117) was grown for 24 days on sterile spruce wood chips at 23°C. About 20g (dw) of chips were extracted by 100ml 25mM Na-acetate buffer, pH 5.5. MnP activity of the extract was 1.02 µkat/l as measured by Mn^{2+}-oxidation method using malonate as a Mn-chelating buffer. Formation of Mn^{3+}-malonate complex was followed at 270nm (Glenn *et al.*, 1986; Wariishi *et al.*, 1992). The extract was concentrated approx. ten-fold by ultrafiltration (10.000d cut-off, Amicon).

The concentrate was loaded onto an anion exchange column (Sepharose Q, Pharmacia) using a fast performance liquid chromatography apparatus (Pharmacia). Proteins were eluted in the linear gradient of 0-0.5 M NaCl in 25mM Na-acetate buffer, pH 5.5 (elution volume 350ml). Fractions containing MnP activity were pooled. The sample was concentrated and desalted by ultrafiltration. Pooled MnP from Sepharose Q was loaded to a Mono Q column (Pharmacia) and eluted in a linear gradient of 0-0.6 M NaCl in 25mM Na-acetate buffer, pH 5.5 (elution volume 30ml). Fractions containing MnP activity were pooled. Pooled MnP was concentrated and desalted by ultrafiltration, and stored at -20°C until analysed.

IEF was prepared as described in Vares *et al*. (1995). MnP activity in the gel was visualised using phenol red as a substrate in the presence of H_2O_2 and $MnSO_4$ (Pease *et al*., 1991).

4 RESULTS AND DISCUSSION

4.1 CELLULOSE DEGRADATION (I, II)

4.1.1 Cellulases produced in carboxymethyl cellulose (CMC) -containing media

When CMC-agar plates were used for the detection of cellulolytic activity in ectomycorrhizal fungi and in *Heterobasidion annosum* P and S types, all investigated fungi produced cellulases (I). However, ectomycorrhizal fungi grew poorly when CMC was used as a sole carbon source in cultures, which indicated that these fungi do not efficiently utilise cellulose under non-symbiotic conditions. Cellulolytic activity was concentrated beneath the older part of the colony, whereas fast-growing *H. annosum*-strains also produced cellulases at the younger mycelial margins of the colonies. These results indicate that mycorrhizal fungi are able to secrete endoglucanases, which diffuse into the growth medium and do not remain tightly bound to the cell wall, as had been previously thought to be the case (Keon *et al.*, 1987). The intensity of the clearing beneath the older part of the *Paxillus involutus* culture indicated that hyphal autolysis could also be involved in liberating cell wall-attached endoglucanases (EGs) into the culture medium (I).

The electrophoretically separated endoglucanases possessed the same mobility in the ectomycorrhizal fungi, *Amanita regalis* and *S. bovinus* (I), which co-localised with the upper EG-band observed in P- and S-strains of *H. annosum*. This indicated similarity of the secreted enzymes between the investigated fungi, when CMC was used as a sole carbon source. In *H. annosum*-isolates and in *P. involutus*, a faster migrating EG-band was also observed. This activity band could be another EG isozyme or a product of partial proteolysis. In gel filtration experiments, cellulases in *H. annosum* also appear as two broad peaks (Ahlgren & Eriksson, 1967).

Cellulose-binding affinities of the proteins secreted to the culture media by ectomycorrhizal fungi and P- and S-strains of *H. annosum* were investigated by gel blotting onto filter paper (Montgomery & Fu, 1988). Several cellulose-binding proteins (CBPs) were detected in P- and S-strains, but they did not exactly correspond with the detected EG-activity bands observed in the CMC-containing gels (I). No CBPs were detected in ectomycorrhizal fungi. CBDs exist in many enzymes hydrolysing cellulose and hemicelluloses, and their structure appears to be highly conserved (Gilkes *et al.*, 1991). Many cellulolytic enzymes contain unidentified non-catalytic domains, which may also be involved in cellulose binding. CBDs do not seem to be essential for enzyme function, but modulate the specific activities of the enzymes on soluble and insoluble substrates (Linder & Teeri, 1997). The CBPs identified in the culture filtrates of *H. annosum* might represent CBD-containing proteins that are not able to hydrolyse CMC, such as CBHs, ß-glucosidases, hemicellulolytic enzymes, and CDHs. The lack of the correlation between the location of CBPs and fast migrating EGs may indicate that the latter proteins are products of partial proteolysis, where the core region of the EG, containing the active site, is cleaved from its tail region including CBD (Ståhlberg

et al., 1988; Kleywegt *et al*., 1997). The catalytic and cellulose-binding domains in cellulolytic and hemicellulolytic enzymes are typically linked with a linker-part, which is susceptible to proteolysis (Van Tilbeurgh *et al*., 1986).

4.1.2 Cellulases in sawdust media

When birch sawdust was used as a carbon source, IEF-analyses revealed several similar EG-bands in P- and S-strains of *H. annosum* (II). The banding pattern between P- and S-types varied at p*I* values below 4.2. An EG isozyme with p*I* 5.9 was restricted to the S-strain of *H. annosum*. The different pattern of enzyme activity of P- and S-strains may be due to differences in the relative intensities of individual isozymes, or different degrees of glycosylation of endoglucanases. Protein glycosylation is known to lower the p*I* values of proteins and cause isozyme heterogeneity (Montenecourt, 1983; Biely & Markovic, 1988; Carder, 1989). In filamentous fungi, glycosylation of extracellular enzymes is a typical feature (Merivuori *et al*., 1984), and four of the five EG-isoenzymes produced *by P. chrysosporium* are glycosylated (Eriksson & Pettersson, 1975). In type P, p*I* values for predominant EGs were 4.2, 3.5 and 3.4, whereas in type S bands with p*I* 4.2, 4.0, 3.8, and 3.4 were the strongest. The constitutively expressed EG-bands in a 1% glucose medium in the S-strain had p*I* values approximately 4.3, 4.0, 3.8 and 3.5. The intensity of these induced bands was higher when birch sawdust cultures were analysed. The results of the present study agree well with those of earlier studies, where p*I* values for *H. annosum* cellulases produced in spruce wood powder medium were reported to be between 3.9-4.2 (Ahlgren & Eriksson, 1967). In *P. chrysosporium*, EGs with p*I*s of 5.7, 4.9 and 4.2 have been characterised (Deshpande *et al*., 1978; Uzcategui *et al.*, 1991b). In *P. chrysosporium* the lowest molecular weight EG, 36 kDa with a p*I* of 5.7, is suggested to represent a product of partial proteolysis (Uzcategui *et al*., 1991b). The detected EG-activity band with a p*I* of 5.9 in the S-strain might have appeared as a result of partial proteolysis as suggested in association for the band with the fastest mobility both in the P- and S-strains with native gel electrophoresis (I). After enzyme purification from *P. chrysosporium*, some CBH-type activity was found in two EGs, whereas an EG with a p*I* of 4.2 resemble *T. reesei* EG2 without any CBH-type activities (Uzcategui *et al*., 1991b).

Fungal endoglucanases rapidly decrease the DP of the cellulose, resulting in a loss of strength of the cellulose fibres in kraft pulp (Pere *et al*., 1995). The low constitutive EG-activity, detected in ectomycorrhizal fungi in the present study, may function in loosening the structure of extracellular material in between the plant root cells, and thus may facilitate the formation of the Hartig net in the intercellular space. The cellulolytic enzyme activity appears to be able to function even in the presence of a high sugar content. As in *S. bovinus* and *P. involutus*, in *Pisolithus tinctorius*, an ectomycorrhizal fungus, cellulolytic activity has been detected (Cao & Crawford, 1993a,b). Interestingly, in senescing mycorrhizas, intracellular penetration has been found in dead root cap cells in *Amanita muscaria-Picea abies* associations (Kottke & Oberwinkler, 1986), which also indicates that ectomycorrhizal fungi are able to produce cellulolytic enzymes.

4.1.3 ß-glucosidase

Low ß-glucosidase activity was observed in the culture filtrates of the P- and S-strains of *H. annosum* in the present study (II), but this activity was not studied further. ß-glucosidase activity in *H. annosum* has been shown to be either intra- or extracellular depending on the culture conditions (Norkrans, 1957; Ahlgren & Eriksson, 1967; Hüttermann & Volger, 1973a). This agrees with studies in other basidiomycete and deuteromycete fungi (Deshpande *et al*., 1978; Sprey, 1986; Gallagher & Evans, 1990; Micales, 1991; Cai *et al*., 1999). *P. chrysosporium* has multiple ß-glucosidases (Deshpande *et al*., 1978, Smith & Gold, 1979), whereas in *T. reesei* there is only one isoenzyme (Chirico & Brown, 1987). The enzymes are large as compared to polymer-hydrolysing enzymes, the sizes vary between 165-182 kDa (Eriksson *et al*., 1990). An extracellular enzyme of 114 kDa was found in the medium of *P. chrysosporium* (Lymar *et al*., 1995). Recently a putative CBD-containing ß-glucosidase gene was isolated from *P. chrysosporium* (Li & Renganathan, 1998). Separate catalytic and cellulose-binding domains were also demonstrated.

4.1.4 Regulation of cellulases

Information on the regulation of fungal cellulase genes mainly comes from studies of *T. reesei* cellulases, but according to structural similarities and enzymatic investigations, many regulatory aspects seem to be common to cellulases from different fungi. Generally the fungal cellulolytic genes are regulated at the transcriptional level: cellulose acts as an inducer, and glucose as a repressor, so separate induction and repression mechanisms control the expression of cellulolytic genes (Tomme *et al*., 1995). Cellulases seem to be trancriptionally regulated in *A. bisporus* (Chow *et al*., 1994; Yagüe *et al*., 1997) and *P. chrysosporium* (Covert *et al*., 1992b; Vanden Wymelenberg *et al*., 1993; Sims *et al*., 1994; Birch *et al*., 1995). In *H. annosum* or ectomycorrhizal fungi no cellulolytic genes have yet been isolated.

In the ectomycorrhizal fungi, *Suillus bovinus* and *P. involutus*, endoglucanase activity was observed in liquid CMC-cultures to which high levels (1% w/v) of glucose were added (I). Thus, cellulolytic activity in these fungi appears to be constitutive, whereas in *H. annosum* repression of endoglucanase activity by 1% glucose led to low constitutive EG-activity both in P- and S-strains (I, II). In *Ischnoderma resinosum* some constitutive EG-activity has been observed (Sutherland, 1986), whereas in *Ceriporiopsis subvermispora* negligible amounts of EGs were produced in the presence of 1% glucose (Sethuraman *et al*., 1998). In *Phanerochaete chrysosporium* (Eriksson & Hamp, 1978) and *Trichoderma reesei* (El-Gogary *et al*., 1989), no endoglucanase activity is detected in the presence of glucose. However, when *T. reesei* was allowed to consume glucose, cellulase expression signals were detected (Ilmén *et al*., 1997). This derepression is possibly a consequence of a formation of an inducing compound, such as sophorose, from glucose (Saloheimo *et al*., 1998). Sophorose, a ß-1,2-linked disaccharide of glucose, induces in *T. viride* strong cellulase expression that is not detected in *P. chrysosporium* (Eriksson & Hamp, 1978). It might be that *P. chrysosporium* cellulases lack the transglycosylation capability that is suggested to

cause natural formation of sophorose from cello-oligosaccharides (Vaheri *et al*., 1979; Claeyssens *et al*., 1990). In addition to *P. chrysosporium*, the effects of sophorose on cellulase induction appears to have been studied only in *Schizophyllum commune*, where sophorose was found not to induce cellulolytic enzyme production (Rho *et al*., 1982). In fungi such as *T. reesei* this system under natural conditions could operate as a way of preventing the constitutive expression of the hydrolase genes but still allow for their rapid induction as soon as the enzymes are required (Saloheimo *et al*., 1998).

A CREA-type glucose repressor gene has been identified, the product of which seems to bind with the promoters of glucosyl hydrolase genes in *T. reesei* and *Aspergillus*-species (van der Veen *et al*., 1994; Ilmén *et al*., 1996; Lee *et al*., 1996). A functionally related CRE1 -repressor gene has been characterised in a basidiomycete *Sclerotinia sclerotiorum* (Vautard *et al*., 1999). A low constitutive CBH-activity has been suggested to be important in the formation of an inducing, low-molecular weight compound (El-Gogary *et al*., 1989). Genes encoding activators responsible for the induction of cellulases in *T. reesei* have been found (Saloheimo *et al*., 1998). In *Aspergillus niger*, CBH-encoding genes require xylose and a xylanolytic transcriptional activator XlnR for their expression (Gielkens *et al*., 1999; see also chapter 4.2.3). Pure xylan also induced EG activity in *H. annosum* (II), thus resembling cellulolytic enzyme regulation in *P. chrysosporium*. A single-copy *cbh*II gene expression in *P. chrysosporium* genome was induced in the presence of xylan, so the gene might be co-regulated with the xylanolytic enzyme system (Tempelaars *et al*., 1994).

In fungi colonising wood, production of cellulases may be regulated by factors other than induction and repression by sugars. The effect of various phenols has been studied extensively (Varadi, 1972, Ander & Eriksson, 1976b, Müller *et al*., 1988; Highley & Micales, 1990; Rogalski *et al*., 1993). Repression of cellulases by various phenols has been demonstrated in *Schizophyllum commune* and *Chaetomium globosum* (Varadi, 1972), but low concentrations of phenols have induced cellulase production in fungi such as *Phlebia radiata*, *Kuehneromyces mutabilis*, *Trametes gibbosa* and *T. versicolor* (Müller *et al.*, 1988) and in brown rot fungi *Postia placenta* and *Gloeophyllum trabeum* (Highley & Micales, 1989). In a pleiotropic phenoloxidase-less mutant of *P. chrysosporium* phenols have been shown to repress endoglucanase production drastically (Ander & Eriksson, 1976). It was assumed that in the mutant lacking phenol-polymerising activity free phenols repressed cellulolytic enzyme production.

In plant-fungus associations that involve attack into living tissues, e.g. *H. annosum*-infections (III), cellulase production may be activated by the presence of phenolics. *H. annosum* appears to be able to detoxify free, growth-restricting phenolics, produced by the plant, into neutral compounds by glycosylation into phenolic glucosides (Stenlid & Johansson, 1987; Asiegbu *et al*., 1993). *H. annosum* is an active producer of laccase, which polymerises phenolics as a detoxification mechanism, and together with CDH may increase the cellulolytic efficiency in this fungus, similarly to that in *T. versicolor*. In *T. versicolor* lignin-related phenolics may indirectly stimulate cellulase production in the fungus (Müller *et al*., 1988). White rot fungi were suggested to produce laccase that oxidise phenolics into their corresponding quinones, which are reduced back to phenols by CDH using cellobiose as reductant. CDH oxidises cellobiose to cellobionolactone which works as a powerful

cellulase inducer (Bruchmann *et al*., 1987). Cellobiose oxidation by CDH relieves CBH I from product inhibition, thus enhancing cellulose degradation in *P. chrysosporium* (Igarashi *et al*., 1998). CDH has been characterised in cellobiose-grown cultures of *H. annosum* (Hüttermann & Noelle, 1982).

Although CDH has been characterised in several fungi, limited information on CDH production is available for ectomycorrhiza-forming fungi. Ericoid mycorrhizal fungi and ectomycorrhizal fungi have been reported to produce carbohydrate oxidases (Burke & Cairney, 1998), and such activity may increase cellulolytic activity. On the other hand, these fungi appear to also produce glucose oxidases in axenic cultures (Burke & Cairney, 1998). The oxidation of glucose by glucose-2-oxidase (pyranose oxidase) will produce gluconolactone (Eriksson *et al*., 1990; Daniel *et al*., 1992, 1994) that might repress the total cellulolytic efficiency. Gluconolactone is a strong ß-glucosidase inhibitor. On the other hand, by producing H_2O_2, glucose oxidase activity may contribute *via* the Fenton reaction the lignocellulosic oxidation (Evans *et al*., 1991; Daniel *et al*., 1994; Cairney & Burke, 1998). The temporal and spatial production of these enzymes in functional mycorrhiza requires further investigation *in situ*. Molecular biological methods have become available for the analysis of the gene expression also in mycorrhizal associations (Tagu *et al*., 1993). The significance of the cellulolytic enzyme system in the pathogenesis process has been studied in *Ustilago maydis*, a dimorphic basidiomycete. Endoglucanase gene *egl*1 is not expressed in haploid, non-pathogenic cells, but highly induced in pathogenic filamentous cells. However, a filamentous mutant lacking *egl*1 remained equally pathogenic, indicating that this endoglucanase did not affect the pathogenic behaviour of *U. maydis* (Schauwecker *et al*., 1995).

4.1.5 Immunodetection of cellulolytic enzymes in *H. annosum*

a. Endoglucanases

In order to further characterise cellulolytic enzymes in *H. annosum* culture filtrates and to determine their molecular weights, polyclonal antibody raised against *T. reesei* EG I was initially utilised (Table IV). Antibody raised against *T. reesei* EG I recognised at least three proteins with molecular weights 42, 55 and 62 kDa in culture filtrates of P- and S-strains (Fig. 8A). Typical fungal EGs have molecular weights ranging from 25 to 67 kDa. In *P. chrysosporium* the molecular weight varies between 36 and 44 kDa (Uzcategui *et al*., 1991b). A band at 55 kDa was only observed with the EG-antibody in P- and S-strains of *H. annosum*, but two other bands (42 and 62 kDa) were also observed with the mannanase antibody in SDS-PAGE blots. A band at 42 kDa was weakly labelled in the S-strain. Obviously this band corresponds to a strongly labelled band in the P-strain in IEF-blots at a p*I* of 4.2, which co-localised with the EG-activity bands in the P- and S-strains (Fig. 8B). Two similar proteins were labelled in P- and S-strains with p*I*s of 3.5 and 3.4, and they co-localised with the strong EG-activity bands in the P-strain. The use of EG I antibody revealed positive labelling of the cell wall extract, indicating that a portion of these proteins remains attached to the cell wall in

H. annosum. Attachment of endoglucanases and CBHs to the cell wall has recently been observed in *Volvariella volvacea* (Cai *et al.*, 1999).

Further characterisation of *H. annosum* endoglucanases clearly requires further purification of the proteins, gene cloning and sequence homology analyses. No endoglucanase genes have been cloned from white rot basidiomycetes. The serological similarity of *T. reesei* cellulolytic enzymes with *H. annosum* endoglucanases and cellobiohydrolases suggest that the cloned *T. reesei* genes could be used as probes to identify the cellulolytic genes in *H. annosum*. In *P. chrysosporium* the *cbh*1-like genes have been suggested to encode proteins with endoglucanase activity (Sims *et al.*, 1994). A cellulolytic protein CEL1 from *A. bisporus* appears to be different from the known EGs, CBHs, or ß-glucosidases (Raguz *et al.*, 1992; Armesilla *et al.*, 1994). However, a novel type endoglucanase from *T. reesei* has been characterised (Saloheimo *et al.*, 1997), which shows a high homology to CEL1, indicating that the CEL1 encodes an endoglucanase in *A. bisporus*.

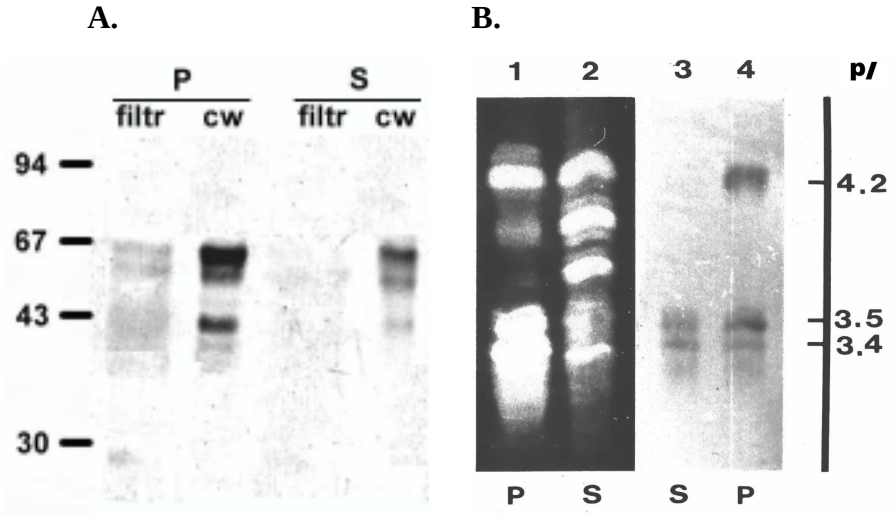

Fig. 8. Immunoblotting of endoglucanases. **A.** Proteins from culture filtrate (filtr) and washed mycelium (cw) were separated by SDS-PAGE, blotted on nitrocellulose, and allowed to react with *T. reesei* EG I antibody. *H. annosum* P- and S-strain samples were from 28d-old liquid cultures with birch sawdust used as a carbon source. **B.** Proteins separated by IEF. EG-activity visualised using HEC-containing agar replicas (lanes 1 and 2) (II), and immunodetection of endoglucanases from the same gel (lanes 3 and 4). Samples from P- and S-strain were at 28-36d cultivation on birch sawdust.

Table IV. Immunodetection of cellulolytic and hemicellulolytic enzymes in the culture filtrates of P- and S-strains of *Heterobasidion annosum*.

Polyclonal antibodies used in the study					*H. annosum* P		*H. annosum* S	
Enzyme	**origin**	**MW**	**p*I***	**Ref.**	**MW**	**p*I***	**MW**	**p*I***
EG I	*T. reesei*	43	4.0	2	42, 55, 62	4.2, 3.5, 3.4	42, 55, 62	3.5, 3.4, (3.2)
CBH I	*T. reesei*	65	4.2-3.6	1	(46), 49, 56, 63, 69	ND*	46, 49, 56, 63, 69	ND
CBH II	*T. reesei*	58	6.3	3	46, 49, 56, 63	ND	46, 49, 56, 63	ND
XYL[a]	*T. reesei*	20	9.0	4	~20	ND	~20	ND
MAN[b]	*T. reesei*	53	5.4	5	42, 60, 62	3.5, 3.4	(42),60, 62	3.5, 3.4

[a]xylanase, [b]mannanase. 1. Nummi *et al.*, 1983, 2. Niku-Paavola *et al.*, 1985, 3. Niku-Paavola *et al.*, 1986, 4. Tenkanen *et al.*, 1992, 5. Ståhlbrand *et al.*, 1993. *ND: not determined.

b. Cellobiohydrolases (CBHs)

Polyclonal antibodies produced against *T. reesei* CBH I and CBH II recognised several proteins in birch sawdust culture filtrates, in which *H. annosum* had grown for 23 days (Fig. 9A,B; Table IV). The polyclonal antibody against CBH I recognised proteins at 46, 49, 56, 63, and 69 kDa in S-strain of *H. annosum*. In the P-strain the 46 kDa protein was only weakly labelled, but the same bands were visualised. Bands with the same molecular weights were observed with the CBH II antibody, except for the 69 kDa protein (Fig. 9B, Table II). Some labelling was observed in proteins that were attached to the sample wells and were not eluted into the gel. Aggregate formation in cellulolytic proteins of *H. annosum* cultures could explain the incomplete elution. For example, in *T. reesei*, EG I has been observed to be able to form aggregates, especially at a pH above 5.5 (Dominguez *et al.*, 1992).

The molecular structure and the catalytic action of *T. reesei* CBHs is well characterised (Divne *et al.*, 1994; Teeri, 1997; Divne *et al.*, 1998). The molecular weights of fungal CBHs typically range from 45 to 65 kDa (Wood, 1992). In *T. reesei*, the reported molecular weight of CBH I varies between 42 and 71 kDa, and that of CBH II between 53 and 58 kDa (Salovuori, 1987). The 69 kDa protein may represent a cellobiohydrolase in P- and S-strains that bears similar epitopes as *T. reesei* CBH I. Other recognised proteins have similar epitopes with both cellobiohydrolases of *T. reesei*. The different banding pattern with EG I, CBHs and hemicellulases indicates that a cellobiohydrolase-type protein is present in *H. annosum*. Multiple bands observed with EG I, CBH I, and CBH II-antibodies may indicate the heterogeneity of the enzymes, or non-specificity in the utilised polyclonal antibodies. Unfortunately, in cellulolytic enzymes, especially the well-conserved CBD-regions form strong antigenic epitopes, and polyclonal antibodies are mainly directed against these epitopes (Aho &

Paloheimo, 1990; Aho *et al*., 1991). Similarities with the glycosylation pattern in different cellulolytic enzymes may also cause cross-reactions with the polyclonal antibodies raised against them.

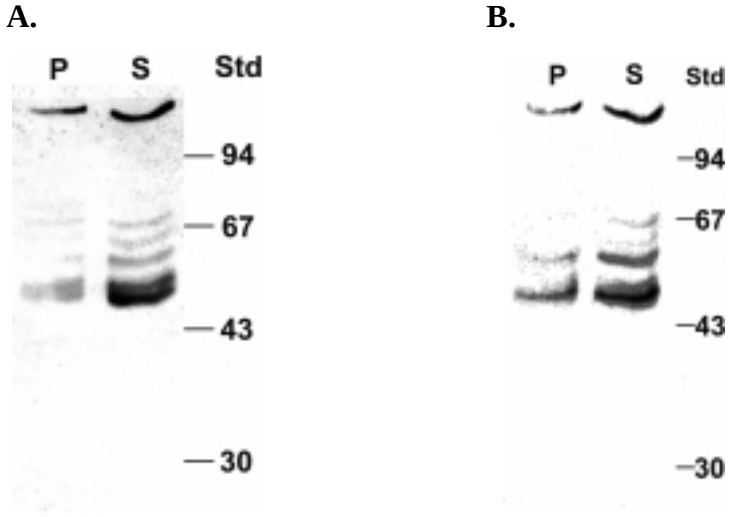

Fig. 9. Immunoblotting of cellobiohydrolases. Extracellular proteins were separated using SDS-PAGE, blotted onto nitrocellulose, and allowed to react with *T. reesei* CBH I -antibody (**A**) or CBH II -antibody (**B**). P- and S-strains of *H. annosum* were grown for 23d on birch sawdust.

In *P. chrysosporium*, three CBHs have been described (Uzcategui *et al.*, 1991a). The dominating CBH and one of the low-activity CBHs resemble *T. reesei* CBH I, whereas the other low-activity CBH appears to be homologous to CBH II of *T. reesei*. In another white rot fungus, *C. subvermispora*, CBH-activity was not detected in various liquid culture conditions (Sethuraman *et al*., 1998). On the basis of structural similarities of cellulase genes, several *T. reesei* CBH-like gene clones have been identified in *P. chrysosporium* (Sims *et al*., 1988; Covert *et al*., 1992a, 1992b; Tempelaars *et al*., 1994). In *Agaricus bisporus*, three cellulase genes have also been isolated, one of which is similar to *T. reesei cbh*1 (Raguz *et al*., 1992; Armesilla *et al*., 1994; Chow *et al*., 1994; Yagüe *et al*., 1997).

4.2 HEMICELLULOSE DEGRADATION (II)

Hemicellulolytic enzymes were characterised in the P- and S-type of *H. annosum* in cultures supplemented with glucose or birch or pine sawdust (II). Hemicellulolytic activities were stronger than endoglucanase activity in the cultures grown with birch sawdust, which may enable the fungus to remove hemicellulose and lignin effectively from the wood substrate without causing significant destruction of cellulose (Hartig, 1878; Blanchette, 1984b, 1991). The results agree well with previous reports on enzymatic activities in white-rot basidiomycetes such as *I. resinosum* (Sutherland,

1986), *Phlebia tremellosa* (Reid & Deschamps, 1991), and *C. subvermispora* (Sethuraman *et al*., 1998), in which the selective removal of lignin is facilitated by hemicellulases.

4.2.1 Xylan degradation

Endo-1,4-ß-xylanase activity occurred in both P- and S-strains of *H. annosum*. The high induction of xylanolytic enzymes was observed in media supplemented with pure beechwood xylan in the P-type, although the fungal growth was poor. The S-type strains appeared to be less able to utilise pure xylan than the P-strain, although levels of xylanase similar to the P-strain were detected in the birch sawdust medium. The function of xylanolytic enzyme system in the S-strain was confirmed by analysing the birch glucuronoxylan hydrolysis products from culture filtrate samples (II, Table V).

Table V. Hydrolysis products of birch glucuronoxylan after incubation with *H. annsoum* S-type culture filtrates of birch sawdust, pine sawdust, or beechwood xylan after 20h of incubation.

	hydrolysis products (mg ml^{-1})				
	X	**X_2**	**X_3**	**X_4**	**X_5**
birch sawdust	0.06	0.35	0.08	0.03	0.01
pine sawdust	0.12	0.23	0.12	≤0.01	≤0.01
beechwood xylan	0.04	0.33	0.11	0.02	≤0.01

X=xylose; X_2= xylobiose; X_3=xylotriose; X_4=xylotetraose; X_5= xylopentaose

In western blotting experiments of *H. annosum* culture filtrate using a polyclonal antibody against *T. reesei* endoxylanase, a positive signal was obtained with a molecular weight around 20 kDa (Fig. 10), which is the typical size of xylanases in many white-rot fungi. Endoxylanase molecular weights from 16 to 75 kDa have been reported. The enzymes also show wide variation in p*I* values (Coughlan *et al*, 1993). *Trichoderma* and *Aspergillus*-species are the most studied fungi, but xylanases have also been characterised in white-rot basidiomycetes, such as *Irpex lacteus* (Hoebler & Brillouet, 1984; Kanda *et al*., 1985), *Lentinula edodes* (Mishra *et al*., 1990), *P. chrysosporium* (Dobozi *et al*., 1992; Copa-Patiño *et al*., 1993), *S. commune* (Jurasek & Paice, 1988), *T. versicolor* (Highley, 1976) and *Stereum sanguinolentum* (Eriksson & Pettersson, 1971). Xylanase activity has also been identified from *G. trabeum*, a brown-rot fungus (Ritschkoff *et al*., 1994). Xylanases in *T. reesei* (Törrönen *et al*., 1994) and in *A. bisporus* (De Groot *et al*., 1998) belong to enzymes that lack a recognisable CBD, which means that they do not bind strongly to xylan, mannan, or cellulose (Tenkanen *et al*., 1995), whereas a xylanase from *Fusarium oxysporum* has a CBD (Christakopoulos *et al*., 1996).

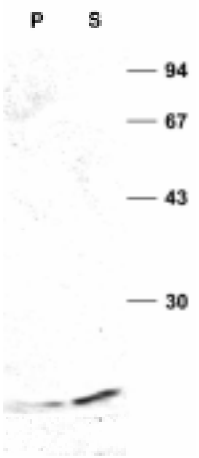

Fig. 10. Immunoblotting of xylanases. Extracellular proteins were separated by SDS-PAGE, blotted onto nitrocellulose, and allowed to react with *T. reesei* xylanase antibody (Table IV). Samples from P- and S-strains were at 28-36d cultivation on birch sawdust.

Endoxylanases induced by beechwood xylan and birch sawdust cultures were analyzed using IEF (II). Xylanolytic activity occurred in the acid region at p*I* 4.4 and 4.1 in the P-strain, and at 5.2 and 4.4 in the S-strain. In beechwood xylan medium, a basic endoxylanase with a p*I* 8.4 was found in the P-, but not the S-strain. The xylanolytic activity at the alkaline pH-region obviously exhibits specific xylanolytic activity, as no endoglucanase activity was detected in that pH region. In *T. reesei*, an alkaline, small molecular weight xylanase has been reported to be induced specifically by xylan oligomers (Herzog *et al*., 1992). In *Trichoderma*-species, only two of the xylanolytic enzymes have been reported to be specific xylanases, while the others are nonspecific endoglucanases (Kolarova & Farkas, 1983; Biely & Markovic, 1988; Claeyssens *et al*., 1990; Tenkanen *et al*., 1992). As both xylan and HEC hydrolysis was identified at acidic p*I* regions, similar non-specificity among *H. annosum* xylanases may be possible. Further purification of the xylanolytic enzyme complex in *H. annosum* is required to clarify the xylanase specificities. In a previous study, two xylanase peaks were separated following gel filtration of *H. annosum* spruce wood powder culture filtrates, and the p*I* values were determined between 4.1-4.6 and around 7.0 (Ahlgren & Eriksson, 1967). The results obtained in the present study agree well with these values.

Xylan substituent-hydrolysing enzyme activities of basidiomycetes have been poorly described. Two types of esterases can be distinguished: non-specific acetyl esterases (E.C. 3.1.1.6) and acetylxylan esterases (AXE). The acetyl esterases release acetate from acetylated xylo-oligomers, acetylxylobiose being the optimum substrate. AXE liberates acetic acid substituents directly from the polymeric xylan. Xylan hydrolysis products of culture filtrates from birch and pine sawdust and xylan media (Table V) indicate complete xylan hydrolysis and the presence of specific AXE-activity in the S-type of *H. annosum*. In *H. annosum* P- and S-strains culture filtrates from beechwood xylan medium, low amounts of AXE-activity was found in the present study. These activities were not studied further.

AXE has been reported from *S. commune* (Biely *et al*., 1985). This enzyme releases acetate from polymeric xylan, but prefers xylo-oligomers as a substrate (Biely *et al*., 1988). In *T. reesei* two AXEs are relatively non-specific, 34 kDa glycoproteins with neutral p*I*s (Sundberg & Poutanen, 1991). They seem to be the products of a

single gene (Margolles-Clark *et al*., 1996), whereas in *Penicillium purpurogenum* two AXE-genes have been characterised (Egana *et al*., 1996).

Similarly, low amounts of ß-xylosidase needed for the hydrolysis of xylo-oligosaccharides to xylose was found in P- and S-strains. ß-xylosidase is analogous to ß-glucosidase in cellulose hydrolysis and is required for complete hydrolysis of wood xylan. The formation of xylose from birch glucuronoxylan in the hydrolysis experiments (II) also points to ß-xylosidase activity, although the main product was xylobiose. Small quantitative differences in the hydrolysis products between the three different culture filtrates analysed may be due to differences in the composition of xylanolytic enzymes induced by the carbon source. ß-xylosidase may be extracellular or cell-bound depending on the organism and culture conditions (Wong *et al*., 1988; Eriksson *et al*., 1990). At least some ß-xylosidases are unable to hydrolyse substituted xylo-oligosaccharides as substrates. In *P. chrysosporium* a ß-xylosidase-type enzyme was characterised, which has activity against xylobiose, but also against cellobiose and laminaribiose ((1→3)-ß-D-glucan) (Copa-Patiño & Broda, 1994).

4.2.2 Mannan degradation

In the present study, mannanase activity was found in P- and S-strains of *H. annosum* (II). Generally mannanase expression in *H. annosum* was relatively high when compared to xylanase or other hemicellulase activities. Similar relationships between mannanase and other hemicellulases have been observed in *Phlebia radiata* (Rogalski *et al*., 1993) and *C. subvermispora* (Sethuraman *et al*., 1998). This is in sharp contrast to low mannanase and high xylanase activities occurring in fungi, such as *S. commune* (Haltrich & Steiner, 1994) and *G. trabeum* (Ritschkoff *et al*., 1994). Endomannanase activity has been investigated in white-rot fungi, including *Irpex lacteus*, *Haematostereum* (= *Stereum*) *sanguinolentum* and *Trametes versicolor* (Johnson, 1990; Johnson & Ross, 1990; Eriksson *et al*., 1990). More detailed studies have been conducted with nonwood decay ascomycetes, such as *Sporotrichum cellulophilum* (Araujo & Ward, 1991), *Thielavia terrestris* (Araujo & Ward, 1990), different *Trichoderma*-species (Torrie *et al*., 1990; Arisan-Atac *et al*., 1993; Ståhlbrand *et al*., 1993, 1995), different *Aspergillus*-species (Christgau *et al*., 1994; Ademark *et al*., 1998) and *Sclerotium rolfsii* (Gübitz *et al*., 1996). Mannanases are usually produced as multiple isoenzymes, with acidic pH optima and p*I* values. It is not known, whether they are produced as a result of multiple structural genes. Molecular biological studies concerning basidiomycete mannanases have not been reported. An endomannanase gene has been cloned from *T. reesei* (Ståhlbrand *et al*., 1995) that shows considerable homology to *T. reesei* cellulases, and contains a CBD. An *Aspergillus aculateus* mannanase gene with CBD was distinguished (Christgau *et al*., 1994).

In the immunoblotting experiments the polyclonal mannanase antibody recognised three bands at 62, 60 and 42 kDa in the washed mycelium of the P-strain. The same bands were weakly labelled in the culture filtrate (Fig. 11). The labelling with the mannanase antibody in the S-strain was weaker than in the P-strain. The band at 42 kDa was not detected in the S-strain. The 60 kDa protein appeared to be specific for the mannanase antibody labelling, but the other two bands in the P-strain were also

labelled with the polyclonal EG-antibody (Fig. 8). In this strain, two other bands at about 80 and 110 kDa were detected in the washed mycelium sample, which could represent dimerized forms of the mannanases or unspecific labelling. In IEF-experiments, strong mannanase activity bands in the P-strain at p*I* of 3.5 and 3.4 co-localised with the mannanase antibody labelling. These bands were also labelled in the S-strain, although only weak activity was seen in the IEF-gel at that pH-region. In the S-strain, no co-localised labelling with the mannanase antibody of the strong mannanase activity band at p*I* 4.2 occurred, but weak labelling was observed in the P-strain, although no mannanase activity was co-localised with the labelled region. The combined data obtained in this study involving activity staining of mannanase and EG and the immunoblotting results indicate that the band labelled at p*I* 4.2 in the P-strain with both the EG and mannanase antibodies represents a 42 kDa endoglucanase. It appears to have some similarities with the mannanase, which is seen as a weak positive signal in the IEF-blots. The mannanases and EGs in the S-strain appear to have less similarities with the *T. reesei* enzymes based on the results of the immunoblotting experiments.

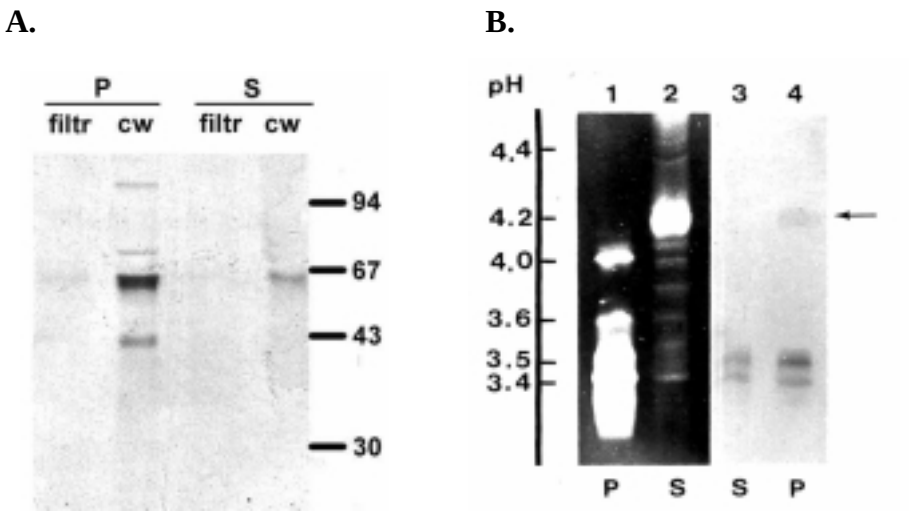

Fig. 11. Immunoblotting of mannanases. **A.** Proteins from culture filtrates (filtr) and washed mycelium (cw) were separated by SDS-PAGE, blotted onto nitrocellulose, and allowed to react with *T. reesei* mannanase antibody. *H. annosum* P- and S-strain samples as in Fig. 8. **B.** Proteins separated by IEF. Mannanase activity visualised using mannan agar replicas (lanes 1-2) and immunodetection of mannanases (lanes 3-4) from the same gel. Weak labeling of one isozyme is indicated by an arrow.

Only negligible amounts of mannanase was found in the presence of 1% glucose, whereas in the P-type only slight repression was noticed, which indicate that mannanase was repressed by glucose in both strains studied, but differently in the P- and S-strains. In the S-strain the repressive effect of glucose on the whole mannolytic machinery appeared to be stronger than in the P-type. Moreover, α-galactosidase activity was not measurable in the S-type in the presence of glucose (II).

Interestingly, in the early stage of growth during the first seven days, mannanases with more basic p*I* values (3.8-6.2) were noticed in the P-strain. These bands were not detected in the S-strain during the first week of cultivation (II). Mannanase bands with p*I* value 4.0-6.2 were replaced by mannanases with p*I* below 4.0 after four weeks of cultivation. In the S-strain mannanase isoenzymes that appeared after four weeks of cultivation had p*I* values comparable to those that appeared in the P-strain during the first week cultivation (II). The presence of mannanase isoenzymes in the P-strain during the primary growth phase might reflect functional differences between P- and S-type during primary colonisation of the plant.

Galactoglucomannan hydrolysis by endomannanase has been shown to depend on the degree of substitution and distribution of the substituents (McCleary, 1991; Tenkanen *et al*., 1997). The *P. purpurogenum* endomannanase was shown to be specific for the cleavage of the glucomannan backbone between mannose-ß-1,4-mannose and mannose-ß-1,4-glucose residues, but not glucose-ß-1,4-mannose residues (Kusakabe *et al*., 1988). Whether this type of specificity is similar in other fungi, is not known.

Of the minor side-branch activities in mannan hydrolysis, α-galactosidase activity was dominant in *H. annosum* cultures (II). α-Galactosidase has been recently characterised in *P. chrysosporium* as a 200 kDa tetramer (Brumer III *et al*., 1999), but generally this enzyme has received little attention. In *A. tamarii* the extracellular form of the enzyme was characterised as a 56 kDa glycoprotein (Civas *et al*., 1984b), and the mycelial forms were 265 and 254 kDa glycoproteins (Civas *et al*., 1984a).

4.2.3 Regulation of hemicellulases

In *H. annosum* xylanase activity is controlled by mechanisms which differ from those controlling EG and endomannanase activities. Similar regulatory functions seem to operate with many hemicellulolytic genes. All hemicellulolytic enzymes investigated appeared to be subject to glucose repression (II), but the induction patterns of hemicellulases were variable. The S-strain of *H. annosum* produced limited amounts of xylanase on pure xylan as compared to the P-strain, but on the woody substrate the xylanase activities were similar in both strains (II). However, the S-strain grown on pure xylan was still able to hydrolyse birch glucuronoxylan into monomers indicating that the complete xylanolytic machinery was also present in this medium (Table V). In *A. bisporus*, a xylanase- encoding gene *xln*A and a cellulase-encoding gene *cel*3 appear to be co-regulated (De Groot *et al*., 1998). As genes *cel*2, coding for cellobiohydrolase, and *cel*4, coding for mannanase, are also co-regulated in *A. bisporus* (Yagüe *et al*., 1997), many genes encoding for enzymes required for lignocellulose hydrolysis are highly likely to be co-regulated in *H. annosum*. Co-regulation between cellulolytic and hemicellulolytic proteins has been also proposed in *Bjerkandera adusta* (Eriksson & Goodell, 1974) and *C. subvermispora* (Sethuraman *et al*., 1998), whereas in *Sclerotium rolfsii*, no common co-ordinated regulatory mechanism was found (Sachslehner *et al*., 1998). A detailed investigation at the molecular level is required for the characterisation of common and separate regulatory systems in cellulolytic and hemicellulolytic enzymes of *H. annosum*.

4.3 SPECIAL CHARACTERISTICS OF P- AND S-TYPE OF *H. ANNOSUM*

H. annosum IS-group S is one of the most important spruce heart rot causing fungi in Europe. IS-group P infects pines, invades living roots and kills sapwood and cambium in the roots and basal portions of the trunk, resulting in death of the host. It is able to survive as vegetative mycelium in the dead roots and stumps for a long time. Growing roots are further infected through root contacts. In infected pines, the P-strain grows mainly in the cambial zone and in outer tissues of the sapwood, but never in the heartwood. The infection induces high levels of host resin production, which may eventually lead to the loss of functional tissue and the death of the tree (Stenlid & Redfern, 1998).

In most of the experiments described here both P- and S-strains were used. This allowed us to compare the two strains and to distinguish some features specific to each IS-group. The results are presented in Table VI. The utilisation of simple sugars, such as glucose and maltose, by actively growing hyphae in liquid cultures supplemented with spruce sawdust was two times faster in the S-strain. This probably results from the faster germination of the S- than the P-strain conidia at the inoculation. This result has been confirmed by other researchers (Johansson *et al.*, 1998).

Table VI. Utilisation of simple sugars from the medium supplemented with birch sawdust.

Feature	*P*	*S*
Utilisation of simple sugars from the medium* by germinating conidia	152 h (red. sugars) 150 h (glucose)	74 h (red. sugars) 63 h (glucose)

*Rate was determined as time required to diminish the amount of simple sugars by 50% as described in Materials and methods.

In birch sawdust or xylan containing liquid media a supporting carrier matrix was needed to retain growth and enzyme secretion in the S-strain at the same level as in the P-strain (II). Whether such differences in growth patterns between the two *Heterobasidion*-isolates are significant for the growth of the P- and S-types on their respective host tree species, needs to be clarified. Laccase and pectinase production and the capacity to oxidise conifer wood phenolics have been shown to be higher in the P- than in the S-strains of *H. annosum*, and these differences have been postulated to affect virulence (Johansson, 1988; Karlsson & Stenlid, 1991; Johansson *et al.*, 1998; Daniel *et al.*, 1998). Nevertheless no clear determinants for the host specificity and virulence differences between the P- and S-strains (Stenlid & Swedjemark, 1988; Lindberg, 1992) has yet been found.

4.4 LIGNIN DEGRADATION (III)

4.4.1 Manganese peroxidases (MnPs) and lignin peroxidases (LiPs)

In the present study the role of MnP in wood colonisation by *H. annosum* was investigated by utilising spruce wood shavings as a growth medium. MnP activity was observed in 24-d old solid state cultures of the homokaryotic *H. annosum* P-strain after the extraction of spruce wood chips with acetate buffer and purification of the extract with Sepharose Q and Mono Q chromatography. No LiP-activity was found. Several partially purified MnP-containing fractions were combined and analysed using IEF. Three acidic isozymes were separated with p*I*s of 3.3, 3.5, and 3.7 (Fig. 12). These values are similar compared to the other fungal MnPs (Hatakka, 1994; Table VI). In *P. chrysosporium*, ten different peroxidases can be separated by Mono Q chromatography, and they are designated from H1 to H10 (Renganathan *et al*., 1985; Kirk *et al*., 1986; Leisola *et al*., 1987). Six of these have veratryl alcohol oxidising activity, characteristic of LiP activity (Farrell *et al*., 1989). Some LiPs have been shown to possess Mn^{2+} oxidase activities, e.g. LiP H2 from *P. chrysosporium* (Khindaria *et al*., 1995a).

Fig. 12. MnP activity of the homokaryotic *H. annosum* P-strain visualised by isoelectric focusing. MnPs were partially purified from the extract of 24d old spruce wood chips culture. Isozymes were detected by enzyme activity staining using phenol red, Mn^{2+}-ions and H_2O_2.

MnPs in white rot fungi have acidic pH-optima and p*I*-values, they are glycosylated and the molecular weights are similar to LiPs. MnPs are common in delignifying fungi, and only few taxonomic fungal groups lack MnP (Hatakka, 1994). Litter-decomposing fungi, such as *A. bisporus*, also produce MnPs (Bonnen *et al*., 1994; Lankinen *et al*., 1999). Because phenolic substructures comprise only about 10% of all lignin subunits, the significance of MnP in lignin degradation has been estimated to be rather limited. However, Jensen *et al*. (1996) showed that MnP is able to oxidise nonphenolic substructures in the presence of unsaturated lipids. MnP promotes the peroxidation of unsaturated lipids in the presence of Mn(II), which results in lipoxyradicals that may

oxidise nonphenolic lignin model compounds. This system can also depolymerise synthetic phenolic and nonphenolic (phenolic groups blocked by methylation) lignins and ^{14}C-labelled wheat straw lignins (Bao *et al*., 1994; Kapich *et al*., 1999), which may indicate that similar degradation mechanism for wood lignin could operate *in vivo* (Srebotnik *et al*., 1994). Non-phenolic lignin or lignin-related compounds have also been oxidised by MnP in the presence of thiol reductants (Forrester *et al*., 1988; Wariishi *et al*., 1989).

Table VII. Isoelectric points of manganese peroxidases from selected white rot fungi.

Species	pI of MnPs detected	Reference
Agaricus bisporus	3.25, 3.3 (straw)	Lankinen *et al*., 1999
Bjerkandera adusta	3.45, 3.35	Heinfling *et al*., 1998
B. adusta	"LiP-hybrid", 3.9	Mester & Field, 1998
Ceriporiopsis subvermispora	multiple, 4.1-4.6 (liquid)	Lobos *et al*., 1994
	3.2, 3.3, 3.4, 3.5 (wood)	
Dichomitus squalens	3.9, 4.15	Périé *et al*., 1996
Lentinula edodes	3.2	Forrester *et al*., 1990
Heterobasidion annosum	**3.3, 3.5, 3.7 (wood)**	**This work**
Nematoloma frowardii	3.2 (MnP2)	Schneegaß *et al*., 1997
Panus tigrinus	2.95, 3.2	Maltseva *et al*., 1991
Phanerochaete chrysosporium	six total, 4.2-4.9	Pease & Tien, 1992; Leisola *et al*., 1987
P. sordida	3.3, 4.2, 5.3	Rüttimann-Johnson *et al*., 1994
Phlebia radiata	3.6, 3.85, 4.85 (liquid)	Moilanen *et al*., 1996
	3.3-5.9 (straw)	Vares *et al*., 1995
Pleurotus eryngii	3.65, 3.75	Martínez *et al*., 1996
P. ostreatus	3.5, 3.7, 4.3 (liquid),	Becker & Sinitsyn, 1993; Sarkar *et al*.,
	3.5, 3.7 (wood)	1997
Rigidoporus lignosus	3.5, 3.7	Galliano *et al*., 1991
Trametes versicolor	multiple, 2.9-3.2	Johansson & Nyman, 1993a
IZU-154	3.7, 4.5, 4.9, 5.1	Nishida *et al*., 1988

The reason for the multiplicity of LiP and MnP proteins is unclear, although some kinetic and substrate range differences have been observed among different isozymes. In LiPs, differences with respect to K_m and K_{cat} values for substrates and oxidants are revealed (Farrell *et al*., 1989; Glumoff *et al*., 1990). Obviously some of these differences may be due to differences in the oxidation-reduction potentials of the different isozymes (Millis *et al*., 1989), or due to slight structural variations at the enzyme active site (Sinclair *et al*., 1995). Access to the heme group might be restricted in some isoforms, due to differences in the flexibility of the surrounding protein (Glumoff *et al*., 1990). In *H. annosum* further characterisation of different isozymes is required to resolve the kinetic and substrate range differences between the identified isoforms. In the IEF-experiments the dye phenol red was used to visualise peroxidase

activity. No dye-oxidising activity was noted in any of the isoforms without exogenous manganese, which indicates that the isozymes secreted into the spruce chips by *H. annosum* P-strain are true manganese-dependent peroxidases, which is in agreement with the obtained nucleotide sequences presented later in the text (see chapter 4.4.3).

MnP oxidises Mn(II) to Mn(III). Non-chelated Mn(III) may disproportionate to Mn(II) and Mn(IV), which forms black deposits of insoluble MnO_2 in the wood The accumulation of MnO_2 is believed to be an indication of the MnP action and is possibly affected by low Mn-chelating activity of α-ketoacids present in wood (Perez & Jeffries, 1992). Black deposit formation is a characteristic feature in wood decay of several basidiomycetes, such as *C. subvermispora* and *H. annosum* (Blanchette *et al*., 1984). MnO_2 deposits can be reduced by cellobiose dehydrogenase (CDH), which prodvides further oxidative power for increased lignin degradation (Roy *et al*., 1994). In the presence of low concentrations of suitable manganese chelators, catalatic activity of MnP is observed to protect the enzyme from inactivation by H_2O_2. This activity is dependent on manganese and is inhibited by oxalate (Timofeevski & Aust, 1997; Timofeevski *et al*., 1998).

When *H. annosum* was grown in the synthetic low nitrogen and high carbon -containing liquid media, low Mn-peroxidase activity was measured. A low veratryl alcohol (VA) -oxidising activity was also noted indicating a possible LiP-activity. Exogenously added VA significantly increased laccase activity in low nitrogen and low carbon containing liquid cultures, but no LiP-activity was induced (III). On the other hand, added VA was rapidly oxidised into veratraldehyde as determined by HPLC, which suggests that some LiP activity was present in the cultures. Perhaps most of the LiP-activity remains attached to the cell wall in *H. annosum*, and is not released into to the culture medium. The presence of LiP-activity in the mycelium was not investigated further in the present study. VA has been reported to increase LiP production in *P. chrysosporium* (Faison & Kirk, 1985) and to stabilise LiP by protecting it from the inactivation by H_2O_2 (Haemmerli *et al*., 1986; Tonon & Odier, 1988; Barr & Aust, 1994). VA is a secondary metabolite known to be secreted by several white rot fungi (Lundquist & Kirk, 1978; Kawai *et al*., 1986; Harper *et al*., 1990; De Jong *et al*., 1994; Jensen *et al*., 1994), but it may also be formed as a result of degradation (Shimada *et al*., 1981). The significance of VA in charge-transfer reactions as a mediator molecule in lignin oxidation seems to be a controversial question (Harvey *et al*., 1986; Schoemaker, 1990; Chung & Aust, 1995; Khindaria *et al*., 1995b).

The presence and absence of MnP and LiP activities in *H. annosum* cultures reported here and previously (Lyr, 1958; Koenigs, 1970; Galliano *et al*., 1986; N.C. Preston & S. Woodward, Aberdeen, unpublished) could be dependent on culture conditions used. Peroxidases from several white rot fungi with unusual characteristics, that can not clearly be attributed to lignin or manganese peroxidases, have been described. These include peroxidases from *Junghuhnia separabilima* (Vares *et al*., 1992), *Dichomitus squalens*, *Pycnoporus cinnabarinus* (Nerud *et al*., 1991), *Panus tigrinus* (Golovleva *et al*., 1993) and *Inonotus weirii* (Mustranta, 1987). *Bjerkandera* sp. BOS55 produces also a manganese-inhibited peroxidase (De Jong *et al*., 1992) and a MnP-LiP "hybrid" enzyme, which oxidises veratryl alcohol and 1,4-dimethoxybenzene without manganese, and which was efficient in the oxidation of Mn(II) (Mester & Field, 1998). Also purified MnPs from *Pleurotus*-species have been

shown to oxidise veratryl alcohol at a slow rate (Martínez *et al*., 1996; Sarkar *et al*., 1997).

4.4.2 Laccase

Laccase appears to be the predominant ligninolytic enzyme in *H. annosum* (Maijala, 1996, III). Together with the data on peroxidases it is also the only ligninolytic enzyme from this species that is presently characterised (Hüttermann *et al*., 1977; Haars *et al*., 1981; Haars & Hüttermann, 1983; Johansson *et al*., 1999). In addition to basidiomycetes causing white rot, laccases are produced by several other taxonomic groups of fungi, such as soil-inhabiting ascomycetes *Chaetomium thermophilium* (Chefetz *et al*., 1998) and *Thermoascus aurantiacus* (Machuca *et al*., 1998), deuteromycetes *Penicillium chrysogenum* (Rodríguez *et al*., 1996), *Aspergillus fumigatus* (Kadam & Drew, 1986), litter-decaying basidiomycete *Marasmius quercophilus* (Tagger *et al*., 1998), *Coprinus cinereus* and other *Coprinaceae*-group species (Heinzkill *et al*., 1998; Schneider *et al*., 1999). Laccase is also described from a rice rhizosphere bacterium, *Azospirillum lipoferum* (Givaudan *et al*., 1993). In ectomycorrhiza-forming fungi *Suillus bovinus* or *Paxillus involutus* no intracellular or wall-bound laccase activity was detected in symbiosis with Scots pine (*Pinus sylvestris*) (Timonen & Sen, 1998), but the presence of laccase has been reported in other mycorrhizal fungi (Lindeberg, 1948; Giltrap, 1982; Hutchison, 1990; Günther *et al*., 1998). No laccase has been reported from brown rot fungi, although in *G. trabeum* a laccase-like gene sequence has been found (D'Souza *et al*., 1996).

Laccases appear to be encoded by complex families of structurally related genes. In fungi, laccase genes have been described in at least from 15 species, and from eight white rot basidiomycetes, numerous genes have been cloned and sequenced. Sequence similarity between different fungal laccases is low compared to similarity among the peroxidases, but the copper-binding regions crucial for enzyme function are highly conserved (D'Souza *et al*., 1996). Reports concerning intracellular forms of laccases do exist (Fåhraeus & Reinhammar, 1967; Blaich & Esser, 1975; Bollag & Leonowicz, 1984; Schlosser *et al*., 1997), but it is not known, if they represent different gene products, or intracellular forms of extracellular laccases. Intracellular laccase, in common with extracellular laccase, might function in the demethoxylation of low molecular weight phenolic products, like methoxyhydroquinone, which leads to the formation of methanol (Eriksson *et al*., 1990; Bourbonnais & Paice, 1992). Three-dimensional structure of the laccase from *Coprinus cinereus* has been recently resolved (Ducros *et al*., 1998).

In *H. annosum*, the laccase isozyme pattern varies depending on fungal isolate, substrate, and incubation period (Johansson *et al*., 1999). In many white rot fungi laccase is produced as several isozymes, which may have different substrate specificities. In S-, P-, and F-groups of *H. annosum* two main laccase isozymes exist with molecular weights 65 and 67 kDa (R. Karjalainen, Helsinki, unpublished; N.C. Preston & S. Woodward, Aberdeen, unpublished). These values are well within the range of the reported sizes (between 60 and 80 kDa) of laccases in white rot fungi.

The regulation of laccase expression varies significantly among species. Exogenously added veratryl alcohol (VA) induced laccase in synthetic liquid cultures of *H. annosum* (III), as has been shown to be the case in other white-rot fungi (Lundell *et al.*, 1990; Rogalski *et al.*, 1991; Périé *et al.*, 1998) and in an ascomycete *Botryosphaeria* sp. (Barbosa *et al.*, 1996). Some of the genes appear to be induced by various chemicals, e.g. copper (Collins & Dobson, 1997; Eggert *et al.*, 1998), others by nitrogen deficiency, whereas some appear to be constitutively expressed (Kirk & Cullen, 1998). In *H. annosum*, phenolics appear to significantly induce laccase activity (Haars *et al.*, 1981; Haars & Hüttermann, 1983). Small receptor-type phenolics-binding structures have been reported from *H. annosum* (Haars & Hüttermann, 1983).

Other low molecular weight molecules, such as 3-hydroxyanthranilate (3-HAA) have gained increasing interest as mediator compounds for the laccase-based degradation of non-phenolic substructures of lignin (Eggert *et al.*, 1996). During the last decade, both natural and synthetic molecules have received much attention as mediators of laccase action in various applications in pulp and paper manufacture (Kawai *et al.*, 1989; Bourbonnais & Paice, 1990, 1996; Call & Mücke, 1997), and in studies of organopollutant degradation (Field *et al.*, 1993; Collins *et al.*, 1996; Johannes *et al.*, 1998). One of such molecules, ABTS, has been utilised as a stain for the detection of laccase activity in wood samples degraded by white rot fungi (Niku-Paavola *et al.*, 1990). Lipid peroxidation has been proposed to be involved in laccase and a mediator -based phenanthrene oxidation (Böhmer *et al.*, 1998). Laccase and ligninolytic peroxidases are too large in size to allow entery into the lignocellulose matrix of the plant cell wall (Srebotnik *et al.*, 1988; Daniel *et al.*, 1989; Evans *et al.*, 1991). The enzyme action is thus restricted to the intimate vicinity of the outer surface of the fungal wall. However, with gradual fungal ingress, lignin removal is often detected even before extracellular enzyme arrival at the target site. This suggests that enzyme-generated low-molecular-weight molecules capable of oxidising lignin must be involved in lignin degradation. Such pattern has also been observed in *H. annosum*-degraded wood (Fig. 1, Meier, 1955; Blanchette, 1984a,b).

4.4.3 Mineralisation of synthetic lignin (DHP) by *H. annosum*

Ligninolytic activity of *H. annosum* was studied by mineralisation of DHP-lignin in the solid-state culture system, where sheets of cellulose, highly bleached wood pulp, was utilised as a growth substrate. ^{14}C-ring-labelled DHP was chosen for mineralisation experiments, because the origin of carbon dioxide, which is measured, inevitably must be from the aromatic lignin backbone indicating lignin depolymerisation. The mineralisation of the ^{14}C-ring-labelled DHP-lignin commenced after two weeks growth, but the rate remained at a low level, reaching 4% during 35 days growth (Fig. 13). In some preliminary experiments, the phenolic DHP was mineralised by *H. annosum* P-strain at over 10% efficiency (not shown). In similar studies done before, only 2% of the applied radiolabelled DHP-lignin was mineralised in 20-d old liquid cultures (Bono *et al.*, 1983; Trojanowski *et al.*, 1985; Boudet *et al.*, 1988). Solid-state pulp culture system used in the present study appear to be superior to liquid cultures in terms of ligninolysis induction in *H. annosum*. ^{14}C-labelled methoxy-groups are more

efficiently mineralised by *H. annosum*, and rates of over 13% has been reported (Trojanowski *et al.*, 1985). In the present investigation, the difference in the mineralisation rates between phenolic and non-phenolic DHP was striking under the culture conditions used. Phenolic DHP was mineralised, but highly limited

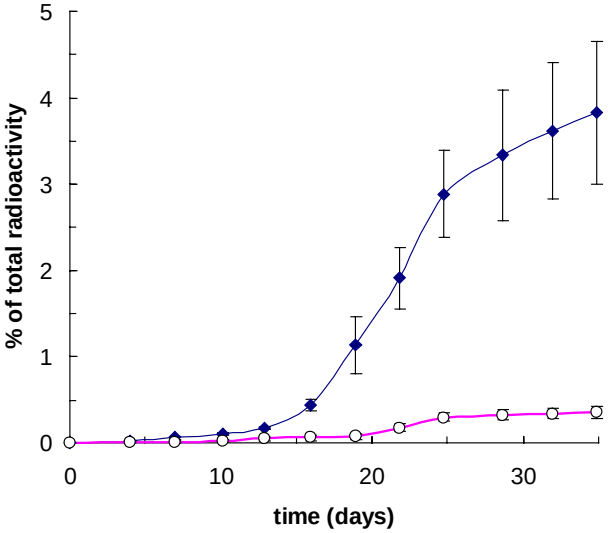

Fig. 13. DHP-lignin mineralisation by *H. annosum* P-strain in the solid-state wood pulp cultures. (♦): Phenolic, ring-labeled DHP, (○): non-phenolic (methylated), ring-labeled DHP. n=3.

mineralisation of the non-phenolic DHP was detected (Fig. 13). Non-phenolic DHP is regarded as being less amenable to oxidation (Kersten *et al.*, 1990), being oxidised by only LiP (Hammel *et al.*, 1993). If no mediating substances are present, the ability of laccase or MnP to oxidise lignin is restricted to phenolic moieties. Thus the results from the mineralisation experiment support the data obtained in the MnP sequence analyses (IV, chapter 4.4.4) which suggest that *H. annosum* and related species contain a small family of genes encoding MnPs.

In these experiments no exogenous carbon was added to the culture media. In laboratory experiments with *P. chrysosporium* and *P. radiata*, enhanced lignin degradation has been obtained by cellulose addition (Ander & Eriksson, 1976; Kirk *et al.*, 1976; Hatakka & Uusi-Rauva, 1983), whereas addition of monosaccharides like glucose repress lignin degradation in several fungi (Hatakka & Uusi-Rauva, 1983; Roch *et al.*, 1983; Reid & Deschamps, 1991). Increased degradation rates after glucose addition have, however, also been reported (Leatham, 1986).

Interestingly, a nitrogen concentration of 22mM in the medium did not repress the phenolic DHP mineralisation efficiency in the pulp culture system of *H. annosum* (data not presented), confirming the earlier findings performed in liquid cultures (Bono *et al.*, 1983; Hüttermann *et al.*, 1984). In *T. versicolor*, nitrogen limitation has been found to have only a moderate influence on lignin mineralisation, while high nitrogen concentrations (over 30mM) suppress fungal ligninolytic activity (Leatham & Kirk, 1983). The amount of added nitrogen has almost no influence on the ligninolytic capacity of *Pleurotus ostreatus*, *Lentinus edodes* (Leatham & Kirk, 1983), *D. squalens* (Périé & Gold, 1991) and *C. subvermispora* (Maijala, 1996). These fungi produce laccase and MnP, but not LiP, or the production of LiP is maintained at a very low level (Hatakka, 1994). In the presence of high nitrogen in synthetic culture conditions *L. edodes* produces laccase, but not MnP (Buswell *et al.*, 1995).

The presence of manganese can also significantly affect lignin degradation in many white rot fungi (Leatham, 1986; Boyle *et al*., 1992; Kerem & Hadar, 1995). Mn-ions are central to the function of MnP, but Mn-ions can also function as regulatory elements in lignin degradation: MnP production is regulated by metal response in *P. chrysosporium* (Brown *et al*., 1990, 1991). Mn-ion concentration also affects growth and interestingly, increase the synthetic, nonphenolic lignin mineralisation by a LiP-negative fungus *C. subvermispora* (Maijala, 1996). In *P. chrysosporium*, elevated Mn-levels in liquid medium cultures have suppressed LiP activities and lignin mineralisation rates (Bonnarme & Jeffries, 1990; Perez & Jeffries, 1992). It has been suggested that this may be due to diminished synthesis rate of veratryl alcohol (Mester *et al*., 1995). The effect of manganese on the mineralisation of DHP was not investigated in this study.

4.4.4 Molecular characteristics of *Heterobasidion* peroxidases (III, IV)

In the present work, degenerative primers were designed on the basis of available peroxidase encoding gene sequences in the databases. A 565bp fragment was successfully amplified, cloned, and sequenced from a heterokaryotic *H. annosum* P-strain (III). The fragment contains the conserved region of the enzyme that includes several important residues for the enzyme function. In further studies, when the comparison of the gene sequences of different strains of *Heterobasidion*-genera was the main objective, homokaryotic strains were preferentially used, in order to avoid problems related to allelic variation of peroxidase genes (IV). The existence of allelic variants have complicated the identification of LiP genes in *P. chrysosporium*, but analysis of single basidiospore cultures have enabled the differentiation of alleles among closely related LiP genes (Alic *et al*., 1987; Gaskell *et al*., 1992).

The genomic fragment was aligned with other fungal peroxidases, and the exon-intron structure could be predicted. The putative 131 amino acid sequence covers amino acids 66 (Gly) to 197 (Thr) out of the typical 340 amino acids that make-up a mature fungal peroxidase protein. The numbering of amino acids follow that of the MnP encoding gene product of *T. versicolor* (Johansson & Nyman, 1996). To analyse the relationships between different intersterility groups of *H. annosum* and some closely related species, the same primers were used to produce additional fragments from 106 isolates of the genus *Heterobasidion* and *Amylostereum areolatum*, *A. chailletii*, *A. ferreum*, *A. laevigatum*, *Bondarzewia montana*, *Echinodontium japonicum*, *E. tinctorium* and *E. tsugicola*. Careful analysis of the nucleotide and amino acid sequences and intron positions suggest that there are in each IS-group of *H. annosum* at least three different MnP-like genes, and four in the American *H. annosum* (P-type) and the S-type (IV). These peroxidases were designated arbitrarily as peroxidase 1a, peroxidase 1b, peroxidase 2 and peroxidase 3. In the paper IV and in the Figs. 14 and 15 the designations of the three different intersterility groups P, S and F of *H. annosum* are replaced by the current species names *H. annosum*, *H. parviporum* and *H. abietinum*, respectively (Niemelä & Korhonen, 1998). In *B. montana* and in different *Amylostereum*- and *Echinodontium*-species at least two MnP-like genes can be separated, and were designated as peroxidase A to E. Using

similar PCR-technique, LiP-like gene fragments have been found from *Phanerochaete sordida* and *C. subvermispora* genomes (Rajakumar *et al*., 1996). A cDNA clone was also amplified from *P. sordida*, but no LiP-transcripts were found from *C. subvermispora*.

Table VIII. Peroxidase sequences characterised from white rot fungi.

Species	Gene product	References
Bjerkandera adusta	LiP	Kimura *et al*., 1991
Ceriporiopsis subvermispora	LiP (PCR fragment)	Rajakumar *et al*., 1996
	MnPs	Lobos *et al*., 1998; Tello *et al*., 2000
Dichomitus squalens	MnPs	Li *et al*., 1999
Phlebia brevispora	LiP (PCR fragment)	Rajakumar *et al*., 1996
Phanerochaete chrysosporium	LiPs	Tien & Tu, 1987; Gaskell *et al*., 1994 (review)
	MnPs	Pribnow *et al*., 1989; Godfrey *et al*., 1990; Mayfield *et al*., 1994a; Alic *et al*., 1997
Phlebia radiata	LiP	Saloheimo *et al*., 1989
Pleurotus eryngii	MnPs	Ruiz-Dueñas *et al*., 1999c
Trametes versicolor	LiPs	Black & Reddy, 1991; Jönsson & Nyman, 1992, 1994; Johansson, 1994
	MnPs	Johansson & Nyman, 1996; AF102515
IZU-154	MnP	Matsubara *et al*., 1996

4.4.5 Intron positions in the MnP fragments of *H. annosum*

All the cloned and sequenced fragments contain four exons separated by three introns designated I, II and III. The position of each intron is highly conserved in all the analysed genes from different *Heterobasidion* IS-groups and the related species (IV). This may indicate functional significance of these introns; they are possibly required for the processing of primary MnP transcripts. The size of introns varies from 49 to 62 bp. In other fungi, LiP and MnP genes contain short, 50-80 nucleotide introns, positions of which are not very well conserved in different species. In the complete lignin peroxidase genes of *P. chrysosporium* the number of introns varies from eight to nine, and the positions of six introns are highly conserved (Brown *et al*., 1988; Schalch *et al*., 1989; Ritch & Gold, 1992). The overall exon-intron structure of the peroxidase-encoding fragments in all *Heterobasidion*-, *Amylostereum*-, *Bondarzewia*-, and *Echinodondium*-isolates appear to be unique among the known fungal peroxidases (IV), although the position of an individual intron was shared in several other peroxidase genes of various white rot fungi.

The coding region of MnP isozyme I in *P. chrysosporium* has elevated GC-content (68%) compared to the genome as a whole (Pribnow *et al*., 1989), whereas the GC-content in LiP-genes is similar to the genomic GC-content (Brown *et al*., 1988;

Broda *et al*., 1989). GC-values from the fragments of the peroxidases 1a, 2, and 3 cloned from *H. annosum* strain EF-2 are 53.7%, 58.0% and 53.4%, respectively (Maijala *et al*., 1998). No information exists on the genomic GC-content of *H. annosum*, but the slightly biased GC-content of the peroxidases is similar or a little lower than corresponding peroxidases reported for the highly expressed peroxidase genes of *P. chrysosporium* (Ritch *et al*., 1991; Alic *et al*., 1997) and *B. adusta* (Kimura *et al*., 1991). The cloned MnP fragments from *Heterobasidion* and other species show the closest similarities to *T. versicolor* peroxidase encoding genes *MPGI* (Johansson & Nyman, 1996) and *pgv* (Jönsson *et al*., 1994) (IV).

4.4.6 Analysis of the structural and functional domains in *Heterobasidion* MnP fragments based on the sequence alignments

The crystal structure of all peroxidases closely resemble each other (Edwards *et al*., 1993; Poulos *et al*, 1993; Sundaramoorthy *et al*., 1994). Peroxidases are divided into two structural domains, distal and proximal, that cover the heme moiety (Banci, 1997). Alignment of peroxidase peptide sequences allows identification of conserved, and hence residues important for a better understanding of the enzyme structure and function (Figs. 14 and 15). Differences in certain amino acid positions may explain the differences in the efficiency of catalysis, steady-state and transient state kinetic properties, pH-optima and glycosylation.

An invariant residue in all known peroxidases has been identified, proximal His177, which forms an axial ligand to the heme (Poulos & Kraut, 1980). This residue is conserved in the sequenced fragments of *Heterobasidion* peroxidases. All known MnP encoding gene sequences contain highly conserved residues Glu37, Glu41, and Asp183 (based on *T. versicolor MPGI*-gene numbering; Johansson & Nyman, 1996), which are important for Mn^{2+} -binding (Kusters-van Someren *et al*., 1995; Kishi *et al*., 1996; Sundaramoorthy *et al*., 1997). In the cloned and sequenced fragments, Asp183 is conserved except in one of the two cloned fragments from *B. montana*, in which Asp183 is replaced by a glutamine. In the other MnP-fragment from *B. montana* the correct Asp183-residue needed for Mn^{2+}-binding is always present.

In LiP encoding genes, but not in MnPs, residues 86, 88, 172 and 195 appear to be invariable: Ile, Leu, Trp and Asp, respectively. These residues in the aligned amino acid sequences of all the amplified PCR-products from the *Heterobasidion*, *Amylostereum*, *Bondarzewia*, and *Echinodontium* -species vary, which suggests that the cloned sequences represent MnP encoding gene fragments. Arg181 appears to be conserved in MnP genes of certain fungi, but not in all fungi. In *Heterobasidion* and the related species this residue is Ala, as in *T. versicolor*, *P. ostreatus* and *P. eryngii* MnP genes (IV). Arg181 is assumed to contribute in maintaining the structural integrity of the Mn^{2+}-binding site of the enzyme by binding to the Mn^{2+}-ion (Sollewijn Gelpke *et al*., 1999b).

The fungal peroxidases are constituted from 10-11 α-helices linked by loops and turns. Enzyme rigidity and correct backbone bending is influenced by some conserved glycine and proline residues, cysteines forming disulphide bridges, and a

buried salt bridge involving Asp108 and Arg133. The latter two residues are highly conserved in the hundreds of sequenced fragments, except in *B. montana* peroxidase B, where Arg133 is replaced by His. Whether this altered amino acid affect the enzyme activity has yet to be clarified.

Two calcium ions in fungal peroxidases are important for maintaining the structure of the active site (Banci, 1997). Ca^{2+} -binding residues at the proximal side domain include Thr/Ser178, Asp195 and Thr197, and at the distal side domain Gly67, Asp69 and Ser71 (Poulos *et al*., 1993; Kunishima *et al*., 1994; Sundaramoorthy *et al*., 1994). These residues are highly conserved in fungal peroxidases. Residue Phe194, which is conserved in LiPs, MnPs, horseradish peroxidase (HRP), and peanut peroxidase, has been shown to affect enzyme stability (Kishi *et al*., 1997).

Based on the crystal structure of LiP and MnP, amino acid residues at the opening of the main heme channel of fungal peroxidases have been identified (Poulos *et al*., 1993; Sundaramoorthy *et al*., 1994). The nature of the residues may affect the substrate specificity of the peroxidases. Phe/Tyr82 and His83 are located on the surface of the protein, at the border of the entrance to the active site channel and form a binding site for aromatic substrates. Together with Pro/Ala84 and Asn85, they form a potential long-range electron transfer pathway in LiP (Schoemaker *et al*., 1994). Glu79 is involved in the stabilisation of the enzyme during the electron transfer and is highly conserved in the known fungal peroxidases (Banci, 1997). Glu79, Phe82 and Asn85 in the aligned sequences are all conserved in the MnP-fragments studied here. The only exception occurs in *B. montana* peroxidase B fragment, in which the Glu79-residue is replaced by an Asn-residue. Another residue at the heme channel that is suggested to be important in substrate binding is aromatic Phe149 (Veitch *et al*., 1995), which is also invariant in the analysed MnP fragments in this study. Residue 86, which is also located at the opening of the heme channel, is in *Heterobasidion*-species peroxidase 1 and 2 and in *B. montana* peroxidase 2 a polar Asn, as in *P. chrysosporium* MnPs, whereas in *P. ostreatus* and *T. versicolor* MnPs it is a hydrophobic Leu. In peroxidase 3 sequences of the *Heterobasidion*-species and in the peroxidase A sequences this residue is Gly, which may change the substrate specificities of these isozymes by opening the channel to the active site.

The binding of aromatic molecules, such as VA, to peroxidases has been proposed to take place near Glu147 or near Trp172 (Ambert-Balay *et al*., 1998; Blodig *et al*., 1998; Doyle *et al*., 1998; Timofeevski *et al*., 1999). The Trp-residue is conserved in all LiPs, but not in MnPs. The significance of Trp172 has been recently shown using recombinant enzymes and site-directed mutagenesis techniques (Asada *et al*., 1995a; Timofeevski *et al*., 1999). The amino acid 172 in the fragments isolated from *Heterobasidion* and *Amylostereum* -species is Ala and in *E. tsugicola* the residue is Ser (IV). This supports the interpretation that the fragments encode MnP without VA oxidising activity.

The relationships between structure and function of fungal peroxidases have been studied by homologous and heterologous expression systems. It has been relatively easy to obtain with MnP a functional homologous (Mayfield *et al*., 1994b) and heterologous expression systems using *Aspergillus oryzae* or *E. coli* as hosts (Stewart *et al*., 1996; Whitwam & Tien, 1996), while with LiP efficient expression of recombinant LiP enzymes has been difficult. The recombinant protein yields of LiP

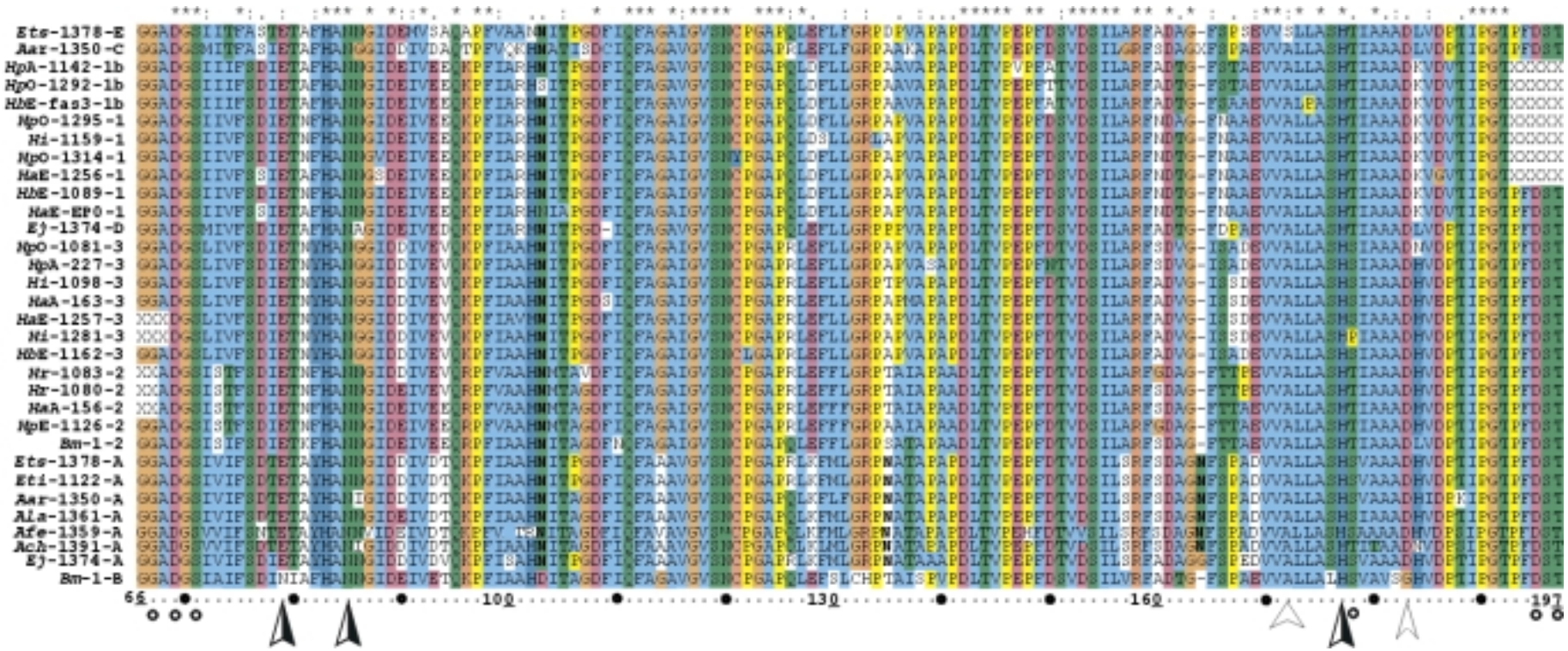

Fig. 14. Sequence alignment of the predicted polypeptide sequences of the cloned MnP gene fragments. Alignment was performed using Clustal X algorithm. Residues are coloured by the program assigning a colour to a specific residue according to the calculated consensus residues at the position. Residues involved in the catalysis are shown by black-and-white arrowheads. Open circles indicate residues involved in Ca^{2+}-binding, long arrowhead Asp173 involved in Mn^{2+}-binding, short arrowhead the position that in LiPs, as a conserved Trp, functions as a veratryl alcohol binding site. Putative N-glycosylation sites are bolded. Abbreviations: *Aar*, *A. areolatum*, *Ach*, *A. chailletii*, *Afe*, *A. ferreum*, *Ala*, *A. laevigatum*, *Bm*, *B. montana*, *Ej*, *E. japonicum*, *Eti*, *E. tinctorium*, *Ets*, *E. tsugicola*, *Ha*A, American *H. annosum* (*H. annosum* ISG P), *Ha*E, European *H. annosum* (*H. annosum* ISG P), *Hb*E, European *H. abietinum* (*H. annosum* ISG F), *Hp*A, American *H. parviporum* (*H. annosum* ISG S), *Hp*E, European *H. parviporum* (*H. annosum* ISG S), *Hp*O, Oriental (Asian) *H. parviporum* (*H. annosum* ISG S), *Hi*, *H. insulare*, *Hr*, *H. araucariae*. Isolate number and the gene type (1,2,3, or A, B, C, D, E) are indicated.

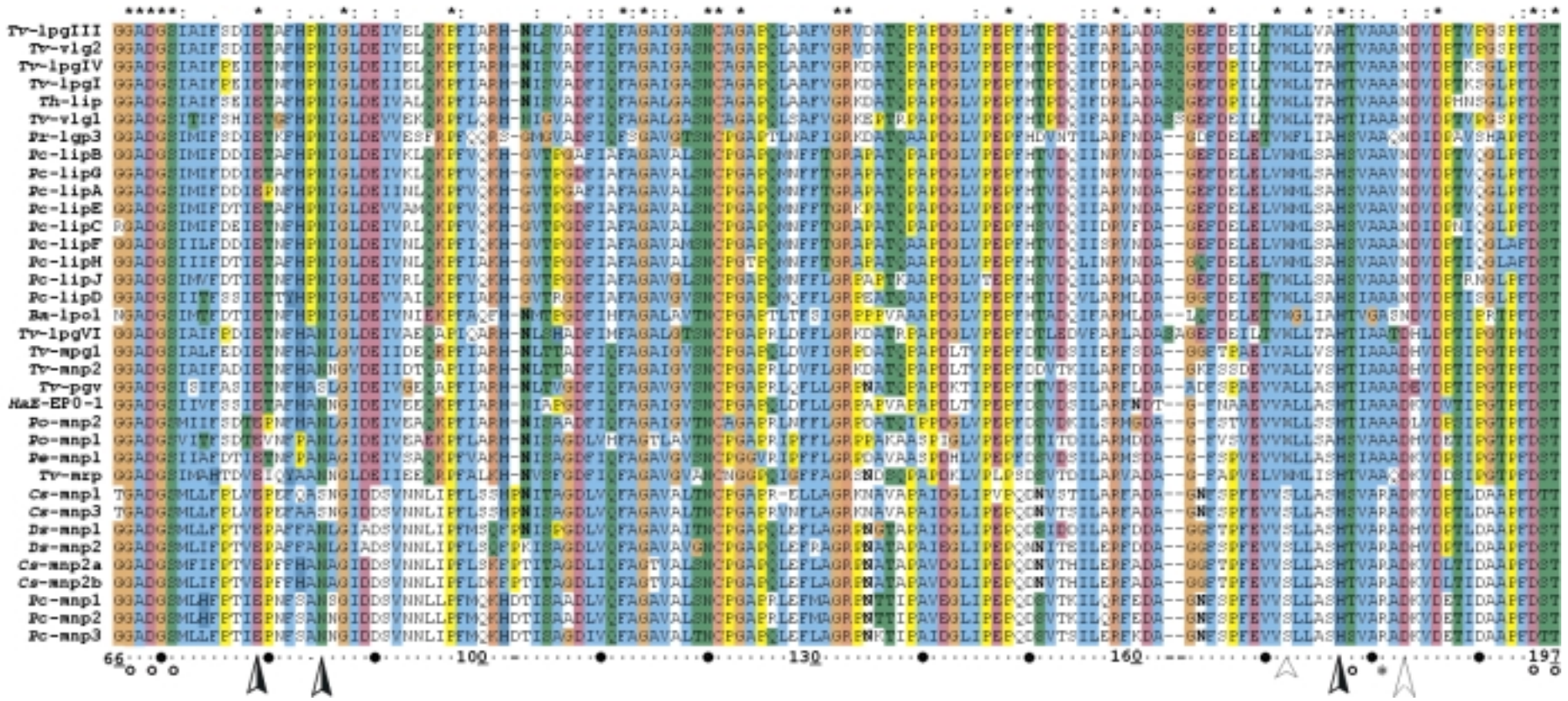

Fig. 15. Sequence alignment of different fungal peroxidase sequences. Only the region characterised in the fragment of this study is shown. Symbols as in Fig. 14. Abbreviations: *Ba*, *B. adusta*, *Cs*, *C. subvermispora*, *Ds*, *D. squalens*, *Pc*, *P. chrysosporium*, *Th-lip*, *T. hirsuta* lip (Unpublished; GeneBank accession no. E07702), *Tv*-mrp, *T. versicolor* manganese repressible peroxidase (Collins *et al*., 1999). The origins of other sequences are explained in the text. The MnP fragment (*mnp1*) of European *H. annosum* P-type isolate (III) (HaE-EP0-1) is included.

have remained low in *E. coli* (Doyle & Smith, 1996; Nie *et al.*, 1998). When LiP H2 was successfully expressed in *E. coli*, it possessed also some MnP activity (Nie *et al.*, 1998). Recently *P. eryngii* versatile peroxidase was expressed successfully in *A. nidulans* (Ruiz-Dueñas *et al.*, 1999b) and a homologous expression system for LiP H8 of *P. chrysosporium* was developed (Sollewijn Gelpke *et al.*, 1999a).

4.4.7 Glycosylation sites of the MnP fragments

Peroxidases have been reported to be glycosylated (Farrell *et al.*, 1989; de Boer *et al.*, 1987; Pease *et al.*, 1989; Pribnow *et al.*, 1989; Schmidt *et al.*, 1990; Poulos *et al.*, 1993; Sundaramoorthy *et al.*, 1994), which is typical for extracellular fungal proteins (Merivuori *et al.*, 1984). The peroxidase gene sequences contain several possible sites that allow either N- or O-type protein glycosylation. Degree of glycosylation modifies the protein conformation and may affect the p*I* of the protein and the stability of the enzyme. Nie *et al.* (1999) proposed that only O-linked glycans may be crucial for the thermal stability of native fungal peroxidases.

Only one potential N-glycosylation site at Asn103 was found in *T. versicolor* MnP (Limongi *et al.*, 1995; Johansson & Nyman, 1996) and this site is also present in almost all *Heterobasidion* peroxidase fragments of this study thus providing a potential N-glycosylation site (Asn-X-Thr/Ser) (Kornfeld & Kornfeld, 1985). All investigated peroxidase fragments from *Amylostereum*, *Echinodontium* and *Heterobasidion* -species contain at least one N-glycosylation site. The only exception is *B. montana* peroxidase B, where no N-glycosylation site was present in the analyzed fragment (Fig. 14). The potential O-glycosylation sites are located in the fungal peroxidases close to their C-termini (Limongi *et al.*, 1995), in a region which is not included in the here analysed fragments.

4.4.8 Clustering of LiP and MnP genes

When homokaryotic P- and S-strains of *Heterobasidion* were analysed using the 565bp PCR-fragment as a probe in Southern-hybridisation analysis, specific hybridisation patterns were observed with *Bam*HI, *Hin*dIII, and *Pst*I-digested genomic DNA (Fig. 16). Similar results were obtained when heterokaryotic P- and S-strains were digested with the same enzymes and the same probe was used (III). Southern analysis of heterokaryons shows clearly that P and S-groups can be distinguished on the basis of restriction fragment length polymorphism (III). Two to three signals were detected indicating the presence of at least two peroxidase genes in P- and S-strains. The *Pst*I-digestions of several P-group isolates were typified by two signals of about 800 bp in size, which agreed with the presence of a *Pst*I site in the cloned peroxidase 1 gene fragment (III).

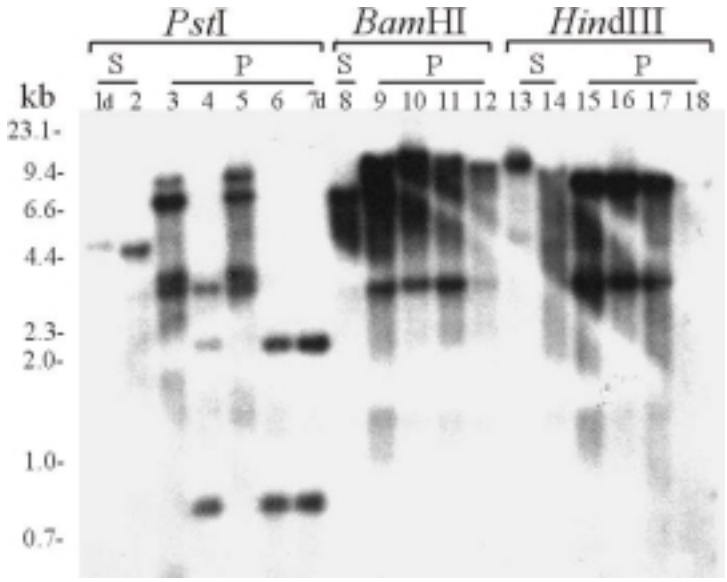

Fig. 16. Southern blot analysis of digested genomic DNA from different strains of homokaryotic P- and S-group isolates of *H. annosum* using a peroxidase 1 - fragment from a European P-type isolate as a probe as in article III. Letter d with the strain numbers 1 and 7 indicate the dikaryon form of the isolate. Size markers are indicated on the left.

Gene clusters of lignin peroxidase and CBH-encoding genes have been identified in *P. chrysosporium* (Raeder *et al*., 1989a, b). In *P. chrysosporium* cosmid and λ-libraries have provided detailed physical maps of gene clusters. *lip*A and *lip*B are tightly connected and *lip*C lies approximately 15 kb upstream of *lip*B (Gaskell *et al*., 1991). In *T. versicolor*, a MnP and two LiPs are clustered within a 10 kb region (Johansson, 1994; Johansson & Nyman, 1996). MnP genes appear to be unlinked to each other and to LiP genes in *P. chrysosporium* (Orth *et al*., 1994; Kersten *et al*., 1995). Using PCR-based methods, linkage relationships of *P. chrysosporium* peroxidases have been refined: ten LiP genes were mapped to three separate linkage groups (Gaskell *et al*., 1994). No clear correlation between *lip*-gene clustering and gene expression level has been noted (Bogan *et al*., 1996; Janse *et al*., 1998). However, in *T. versicolor*, co-ordinated expression of *lip*- and *mnp*-genes was observed under certain culture conditions (Johansson, 1994; Johansson & Nyman, 1996).

4.4.9 Control of LiP and MnP gene expression in wood

In the study presented, spruce wood chip cultures were utilised as a growth substrate for *H. annosum*, in order to express those ligninolytic enzymes which are relevant for ligninolysis in natural conditions. Three MnP isozymes were identified. Similarly, a culture system was developed for the synthetic lignin degradation experiments in order to investigate ligninolysis close to natural conditions. Recently, transcript levels of LiP, MnP, and GLOX-genes of *P. chrysosporium* have been studied in soil (Bogan *et al*., 1996) and in aspen wood (Janse *et al*., 1998) with the RT-PCR technique. The

transcription patterns were substantially different from those obtained from more defined media: Highly expressed *lip*D and *lip*E genes in liquid cultures (Reiser *et al*., 1993) were present at very low levels in wood, whereas *lip*I, *lip*J were expressed at a high level, as was *lip*F in aspen wood.

The expression of all MnP genes was high in wood (Janse *et al*., 1998) and MnP was the dominant enzyme in wood decayed by *P. chrysosporium*, but not in a defined medium (Datta *et al*., 1991). These results indicate great differences between enzyme production in wood and in defined media. On the other hand, the stability of MnPs is higher than that of LiPs in *P. chrysosporium* (Pease & Tien, 1992), which may have influenced the activity detected in wood. MnP isozymes characterised in spruce wood chips cultures of *H. annosum* are possibly encoded by the identified MnP encoding genes. To confirm this, purification of the MnPs, followed by N-terminal peptide sequencing, is necessary, together with the cloning of full-length MnP gene sequence(s) of *H. annosum*.

4.5 PHYLOGENETIC ANALYSIS USING PEROXIDASE ENCODING DNA SEQUENCES

The genus *Heterobasidion* is wide spread on all continents in the Northern Hemisphere and is divided into different intersterility groups, based on laboratory mating tests (Korhonen, 1978; Chase & Ullrich, 1988; Capretti *et al*., 1990). Additional molecular data using various methods, such as RFLP-analysis of ribosomal, mitochondrial and nuclear DNA, RAPD profiling, rDNA sequencing, and isoenzyme analysis, has been used to analyse polymorphism within and between populations of *H. annosum* (Mitchelson & Korhonen, 1998). The use of these tools reveals that the P group of *H. annosum* is closer to the *Heterobasidion*-species *H. insulare* and *H. araucariae* than the S- and F-groups. The S- and F-groups are closely related to each other. The physiological and biochemical characteristics, in addition to different morphology and mating types, has led to the separation of the three intersterility groups P, F and S into *H. annosum*, *H.abietinum* and *H. parviporum*, respectively (Niemelä & Korhonen, 1998). Through the application of nuclear and mitochondrial small subunit rDNA fingerprints, phylogenetic relationships within the *Polyporaceae* have been established (Hibbett & Donoghue, 1995; Hibbett *et al*., 1997). In such analyses *Heterobasidion* spp. show close affinities with *Bondarzewia*, *Hericium*, *Echinodontium*, *Russula*, and *Stereum*. In striking contrast, ligninolytic species from genera *Trametes*, *Phanerochaete*, and *Phlebia* appear to be only distantly related to *Heterobasidion*.

The cloned fragments of MnP encoding genes obtained in this study appear to provide a useful set of markers to help clarify further the relationships both between the intersterility groups of *H. annosum* and between some species, which are suspected to be related to *H. annosum* (IV). Few analyses have been performed using sequence data from functional genes. However, the amount of sequence data available in databases has increased the possibilities to use functional nuclear genes also as targets in phylogenetical studies. The degenerative primers designed for PCR amplification of fungal peroxidases allowed the detection of several peroxidase genes in the

Heterobasidion haploid genome, which could have originated through gene duplications. This is in agreement with the situation in *P. chrysosporium*, in which several closely related peroxidase genes have been identified (Gaskell *et al*., 1994). The sequence analysis suggested that three MnP encoding genes are present in 106 different *Heterobasidion* isolates, and many of them contain four distinct MnP-genes (IV). Each of the four MnP fragment sequences was clearly specific for the P- (*H. annosum*), S- (*H. parviporum*) and F- (*H. abetinum*) intersterility group of *H. annosum*, although at the amino acid level in *mnp1a* fragment *H. parviporum* and *H. abietinum* were not distinguished. The S-group could be separated in *mnp2* and *mnp3* according to the geographic origin and distribution. These findings are in accordance with similar sequence analysis of ITS or IGS regions from rDNA (Harrington *et al*., 1998), although the European S and F types have identical ITS and IGS sequences. The MnP sequence phylogeny presented here strongly support the different ecology and low, 24%, interfertility based on dikaryon formation between the European S and F types (Korhonen *et al*., 1992).

The European S and F types were clearly separated according to their MnP gene fragment sequences. The phylogenetic divergence, based on MnP encoding gene fragments, between the S- and F-strains supports present assumptions concerning evolutionary trends within the genus *Heterobasidion*. It is suggested that the common ancestor of the S and F group originated in East Asia (Korhonen *et al*., 1997), which later diverged through different migration patterns. The S group may have followed the host species (*Picea*/*Abies sibirica*) migration route through Siberia to Europe whilst the F group followed a southern route along with the *Abies* hosts to Europe. During the migration, the rDNA ITS sequences of the S- and F-type appeared to have come under less evolutionary selection pressure than the MnP sequences and gene sequences regulating sexual reproduction. The differences in the MnP genes evolved during migration might have resulted from the selective effect of growth in different hosts. Alternatively, the identical rDNA spacer regions in the S- and F-groups may have resulted from a hybridisation event in Europe, and along with the concerted evolution and homogenisation of the nuclear ribosomal genes, only one of the parental ITS types has emerged.

Gene *mnp1b* was identified only in the American, but not in the European *H. annosum* (P-type), indicating that two phylogenetically distinct species exist. Based on the peroxidase data presented in this study, it seems possible that the ancestral species could be *H. parviporum* (S-type), from which the American *H. annosum* and the F-type (*H. abietinum*) have subsequently diverged.

The identical intron positioning in the MnP fragments of *Amylostereum*, *Bondarzewia*, *Echinodontium* and *Heterobasidion*, and the high similarity of the investigated MnP sequences indicate a close relatedness between these species, and supports the previous phylogenetic positioning based on nuclear rDNA and mitochondrial rDNA sequence analyses (Hibbett & Donoghue, 1995; Hibbett *et al*., 1997).

5. CONCLUSIONS AND FUTURE PROSPECTS

All the enzymes investigated in the present study (Table IX) are important for wood degradation by the white-rot fungus *H. annosum*. Fungal attack, with concurrent extracellular enzyme production by the colonising hyphae, changes wood ultrastructure and causes considerable losses in timber yield particularly in circumpolar boreal forests. *H. annosum* P- and S-types are respectively the most destructive pathogens of Scots pine and Norway spruce. Multiple isoenzymes of endoglucanases, xylanases and mannanases were produced in the investigated P- and S strains. A differential response of xylanase and mannanase production in P- and S-strains was observed.

Table IX. Hydrolytic and oxidative enzymes in P- and S-type of *H. annosum* investigated in the study.

Enzyme	*P*	Method of detection	*S*	Method of detection
Cellulolytic enzymes				
EG	++	E, AB	++	E, AB
CBH	+	AB	+	AB
ß-glucosidase	+	E	+	E
Hemicellulolytic enzymes				
xylanase	++	E, AB	++	E, AB
ß-xylosidase	+	E	+	E
AXE	+	E	+	E
acetyl esterase	+	E	+	E
mannanase	++	E, AB	++	E, AB
α-galactosidase	+	E	+	E
Ligninolytic enzymes and ligninolytic activity				
laccase	++	E	ND	-
MnP	+	E, G	+	G
LiP	±	E	ND	-
DHP	+	$^{14}CO_2$	ND	-

+: low activity detected, ++: high activity detected, E: biochemical assay detection, AB: enzyme detected using polyclonal antibodies, G: homologous gene fragments identified, DHP: Synthetic lignin (DHP) mineralisation, ND: not determined.

The complex set of cellulolytic and hemicellulolytic enzymes identified in the present study are likely to facilitate the degradation of lignin by *H. annosum*. White rot fungi are unable to utilise lignin as the sole source of carbon, although the carbon content of lignin is high (Kirk *et al*., 1976; Reid, 1979). The studies presented strongly support the view that *H. annosum* is a member of the ligninolytic group of fungi. This was confirmed by three different means. Firstly, ligninolytic enzyme activity was investigated in liquid and wood chip cultures. Secondly, the presence of ligninolytic enzyme encoding genes in the *Heterobasidion* genome was screened by PCR-amplification with degenerative primers based on known LiP and MnP genes. Whether

the three MnP isozymes detected from the *H. annosum* P-strain grown in the spruce wood chips represent the products of the genes described in this study requires further investigation. Construction of genomic and expression libraries from *H. annosum* will be an essential step towards completion of molecular analysis of ligninolytic peroxidases in *H. annosum*. Interestingly, three genomic MnP gene sequences and three MnP gene products were identified from wood chips cultures. Thirdly, ^{14}C-DHP-lignin mineralisation experiments showed that *H. annosum* has a moderate ability to mineralise lignin under laboratory conditions.

Protein purification is an essential prerequisite for the detailed investigation of a certain isoenzyme. Purified enzyme components from *H. annosum*, such as endoxylanase, could be used to identify the cellulose-binding capacities of the enzyme with the method described. A combination of PCR-based methods, adopted for MnP analysis in this study, with N-terminal sequencing of a purified isozyme, will aid in the clarification of the multiplicity and differential expression of cellulolytic and hemicellulolytic enzymes in P- and S-types of *H. annosum*.

Targeted enzyme investigations using the preferential host wood substrate, i.e. pine wood for the P-type, and spruce wood for the S-type, may give clues on the specific expression of a certain isozyme on this substrate. The recognition of such an isozyme gives new insights on the functional significance of this enzyme in pathogenesis. For this purpose, studies must be extended to include molecular analysis of the encoding genes. Functional transformation systems and *in vitro* mutagenesis techniques for white rot fungi are already available. The role of individual cellulase, hemicellulase, or peroxidase isoenzymes in pathogenesis of *H. annosum* requires expression studies of these proteins *in planta*, as has recently been achieved (Hardham & Mitchell, 1998; Janse *et al*., 1998). Symbiotic ectomycorrhizal fungi also penetrate and colonise host tree roots, in a similar manner to *H. annosum*, without causing any damage to the root structure. By careful comparison of pathogenic and symbiotic systems, molecular mechanisms that control the respective infection processes can be better characterised and compared.

The solid-state cultures could be further modified to parallel conditions found in wood. When growing in wood or litter, fungi are able to translocate and reuse nitrogen from older parts of the mycelium (Cowling & Merrill, 1966; Thompson, 1984). This complicates the interpretation of the effect of nitrogen availability on fungal growth and lignocellulose degradation based on laboratory experiments. Pine and sprucewood blocks, supplemented with investigated compounds, e.g. detergents or phenolics, could be respectively used as the base for *H. annosum* P- and S-strain inocula. To study the influence of rate-limiting compounds, such as manganese, on peroxidase induction and ligninolytic activity, pulp medium covering the wood blocks may be utilised

Scots pine and Norway spruce, the respective host trees of P- and S-type of *H. annosum*, comprise a clear majority of all harvested wood used by the Finnish forest industry. Understanding of the enzymology in wood degradation processes caused by *H. annosum* forms a basis for the development of effective means to control the root and butt rot disease. Moreover, detailed knowledge about the enzymology during wood decay processes is required for the potential use of *H. annosum*, or other white rot fungi, and their enzymes in modern biotechnological applications, such as in the

pretreatment of wood chips for downstream processes by the pulp and paper industry (Akhtar *et al*., 1998). The findings obtained in the present study provide a framework for continued studies, in elucidating, and later, developing applications based on ligninolytic enzyme capacity of *H. annosum*.

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