The Characterization of Wood and Wood Fibre Ultrastructure Using Specific Enzymes

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Abstract

Wood and wood fibre ultrastructure is of key importance for the pulp and paper making industry. In this thesis there has been a focus on development of methods for studying wood degrading enzymes. The methods have been used in different ways to render new information on wood and wood fibres. Wood degrading enzymes are chosen since they are tailor made to specifically degrade certain substructures in the wood bio-polymer, e.g. cellulose.

In the first part an α-amino group of a carbohydrate binding module (CBM) from *Phanerochaete chrysosporium* cellulase Cel7D was covalently labelled with fluorescein isothiocyanate. The fluorescein labelled CBM showed specific binding only to cellulose and not to mannan and xylan. The labelled CBM was successfully used as a probe for detection of cellulose in lignocellulose material such as never dried spruce, birch wood and processed pulp fibres.

Furthermore, the endoglucanase Cel5A from *Trichoderma reesei* and monocomponent endoglucanase from *Aspergillus sp* (Novozyme 476™ from Novozyme A/S) were evaluated as probes for the surface properties of soft- and hardwood chemical pulp fibres. The empirical kinetic degradation parameters correlated with the tensile index, relative bonded area and z-strength of paper. All paper properties showing a correlation with enzyme kinetic parameters were related to fibre-fibre interactions. Fluorescence labelling of the reducing end groups in pulp fibres was also used to further characterize the degradation.

Finally, *in vitro* model studies as well as studies on intact wood samples were performed with different enzymes to characterize a fluorescent substance found in tori of water-sprinkled spruce logs. The model experiments showed that tannic acid can bind to pectin and that its presence inhibits pectinase action. Furthermore, it was shown that tannin acyl hydrolase can degrade tannic acid adsorbed to pectin. Neither pectinase nor tannase could, however, remove the discolouring substances from pit membranes in intact spruce samples. Manganese peroxidase had a minor but documented effect which, together with HCl/vanillin labelling experiments supported the conclusion that the discolouring substance corresponds to condensed tannin.

Keywords: post harvest, fibre surface, quality control, fungal, paper strength, discolouration, decay, fibre-fibre interactions

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Wood Ultrastructure Research Centre (WURC)

List of abbreviations and definitions

AA	Anthranilic acid
ABTS	2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)
BMCC	Bacterial micro-crystalline cellulose
CBD	Cellulose binding domain
CBH	Cellobiohydrolase
CBM	Carbohydrate binding module
CBM-FITC	Fluorescein isothiocyanate derivatized carbohydrate binding module
Cel	Cellulase
DNS	3,5-dinitrosalicylic acid
EG	Endoglucanase
FE-SEM	Field emission scanning electron microscopy
FITC	Fluorescein isothiocyanate
GH	Glycosyl hydrolase
MFA	Microfibrillar angel
N476	Novozym 476
PW	Primary cell wall
RP-HPLC	Reversed phase HPLC
S	Secondary cell wall
S1	Outer layer of secondary cell wall
S2	Middle layer of secondary cell wall
S3	Inner layer of secondary cell wall
SEM	Scanning electron microscopy
Ultrastructure	Defined as features which are rendered visible by methods which are able to resolve structures at higher resolution than light microscopy. Ultrastructural features are measured in nanometers.

Appendix

Papers I-IV

The thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Hildén, L., Daniel, G. and Johansson, G. (2003) Use of a fluorescence labelled, carbohydrate-binding module from *Phanerochaete chrysosporium* Cel7D for studying wood cell wall ultrastructure. Biotechnology Letters 25, 553-558.

II. Hildén, L. and Johansson, G. Recent developments on cellulases and carbohydrate binding modules with cellulose affinity. Accepted for publication in Biotechnology Letters.

III. Hildén, L., Väljamäe, P. and Johansson, G. Surface character of pulp fibres studied using endoglucanases. Submitted.

IV. Hildén, L., Zhang, J., Persson, E., Johansson, G. and Brändström, J. Allocation and characterisation of discolouring substances appearing in Norway spruce (*Picea abies* L. Karst.) pulp wood stored with water-sprinkling. Manuscript.

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Related publications

i. Hildén, L., Johansson, G., Pettersson, G., Li, J., Ljungquist, P. and Henriksson, G. (2000) Do the extracellular enzymes cellobiose dehydrogenase and manganese peroxidase form a pathway in lignin biodegradation? FEBS Letters 477 79-83.

ii. Hildén, L., Eng, L., Johansson, G., Lindqvist, S.-E. and Pettersson, G. (2001) An amperometric cellobiose dehydrogenase-based biosensor can be used for measurement of cellulase activity. Analytical Biochemistry 290 245-250.

iii. Henriksson, G., Hildén, L., Ljungquist, P., Ander, P. and Pettersson, B. (2001) Cellobiose Dehydrogenase as a Ligninase. ACS Symposium series 785 456-473.

Introduction

1.1 Background

The Swedish forest industry used ca 84×10^6 m^3 of roundwood and the Swedish pulp and paper industry produced ca 4.1×10^6 tons of pulp during 2003 (according to the National Board of Forestry and the Swedish Forest Industries Federation). Wood fibres from spruce and pine account for most of the pulp, although birch is also used. Another important source is recycled paper. Wood fibres are not only used for printing paper but also for protective structures, packaging, in composites, and can be laminated to store liquid. In summary wood fibres are important for everyday life for most people in the world and Sweden is certainly no exception.

Traditionally the comparatively long fibres (2-3cm) of e.g. hemp were used, but in the 1850's a method for making paper out from wood fibres was developed. Although non-wood fibres are still used, for example in bills, wood currently dominates as a source of fibre for pulp and paper.

Naturally many, if not all the properties of paper are dependent on the raw material used. The fibre contribution can be divided into two main parts - contributions from the interior strength of the fibre and from surface properties. The interior properties of a fibre can be studied either by analysing a large group of fibres together, which is the case when for example paper strength or intact wood is examined, or by studying single fibres alone or in sections of intact wood. Properties of fibre surfaces can be studied in many ways. Originally light microscopy was used. Later many different types of microscopy such as scanning electron microscopy (SEM), field emission scanning electron microscopy (FE-SEM), polarised light microscopy, transmission electron microscopy (TEM) etc have been used. In addition a large number of different markers for fibre surface components are available. These include dyes, antibodies, enzymes and enzyme fragments. A problem with all labelling methods is non-specific labelling since many components in the wood resemble each other in their microstructure. This is especially true for carbohydrates.

The present study focuses on the utilisation of specific enzymes for studying wood and wood fibre ultrastructure. Since enzymes have a high specificity for a certain type of substrate, observations on their binding to wood fibres or products of their activity can give important clues one the native ultrastructure of wood and fibres.

1.2 Objectives of the study

The objective of this thesis was to develop tools for characterization of wood and wood fibres with specific enzymes as well as to render new information by using those tools.

1.3 The structure of wood

In a tree, only a few cells are actually living and proliferating. They are mainly situated in the vascular cambium just beneath the bark where the cambium mother cells divide repeatedly. They divide in their x and y directions to form bark and phloem cells. As a tree grows older the innermost fibres stop conducting water. The fibres shut by the closure of pits (i.e. aspiration of pis) and are made resistant by an increase in extractives and phenolic substances. The wood formed in this process is referred to as heartwood while the conducting wood is referred to as sapwood. In Scandinavia, cell proliferation occurs only in spring and summer. The earlyywood cells produced in spring are thin-walled with large cell lumen for efficient water transport while the latewood fibres produced in summer are dense with very thin or almost absent cell lumen. Latewood fibre adds strength to the tree. The annual cycle affected growth rate is the reason that trees from temperate zones always have annual rings while wood from trees growing in tropical zones may have a more homogenous character. At the top of the tree and in the tips of branches and roots the cells also divide in their z-direction to elongate the tree (Fig. 1) (Sjöström, 1981).
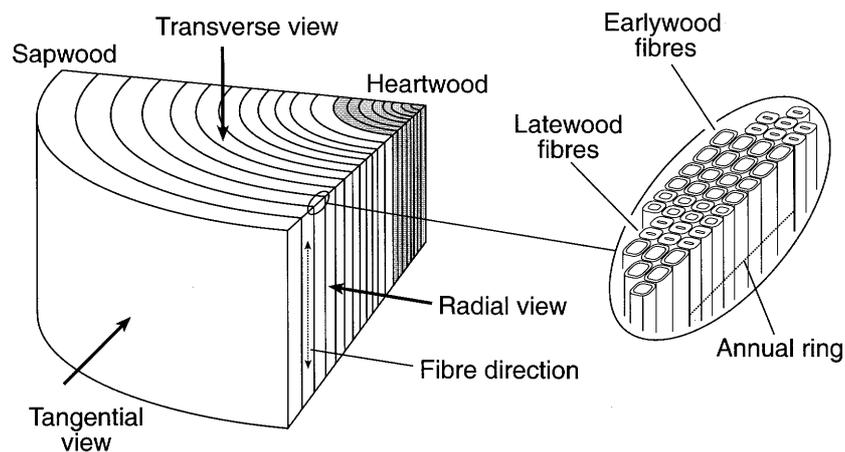
Figure 1. Diagram showing the basic wood terminology.

A typical mature wood fibre is a tube-like, hollow, lignified and dead cell. A spruce (softwood) fibre is 2-4 mm long and 20-40 μm wide, while a typical birch (hardwood) fibre is 1.1-1.2 mm long and 14-40 μm wide (Sjöström, 1981). In softwoods the fibres perform both supporting and conducting roles. Hardwood ha some shorter and wider cells known as vessel elements which act as conducting cells. Adjacent wood cells are held together by the middle lamella, which is rich in lignin and acts like a glue.

A typical softwood fibre is built up of several layers. Each layer consists of microfibrils composed mainly of cellulose chains. The first model on how fibrillar structures are formed by cellulose chains was put forward by Frey-Wyssling in 1953/54 (Frey-Wyssling, 1953; Frey-Wyssling, 1954). Later, a wood microfibril

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model where 30 Å wide cellulose elementary fibrils were ordered 4×4 in 120×120 Å bundles surrounded by hemicellulose was suggested. These bundles were in turn packed 4 x 4 and surrounded by lignin to form a 250×250 Å microfibril (Fengel, 1970) (Fig. 2). This model remains well accepted although interactions between the different polymers in microfibrils are better understood today. Glucomannan, for example seems to be closely associated with cellulose whereas xylan seems to be more associated with lignin (Salmén & Olsson, 1998).

In wood cells the microfibrils are well ordered in the different cell wall layers. The outermost layer is called the primary wall (PW); thereafter comes the secondary wall (S) which is composed of three sub-layers denoted S1, S2 and S3. The S2-layer is always the thickest layer in the cell wall (Fig. 2). The warty layer lines the cell lumen wall. The cellulose microfibrils have different orientations in the different layers. The angle between the z-axis of the fibre and the microfibrills is referred to as the microfibrilar angle (MFA). In the PW the microfibrils have a random orientation, in the S1 layer they are almost perpendicular to the z-axis of the fibre, in S2 they are rather parallel to the z-axis, while in the S3 layer they are again almost perpendicular to the z-axis (Sjöström, 1981). In secondary wall layers the microfibrils form helical structures in the cell wall layers. Furthermore, the S3-layer is reported to be composed of several sub-layers with different helical (S and Z) orientations. It is also reported that there is a gradual transition from S1 to S2 and from S2 to S3 layers (Brändström, 2002) (Fig. 2).

Water transport between adjacent wood cells is made possible by pits. A pit is an opening between two cells provided with a permeable membrane. In softwoods, this membrane consists of two parts known as the torus and margo. The torus is an elliptical-shaped body in the centre of the membrane made up of mostly pectin. The margo, the permeable part of the membrane, is a, thin circular structure holding the torus in place (Fig. 3). In softwoods the tori are often bordered by a pit chamber (surrounded partially by the cell wall) which means that if the non-permeable torus is aspirated (i.e. due to pressure pushed against one of the openings) the pit chambre will be closed. Both half-bordered and non-bordered pits are also present in some wood species.

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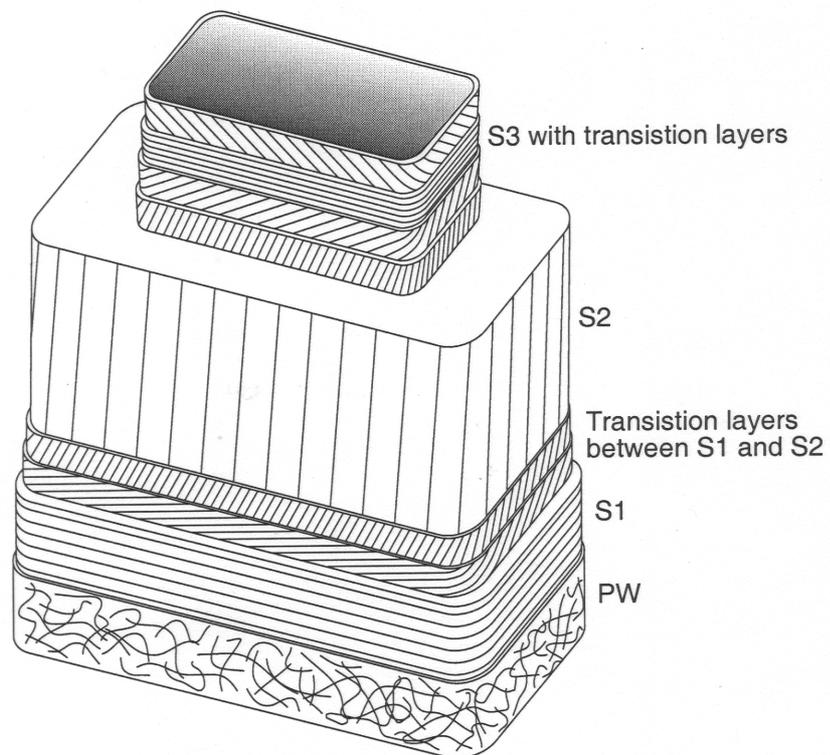
Figure 2. Diagram showing the sub-layers of a wood fibre as suggested for a latewood fibre from mature wood. From the bottom: the randomly ordered PW, the S1-layer with microfibrils perpendicular to the z-axis, two thin transition layers, the thick S2-layer with low MFA and finally sublayers of the S3-layer where the mean MFA is more or less perpendicular to the z-axis.
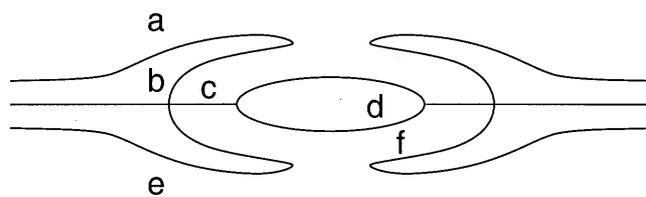
Figure 3. Diagram showing a bordered pit of spruce. a: lumen of one cell, b: pit chamber wall, c: margo holding the torus, d: torus, e: lumen of adjacent cell and f: pit chamber

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1.4 The structure of pulp fibres

There are two main types of pulp fibres - mechanical and chemical. Mechanical pulps are made by separating wood fibres using a grinding procedure resulting in "fines" (small fractions from fibres) and broken fibres. Often heat is applied during the grinding procedure and the pulp is then referred to as thermomechanical. All pulps used in this work were chemical pulps.

1.4.1 Chemical pulps

There are two main types of chemical pulping known as sulphite and sulphate processes. Sulphite pulping is an acidic method which is not used so often today. The dominating process used today is sulphate pulping; a basic method. Chemical pulping disintegrates the fibres by dissolving the middle lamellae. Chemical pulp fibres are more intact in their structure as compared to mechanical pulp fibres. When studied with light microscopy and even when studied with SEM the surface of chemical pulp fibres appears smooth. However, when studied with the better resolving FE-SEM technology it is obvious that the fibre surface, at least in its hydrated state, displays a network-like structure with lots of pores. The pores have sizes up to ca 100 nm in diameter as shown in the literature (Duchesne & Daniel, 1999; Duchesne & Daniel, 2000). The PW, and also parts or all of S1 layer are often missing from pulp fibres after chemical pulping (Duchesne & Daniel, 2000).

1.5 Wood degrading enzymes

Wood is an enormous resource of energy. However this energy is "bound up" in a highly crystalline and well-protected structure. Animals do not generally have the ability to utilise wood cellulose unless they receive help from bacteria or fungi. Some of these organisms have an ecological niche as specialised in attacking wood. However, these organisms need also to degrade the cellulose and hemicellulose to glucose and other monosaccharides in order to utilise this energy. To do so these organisms use enzymes. The most important enzymes in the diverse array produced by micro-organisms are the cellulases, but hemicellulose-, pectin- and lignin-degrading enzymes are also used.

1.5.1 Cellulases

Cellulases hydrolyse β-1,4 glucosidic bonds in cellulose. They are found in all kingdoms (Lawrence, 1997; Watanabe & Tokuda, 2001), although most predominantly in the *Prokaryotae* and *Fungi*. Cellulases belong to the *O-* and *S-*glycosyl-hydrolases (GHs). Other members of the GH-family are for example xylanases and mannanases. Closely related to the GHs are the transglycosidases. The ExPASy-server (http://us.expasy.org/cgi-bin/get-enzyme-entry-unprecise?3.2.1.-; 29-Aug-2004) (Appel, Bairoch & Hochstrasser, 1994) currently lists 151 different types of GHs. These can be divided into three different types with regard to topological arrangement of the active site: a tunnel, a crater/pocket, or a cleft (Davies & Henrissat, 1995). The different shapes are optimal for different types of substrates. The crater is mostly suited for degradation of substrates by

"end-on-attack". The cleft is most commonly found on GHs degrading polysaccharides in an endo-manner, i.e. in any position along a polysaccharide chain. The tunnel is found in GHs degrading one polysaccharide chain processively. Processivity is defined as then number of catalytic events per nuber of binding events. An enzyme that binds and then performs a large number of catalytic events before it becomes unbound has a high processivity

Cellulases have traditionally been divided into exoglucanases (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91) which sequentially release sugar from the ends of the cellulose chain and endoglucanases (1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) which attack the cellulose chain at internal positions (Webb, 1992). However, other enzymes like xylanases and β-glucosidases can also use cellulose as an alternative substrate. There is no sharp distinction. There is not even a sharp distinction between the two types of cellulases. Between the two extremes endoglucanases and exoglucanases one can for example find endo-processive cellulases (Teeri, 1997). The activity pattern is determined by how open the tunnel, or how closed the cleft is (Warren, 1996).

A striking feature of many cellulases and also some other GHs is a structural architecture based on one or more catalytic domain(s) and one or more carbohydrate binding module(s) (CBM) held together by linker domains (Davies & Henrissat, 1995) (Fig. 4). Carbohydrate-binding proteins are divided into two main groups: type I has a "deep lying" or "buried" binding site and type II has a binding site on or near the surface (Quiocho, Spurlino & Rodseth, 1997). Catalytic domains of the cellulase type are found in both groups, but CBMs are only found in the type II group. Only few papers have delt with the importance of the linker domain, but it has been shown that its contribution to the spatial separation of catalytic domain and CBM is important (Srisodsuk *et al.*, 1993).
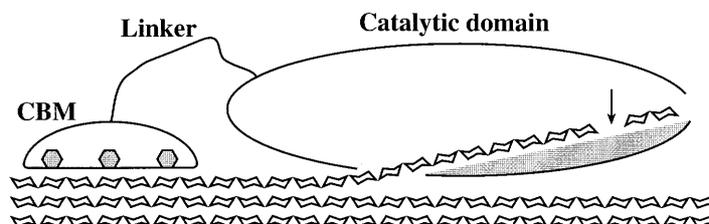
Figure 4. A diagram showing the basic modules of a cellulase situated on a cellulose surface

The name of a GH is based on its preferred substrate, its family number and its order of discovery. For example: the first discovered GH family 7 cellulase from *Trichoderma reesei* (*T.r.*) is designated Cel7A. The second is designated Cel7B. An organism can have genes present in the genome from which no enzyme has yet been found. This is the case for example with some *Phanerochaete chrysosporium* (*P.c.*) Cel7 enzymes. There are genes for Cel7A -Cel7F, but only Cel7C and Cel7D

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has been observed expressed by *P.c.* in cultivation (Uzcategui *et al.*, 1991b; Munoz *et al.*, 2001a).

1.5.2 Carbohydrate binding modules (CBM's)

It is beneficial for cellulases to be located in close proximity to their substrate and as a consequence carbohydrate binding modules (CBMs, formerly denoted CBDs, cellulose binding domains) have been developed. CBM's are structural units that bind to a specific carbohydrate, without performing hydrolysis (Tomme *et al.*, 1998). CBMs range in size from 4 to 20 kDa. They are often located at one of the termini in the protein sequence, but they can also be internally located. With few exceptions, the CBM is connected to the catalytic module via a highly glycosylated flexible linker domain. All CBMs share one common feature independent of their size and 3D-structure: they use aromatic and often polar residues for interactions with their binding target (Lehtiö, 2001). The aromatic residues are responsible for binding to the pyranose rings of the glucose units of cellulose, "stacking" on them via dispersion forces whereas the polar residues form hydrogen bonds to the OH-groups (Din *et al.*, 1994; Tormo *et al.*, 1996; Notenboom *et al.*, 2001).

CBMs binding to crystalline cellulose display a certain positioning of aromatic residues, typically tyrosine and tryptophan; in this interaction the cellulose surface interacts with the flat faces of the aromatic rings. The presence of the aromatic residues is crucial for binding (Reinikainen *et al.*, 1992). The aromatic residues are separated by a distance corresponding to the length of the repeating unit in cellulose (Kraulis *et al.*, 1989; Tormo *et al.*, 1996; Lehtiö, 2001). Polar residues hydrogen bond to adjacent cellulose chains (Tormo *et al.*, 1996). All these features make such CBMs perfectly adapted for binding to a crystalline cellulose surface. They are surprisingly specific, CBMs of family 1 and 3 displays binding predominantly to the corners of cellulose microfibrils that display a hydrophobic face (Lehtiö *et al.*, 2003). These types of domains were the first to be discovered, thus the previous name CBD (Bhikhabhai, Johansson & Pettersson, 1984; Lehtiö, 2001). It is noteworthy that the small size of family 1 CBMs allows their synthesis *in vitro* (Johansson *et al.*, 1989). Although CBMs may have a high affinity for their substrate it is obvious that a irreversible binding to the substrate would be fatal for the enzyme bearing the CBM and indeed movement of these types of CBMs has been demonstrated (Jervis, Haynes & Kilburn, 1997).

1.5.3 Other enzymes used for degrading lignocellulose

To get access to cellulose, wood degrading organisms need to remove lignin. This is achieved with lignin degrading enzymes. There are three types of lignin degrading enzymes known as lignin peroxidases, laccases (polyphenol oxidases) and manganese peroxidases reviewed (Kirk & Farell, 1987). Ligninases are H_2O_2-requiring oxygenases (Gold *et al.*, 1984). Laccases are O_2-dependant phenol oxidases. Manganese peroxidase, the enzyme used in this work, reduces H_2O_2 and oxidizes Mn(II) to Mn(III). Mn(III) is able to perform a one-electron oxidation of phenolic, but not non-phenolic lignin (Glenn, Akileswaran & Gold, 1986; Paszczynski, Huynh & Crawford, 1986). However, non-phenolic lignin can be converted to the phenolic type by action of cellobiose dehydrogenase (Hildén *et*

al., 2000; Henriksson *et al.*, 2001). The manganese peroxidase system can thus oxidize lignin in an indirect mode without direct contact between the lignin and the enzyme. This is important, since lignin makes the structure of a wood cell wall so compact that large molecules, such as a high molecular weight proteins, can not penetrate into the wall and obtain access to the substrate. Manganese is present in most wood tissues (Young & Guinn, 1966). All of the above mentioned enzymes do not attack lignin *in situ* but rather by instead produceing an aggressive species such as Mn(III). Recently, however it was shown that enzymes degrading lignin *in situ* do exist (Otsuka *et al.*, 2003).

Tannin acyl hydrolase (tannase) is an enzyme that is able to hydrolyze ester bonds in hydrolysable tannin (Lawrence, 1997; Lekha & Lonsane, 1997; Aguilar & Gutiérrez-Sánchez, 2001). One common source is the fungus *Aspergillus niger*. Tannase cannot degrade condensed tannins since they contain no ester bonds. In fact, knowledge about enzymes degrading condensed tannins is very scarce (Lekha & Lonsane, 1997).

Pectinases are a group comprised of several different types of pectin degrading enzymes. The group consists of polygalacturonases, pectin esterases, pectin lyases and pectate lyases. The most commonly used, and that responsible mainly for pectin degradation is polygalacturonase (poly-$(1,4-\alpha$-D-galacturonide) glycanohydrolase) (Zhang, Henriksson & Johansson, 2000). As indicated by the name it attacks the main, smooth regions of pectin. In this work a polygalacturonase from Aspergillus niger was used. Pectin esterase simply removes the methyl ester groups, and the lyases cleave the polygalacturonan chain by an elimination reaction resulting in a double bond between the C-4 and C-5 carbons. The lyases can be both endo- or exo-acting types.

1.6 Strength properties of pulp

A number of parameters for describing the properties of a paper exist. Z-strength (kPa) is a measure of the paper strength in the z-direction i.e. 90° to the paper's plane. It is measured by placing the paper between two adhesive plates and pulling until the paper delaminates. Zero-span (Nm/g) represents the interior strength of the fibres without addition of interfibre bonds. It is measured by placing a piece of the paper between two clamps, with a span as close to zero as possible between them. The paper id then pulled apart. To ensure minimal contribution from fibre-fibre bonds the paper is often re-wetted before testing. Tensile index (Nm/g) is measured as zero-span but with a distance of 100 mm between the clamps. This includes the strength of the bonding between the fibres as well as the relative bonded area. Relative bonded area (%) is the amount of contact points between a fibre and a glass plate when the fibre is pressed against the plate under specific conditions. Wet fibre flexibility is the ability of a single wet fibre to bend (m^2/N). Fibre length (mm) and width (μm) simply describes the spatial extension of the fibre. Fibre shape factor (%) is the relation between the exposed length and a the true length of the fibre. Coarseness (μg/m) is the relation between mass and total length of a portion of fibres.

1.7 Constituents of wood

Wood is a biological composite material consisting mainly of cellulose, hemicellulose, lignin, pectin and various extractives (defined below). The highly crystalline cellulose gives strength to the tree but cellulose alone, without lignin - a highly branched phenolic polymer - would be very brittle. Hemicellulose acts primarily as an important linker between cellulose and lignin. The extractives are more or less poisonous and are found in the protective layer of the tree known as the bark. The frequently used term lignocellulose simply refers to a plant substance containing lignin, cellulose and hemicellulose. Apart from wood, hemicellulose can be found in grass-like plants such as bagass (the sugar depleted stems of sugar canes).

1.7.1 Cellulose

Cellulose composes ca 40-50 % of spruce wood. Cellulose is an unbranched homopolymer, and depending on its source, is composed of up to 15,000 D-glucose units linked with β-1,4-glucosidic bonds (Sjöström, 1981). The smallest repeating unit is cellobiose, a dimer consisting of two glucose units rotated 180° with respect to one another. A cellulose chain as an entity has one non-reducing and one reducing end. The reducing end is a potential aldehyde group. The distance between the centres of two pyranose rings is 5.15 Å and the length of the unit cell is the length of one cellobiose unit, i.e. 10.3 Å (Sponsler & Dore, 1926; Meyer & Mark, 1928) (Fig. 1).
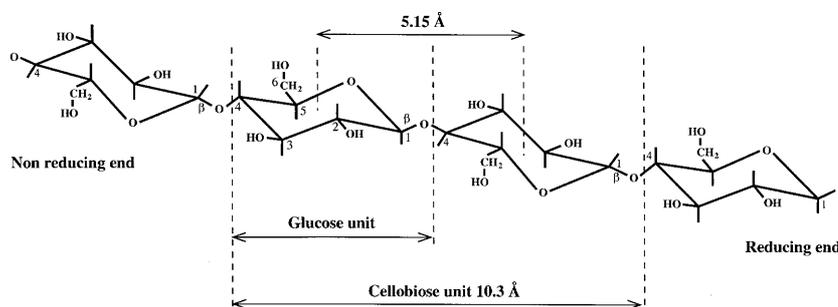
Figure 5. Cellulose chain with its repeating units and reducing and non-reducing ends.

Cellulose has both hydrophilic (i.e. HOC-groups) and hydrophobic (i.e. HC-groups) character (Janado *et al.*, 1979; Sundari & Balasubramanian, 1997). Strong intra- and intermolecular O—H···O bonds retain the chains straight and stacked in a sheet-like structure (Sjöström, 1981; Nishiyama, Langan & Chanzy, 2002). Within one sheet, the cellulose chains are connected in a parallel manner, i.e. all their reducing ends are pointed in the same direction (Gardner & Blackwell, 1974; Sarko & Muggli, 1974) (Fig. 2).

Figure 6. Two adjacent cellulose chains in one sheet and the carbohydrate bonds holding them together. Note the parallel arrangement with the reducing ends aligned in the same direction.
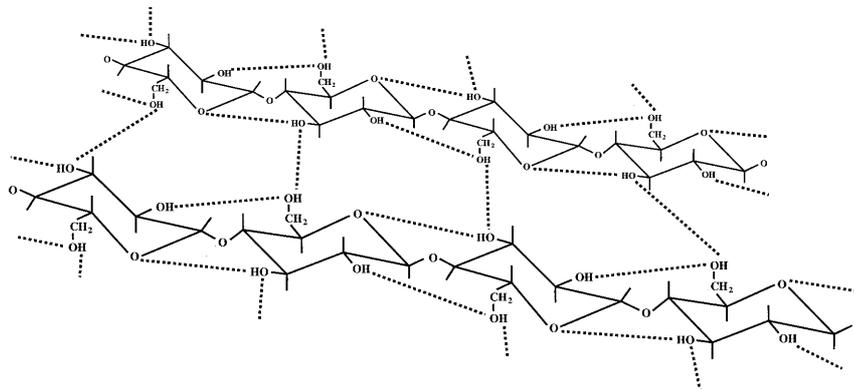
The sheets interact with each other via van der Waals forces and in cellulose Iβ (defined below) also with weak C—H···O bonds. This results in a highly rigid structure (Viëtor *et al.*, 2000; Nishiyama, Langan & Chanzy, 2002). A single isolated chain is more flexible but with an unususal rigidity due to the intra-chain hydrogen bonds. There are several forms of cellulose (I, II, III$_1$, III$_{11}$, IV$_1$ and IV$_{11}$) but in nature only I is found (O´Sullivan, 1997). It is divided into two subforms, Iα and Iβ, where Iα is the less stable one that can be converted into Iβ. The two forms are most often mixed although some rare examples of almost pure forms exist (VanderHart & Atalla, 1984; Sugiyama, Persson & Chanzy, 1991; Imai *et al.*, 1999). Due to its less ordered structure Iα is also assumed to be more reactive (O´Sullivan, 1997). The degree of crystallinity in a cellulose can be determined with x-ray diffraction and is calculated by comparing response from crystalline and non-crystalline cellulose in one sample (Segal *et al.*, 1959). In naturally occurring celluloses the degree of crystallinity varies between 40 and 90 %. The remainder of the cellulose is referred to as non-crystalline or "amorphous" (O´Sullivan, 1997). Interestingly, cellulose chains at the surface of a crystalline microfibril have a different environment then a deeper lying chain and will thus appear as two separate peaks (in the amorphous region) in NMR studies of cellulose materials, e.g. (Larsson, Wickholm & Iversen, 1997). Amorphous cellulose has not been widely studied but it is thought to be composed of cellulose chains randomly connected with hydrogen bonds between the C2, C3 and C6 hydroxyl groups (Kondo & Sawatari, 1996).

1.7.2 Hemicellulose

Hemicelluloses is used as a common name for a group of polysaccharides associated to cellulose. While cellulose is an unbranched homopolymer of glucose, the hemicelluloses are often branched heteropolysaccharides, i.e. they consist of more than one type of saccharide. Apart from glucose, they may contain mannose,

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xylose, arabinose and to some extent rhamnose. Hemicelluloses generally have a much lower degree of polymerisation than cellulose, i.e frequently around 200 (Sjöström, 1981). The most important hemicellulose (ca 20 % of dry weight) found in softwoods is galactoglucomannan. As the name indicates it consists of galactose, glucose and mannose (Sjöström, 1981).

1.7.3 Pectin

Pectin is a branched polysaccharide and in this respect resembles hemicellulose. There are however at least two differences. The main difference is that pectin is acidic since it has galacturonic acid, with a carboxylic group attached to C-5, as its main constituent. The carboxylic group may also be methylated ($COOCH_3$ instead of COOH). Pectin also contains rhamnose, which has a methyl group attached to C-5. Pectin without rhamnose forms straight narrow helices, known as "smooth regions". When rhamnose is present the structure becomes branched and is referred to as "hairy regions" (Thakur, Singh & Handa, 1997). The second difference from hemicellulose is that pectin may have Ca^{2+}-ions crosslinking the smooth regions provided they are rich in carboxylic groups, i.e. not methylated. Removing Ca^{2+} weakens the structure (Thakur, Singh & Handa, 1997; Zhang *et al.*, 2003).

1.7.4 Lignin

Lignin differs considerably from polysaccharides in that it consists of aromatic groups and is highly branched/cross-linked. The three basic lignin precursors are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Fig. 7). In principle, there is no known limit for the size of the lignin polymer.
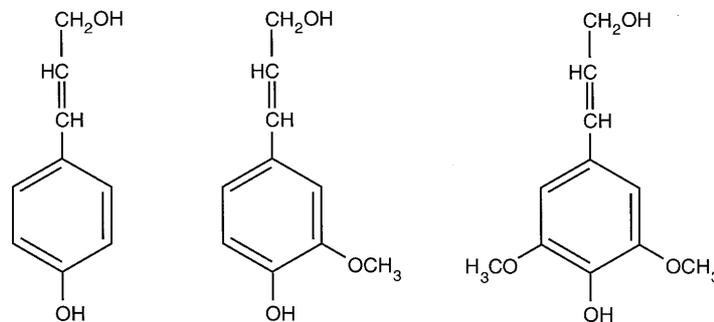
Figure 7. The three basic precursors of lignin: 1. *p*-coumaryl alcohol, 2. coniferyl alcohol and 3. sinapyl alcohol. The precursors bind to each other in numerous ways via their substituents.

1.7.5 Extractives, especially tannin

Extractives are components in wood that can be removed with, for example, acetone extraction. This group contains fats and terpenes but mainly different phenolic compounds such as flavonoids and tannins. Two main types can be

identified, hydrolysable and condensed. Hydrolysable tannins have glucose as their central component. The glucose molecule is frequently derivatised with gallic acid (3,4,5 trihydroxy benzoic acid) or di- or trigallic acid. The gallic acid residues are connected to glucose and to each other (in di- and trigallic acid) via ester linkages (Fig. 8) (Lekha & Lonsane, 1997). Condensed tannins are composed of flavon-type phenols connected to each other via C-C-bonds (Fig. 8).
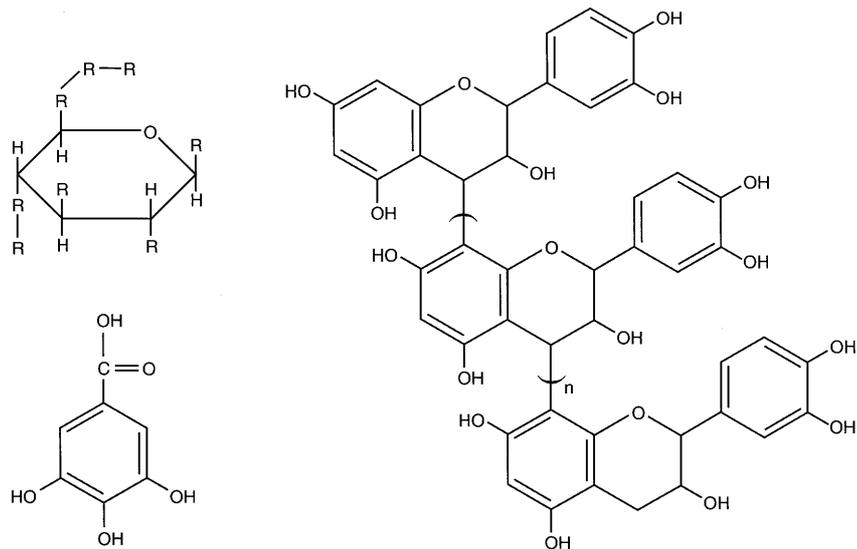
Figure 8. Typical hydrolysable tannin (to the left) and condensed tannin (to the right). Note the sugar residue being the base for the hydrolysable tannin. The substituent (R) on the hydrolysabel tannin is gallic acid. Redrawn from (Taiz & Zeiger, 1991)

1.7.6 Inorganic materials

Apart from different carbon-based constituents, wood also contains inorganic constituents often referred to as ash. They generally compose 0.1-0.5 % of the dry weight of wood (Panshin & de Zeeuw, 1980). Metals represent an important part of this fraction. In black spruce the five dominating ions found are Ca, K, Mg, Cl and Na. The amount of inorganics may differ considerably between different structures in the wood, from 2 % in the torus (where Ca dominated) to ca 0.1 % in the secondary cell wall (Saka & Goring, 1983).

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Materials and methods

2.1 Fluorescein isothiocyanate labelling of CBM

P.c. CBM1$_{Cel7D}$ was prepared by papain cleavage of Cel7D from *P. chrysosporium* culture filtrate (Tomme *et al.*, 1988) and chromatographic separation. FITC in a 10-fold molar excess relative to CBM in the reaction mixture was dissolved in dried N,N-dimethyl formamide. CBM was immediately added to this solution. The reaction mixture incubation was carried out on a vertical rotating disc (end-over-end) mixing at room temperature. CBM-FITC (Fig. 9) was separated from FITC either by filtering or reversed phase HPLC (RP-HPLC). The main peaks were pooled, desalted on a PD-10 column, freeze-dried and re-suspended in buffer. For identification of the peaks, reference runs with only unmodified CBM and FITC, respectively were conducted.
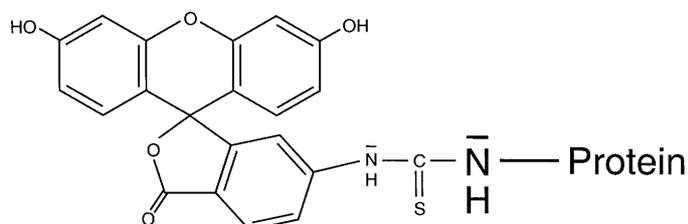

Figure 9. Diagram FITC bound to the N-terminal of a protein, in this case the CBM

2.2 Wood sectioning and incubation with CBM-FITC and FITC

In papers I and IV, 20 μm thick wood sections were cut using a sledge microtome. Sections were cut either tangential, longitudinally or radial to the fibre axis. In paper I, sections and pulp fibres were incubated with CBM-FITC, with FITC as a control.

2.3 Pre-treatment and incubation with enzymes

In paper IV, three types of spruce wood were used in the investigation: fresh spruce sap- and heartwood, tannic acid incubated fresh spruce sapwood and water-sprinkled spruce logs. Initially, fresh and sprinkled spruce sapwood were studied with regard to the effect of pectinase and H$_2$SO$_4$-pretreatment on tori breakdown. The analysis was done with single samples of spruce in a large array with varying wood samples, varying H$_2$SO$_4$-pretreatment and pectinase concentrations. Secondly, pectinase and tannase action on fresh spruce and tannic acid treated fresh spruce were analyzed. Tannic acid-treated sections were obtained by incubation of fresh sections in a large excess of tannic acid over night at room

temperature. Third, the effect of tannase as well as pectinase on tannic acid-treated spruce and sprinkled spruce was tested. Fourth, manganese peroxidase was applied to water-sprinkled and fresh spruce. To maintain the H_2O_2-dependent conversion of Mn^{2+} to Mn^{3+}, which in turn attacks phenolic structures in lignin (Glenn, Akileswaran & Gold, 1986), small aliquots of H_2O_2 were applied over several hours.

2.4 Fluorescence microscopy

The principle for the fluorescence microscopy used in papers I and IV was the same. Samples were irradiated with exciting light. The exciting light was generally filtered to avoid unnecessary excitation of lignin which occurs around 280 nm (Albinsson *et al.*, 1999). Thereafter the fluorescence of the samples was studied after passing a filter that cuts away most of the background fluorescence. In paper I, sections were mounted on slides and examined using an Olympus BX50 fluorescence microscope equipped with a CCD-camera or an Olympus BH2 fluorescence microscope (for the birch samples) equipped with a conventional camera. Samples were irradiated with light using a 450-480 nm excitation filter and a 515 nm emission filter. Birch samples were examined with a 455 nm excitation filter but without emission filter. In paper IV, sections were examined with a Leica DM RE fluorescence microscope using a green filter (N2.1, excitation range green, excitation filter BP 515-560). A Leica DC300F digital camera was attached to the microscope.

2.5 Binding isotherms

In papers I, III and IV, binding isotherms were used for characterisation of the interaction between two species. At a constant excess of binding target (e.g. BMCC) an increasing amount of ligand (e.g. CBM) was added. After equilibration the binding target (which must be solid) was centrifuged down and the remaining concentration of ligand was determined. Eqation 1 describes the binding and can be adapted to the data points. (Fig. 10).

$$B = \frac{B_{\max} \times [F]}{K_D + [F]}$$

Equation 1. Description of a binding isotherm. B is bound amount, $B_{\max}$ is the maximum amount bound, [F] is the concentration of free ligand and K_D is the dissociation constant.

$$K_D = \frac{k_{off}}{k_{on}}$$

Equation 2. The components determining the dissociation constant K_D
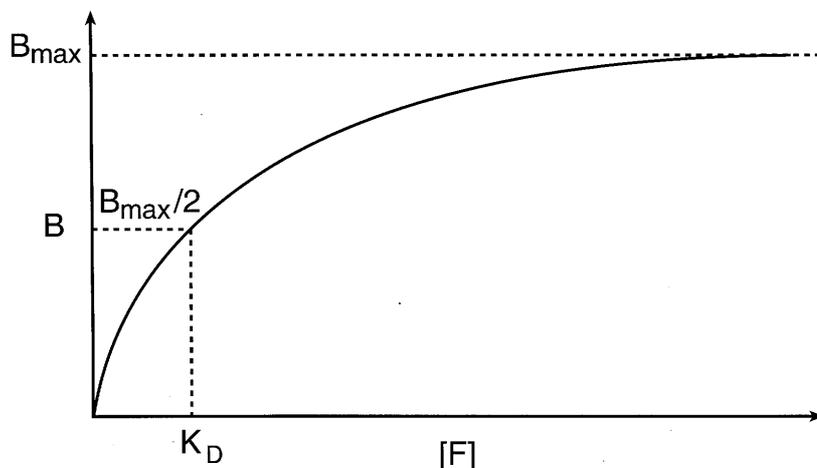
20

Figure 10. Diagram explaining the different paramaters in Equation 1.

K_D in Equation 1 is perhaps the most interesting parameter since it reveals the strength of the binding. One should note the fact that K_D reflects k_{off} according to Equation 2. Since k_{on} can be assumed to depend on diffusion of the binding species (which is the same for particles of similar size), a difference in magnitude of K_D reflects a difference in magnitude of k_{off}, and thus the lifetime of the complex.

2.6 Sugar analysis - different assays

The release of sugar into solutions can be measured in various ways. Sugars do not show absorption of light and no fluorescence upon excitation. There are methods to measure sugar directly, e.g. by refractometry. However in all cases in this work indirect methods involving colour-generation were used.

The anthrone method (Hörmann & Gollwitzer, 1962) (used in paper III) is based on conjugation of anthrone (9(10H)-anthracenone) to sugar via a condensation reaction performed under highly acidic conditions. Analysis of sugar was done using cellobiose as a standard and absorption measurement were made at 585 nm. This wavelength was chosen to minimize interference from any anthrone-tryptophan conjugate, which at that wavelength has an isobestic point in its spectrum (Hörmann & Gollwitzer, 1962).

The 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay (used in paper III) is composed of several steps (Gruno *et al.*, 2004). In summary ABTS was added to the sugar containing solution which was then incubated with β-glucosidase (which converts all sugar to glucose), glucose oxidase which converts glucose to gluconolactone and thereby stochiometrically produces H_2O_2 and horseradish peroxidase which catalyses H_2O_2-oxidation of ABTS. The presence of sugar was reflected in a green colour which absorbs light at 420 nm. A_{485}-values (no green colour absorption) were, as an estimate of any background from proteins

(some minute precipitation can occasionally occur), withdrawn from the A_{420}-values.

In the 3,5-dinitrosalicylic acid (DNS) method used in paper IV, DNS was reduced at high pH by any reducing sugars present. A yellow/red colour then developed and the absorbance recorded in the samples reflected the sugars present (Sumner, 1921; Miller, 1959).

2.7 Handling of pulps

The pulps used in papers I and III were never dried. Pulp is a non-homogenous material with a distribution of fibre/particle length/size. The smallest particles, fragments of broken fibres, are generally referred to as fines. In paper III, fines were defined as all particles passing through a net with 140 µm wide openings (ca 110 mesh) upon repeated washing. Analyses in paper III were done both with and without fines.

2.8 Determination of suitable enzyme/substrate ratios for incubation of pulps with enzyme

In order to ensure a surplus of enzyme as well as an easily detectable amount of released sugar in paper III, a number of tests were performed. The maximum amount of pulp fibres possible was limited by the fact that at a pulp concentration over ca 10 g/l the pulp suspension becomes too viscous to allow sufficient mixing in the syringes (described below). Two different ways of analysis were used. Enzyme (*T.r.* Cel5A) at a given concentration was incubated with different amounts of substrate, or alternatively a suitable amount of substrate was incubated with increasing amounts of enzyme (N476). Any decrease in activity over time due to non-productive binding or degradation of the enzyme was investigated by doubling the enzyme concentration at a later stage of incubation.

2.9 Enzymatic degradation of pulps - incubation in syringes

Since pulp does not form stable pellets in the same way as e.g. bacterial microcrystalline cellulose (BMCC) does upon centrifugation, the pulps in paper III were in most cases incubated in syringes equipped with a filter. Small samples were taken at desired times during enzyme incubation and as the sample passed through the filter the reaction was stopped since virtually all of the substrate was bigger than the filter pores. There was a rather small and constant leakage of sugar which was later compensated for in the analysis. Since enzyme was present in excess, the assumption was made that when samples were taken, the enzyme concentration remain rather constant while the substrate concentration increased. The increased sample concentration and decreased volume was also compensated in the analysis. Small background readings for sugars from the glycosylated proteins was withdrawn from the achieved values. Incubations were carried out end-over-end or on a swaying table, both in a thermostated heating chamber. Pulps with different mechanical properties were analysed.

2.10 Scanning electron microscopy (SEM)

SEM is a microscopic method which can be used for analysis of surfaces. Samples were freeze-dried and mounted on a small metal stub using double sided cellotape. Thereafter, samples were coated with gold using an argon filled Polaron E5000 sputter coating device. Following coating, samples were observed in a Philips XL30 SEM operated at 15 kV using the secondary electron mode with images collected digitally.

2.11 Labelling of reduced ends with anthranilic acid

A bleached softwood pulp was labelled by reductive amination of reducing sugars with anthranilic acid (2-aminobenzoic acid, AA), a technique originally developed for analysis of soluble reducing sugars with HPLC (Anumula, 1994) (Fig. 11). The labelled pulp was washed extensively with ethanol/water, buffer and water and thereafter stored in a moist condition. Release of AA-labelled carbohydrates by N476 degradation was studied by syringe-incubation with N476. AA-fluorescence analysis of the released labelled sugar was done with a spectrofluorimeter at $\lambda_{excitation}$= 330 nm, $\lambda_{emission}$= 425 nm and with pure AA as reference.
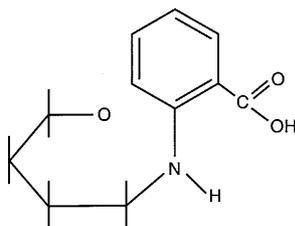
Figure 11 Reducing sugar labelled with anthranilic acid

2.12 Detection of condensed tannins - Vanillin/HCl analysis

The vanillin/HCl test for localisation of condensed tannins performed in paper IV was based on that vanillin (4-hydroxy-3-methoxybenzaldehyde) forms a red adduct with flavon-type structures present in condensed tannins (Gardner, 1975). The reaction, a H^+-catalysed condensation, was carried out at high HCl concentration. Fresh and water-sprinkled spruce were analysed with 50 % HCl treatment as control. Samples were photographed with normal light microscopy.

Present investigations

3.1 Enzymes as qualitative tools - a fluorescent CBM

3.1.1 Background to the results

Currently there is no specific dye known for cellulose. Some dyes, e.g. Congo red, interact non-covalently with carbohydrates but simultaneous labelling with other carbohydrates (hemicellulose) when studying native lignocellulosic substrates is common (Wood, 1980)(Taylor *et al.*, 1996). This is probably because the different carbohydrates in lignocellulose in many aspects resemble each other (e.g.,hydrophilic, OH rich "edges" and hydrophobic carbon rings). Another possible approach is to use antibodies against the sugar under study. However, antibodies are comparatively large molecules and may also display non-specific binding to other sugar motifes.

A way around these problems is to use specific enzymes tailored by nature to work on native lignocellulose substrates and recognize specific types of carbohydrates such as cellulose, in which case the enzyme should be a cellulase. One problem with using intact cellulase is that they are inclined to degrade the substrate. A cellulase is also quite a large compared to the substrate so labelling may be sparse. A CBM is a more appealing alternative, especially fungal CBMs of family 1 due to their small size of ca $3 \times 2nm$. Another possible candidate, bacterial CBMs of family 2, that are also known to bind very firmly to cellulose were rejected since they are larger and their binding surfaces are known to interact with each other in solution at higher concentrations (Tomme *et al.*, 1998).

One obvious family 1 candidate would be the CBH Cel7A from the easily grown fungus *T. reesei* (CBM1$_{Cel7A}$). However, this CBM had already been rejected as a good candidate for labelling of cellulose in native lignocellulosic samples (Taylor *et al.*, 1996; Jervis, Haynes & Kilburn, 1997). Most likely it was a poor label since its three conserved aromatic residues are all tyrosins resulting in weak substrate binding. In paper I, we selected CBM1$_{Cel7D}$ from *P. chrysosporium* because the tyrosine in position 5 is replaced by a more hydrophobic tryptophan (Linder *et al.*, 1995). This gives it a lower k_{off} and since k_{on} is the same as for the *T. reesei* CBM1$_{Cel7A}$ (k_{on} is diffusion controlled and the two CBMs are of similar size) it has a higher K_D, i.e. it is somewhat firmer attached to cellulose. The result is decreased problems with washing times etc. It has also been found to bind more than twice as firmly to cellulose than *T. reesei* CBM1$_{Cel7A}$ (Johansson *et al.*, 1989). While the catalytic module of cellobiohydrolase Cel7D (CBH 58) from the whit-rot fungus *P. chrysosporium* is roughly 6 nm long and is connected to the CBM via a rather long loop, the CBM itself is only 3 nm long (size determined for an analogue CBM from *T. reesei*)(Sims, James & Broda, 1988; Kraulis *et al.*, 1989; Linder *et al.*, 1995; Munoz *et al.*, 2001a). They are also specific for crystalline cellulose due to their flat area of exposed aromatic rings.

3.1.2 Labelling of the CBM and use of the conjugate

The CBM was isolated from intact Cel7D by papain cleavage. It was then purified and characterised by binding isotherms to possible substrates in lignocellulose, i.e. cellulose (BMCC), mannan and xylan. It was found to bind only to cellulose with a K_D of 7.6 μM. This figure can be compared to *T. reesei* $CBM1_{Cel7A}$ which has a higher K_D on BMCC (roughly 10-15 μM) reflecting its weaker binding (Linder *et al.*, 1996) (Tomme *et al.*, 1998). In order to facilitate microscopic studies of bound CBM (as is the case with all proteins) it must be labelled. We chose labelling with fluorescein isothiocyanate (FITC) in order to maintain a small size of the probe. FITC normally binds to lysins but since *P. chrysosporium* $CBM1_{Cel7D}$ lacks lysins the only possible binding site is the α-amino group. The labelled CBM-FITC was separated from the surplus of FITC by either filtering (for analytical use on lignocellulose samples) or with reversed phase HPLC (for analysis of the conjugate). The CBM-FITC conjugate was found to have a similar binding capacity to cellulose as the unmodified CBM.

Fresh wood sections and pulp fibre samples were incubated with a solution containing CBM-FITC for at least 4h. The samples were thereafter washed quickly in a large excess of water for 2-5 min. The short washing time is important since the CBM despite stronger binding than its analogue from *T. reesei*. is for all practical purposes considered as a reversible binding probe. This was also evident during microscopy. Directly after washing, the samples had high contrast which then faded quite rapidly and finally ended at a stage where the most dominating structures were apparent but the background makede detailed analysis impossible. The labelling for both spruce and birch was stronger in areas showing damage and to areas known to have microfibrils horizontal to the cut surface (Fig. 12 and 13). In radially cut samples of spruce, a lamellar-like patterns was evident (see paper I). This is interesting since there is still an ongoing debate on whether the microfibrils in the S2-layer have a concentric or radial sub-orientation, e.g., (Sell & Zimmermann, 1993). Our results indicate a concentric orientation. With unbleached pulp fibres (4 % residual lignin) the CBM-FITC showed a homogenous pale colouring with the exception for enhanced binding to damaged fibre regions (see paper I), the pale colour considered to reflect background (Fig. 14).

3.1.3 Conclusions - other conjugates with the CBM are also possible

In conclusion, the CBM-FITC probe gave promising results. The single binding position for FITC on the native CBM has the side effect that the CBM-FITC is a mono derivative which is crucial when using the probe for quantitative determination of exposed cellulose on pulp fibre surfaces. CBMs of this small size can be synthesized *in vitro* (Johansson *et al.*, 1989) allowing changes of the amino acid sequence without using genetics. Examples of what may be possible is insertion of lysine on the "back" of the CBM. Lysines are tags for connecting for example FITC as well as biotin. Biotin in turn would allow the CBM to be used as a scaling tool since avidin or streptavidin (proteins binding to biotin) with differently sized attached groups are commercially available. More then one lysine would most likely cause structural problems to the small CBM. Cysteine, another

possible tag would on the other hand be likely to cause structural problems, since the CBM already contains four cysteins, in critical S-S-bonds.
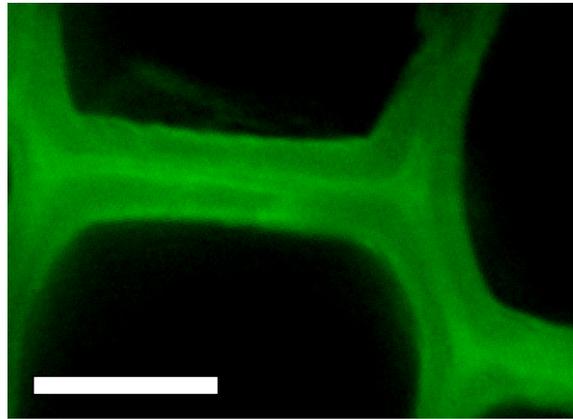

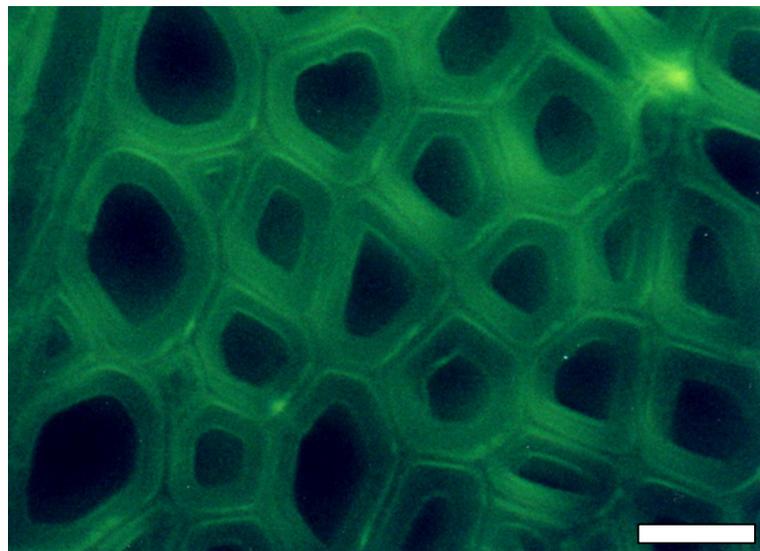Figure 12 CBM-FITC binding to transversially cut spruce. S1 and S3 is indicated with arrows. Scale bar is 10 μm.

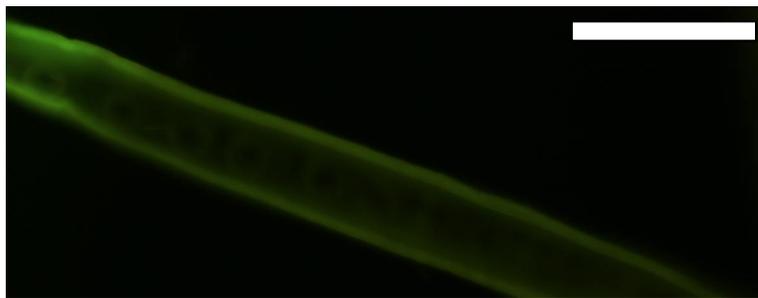Figure 13 CBM-FITC binding to transversially cut birch. Note enhanced binding to, especially S1. Scale bar is 10 μm.

Figure 14 CBM-FITC binding to unbleached pulp fibre. Only weak labelling. Scale bar is 100 μm.

3.2 A general view of enzymes with cellulose affinity

3.2.1 A few comments to the review - paper II

In all papers in this thesis as well as the additional papers cellulases, CBMs or cellulase related enzymes have been used. In paper II the current status of cellulase and CBM research was reviewed. The cellulase research emanates from solubilization of pharmaceutical products containing carboxymethyl cellulose (CMC). It was soon found that the problems were caused by the fungi *Aspergillus niger* and *Aspergillus flavus*. At an early stage a gram-negative bacteria able to degrade CMC was also isolated (Reese, Siu & Levinson, 1950). Since the 1950's the research field has grown considerable but was still in 2002 dominated by fungi, e.g. *T. reesei*. When studying papers published during 2003 a trend change was noted and the majority of the papers dealing with cellulases in a fundamental way (i.e. not optimal conditions for ethanol production etc) were based on bacteria as enzyme sources.

However, recently the genome of the white-rot fungus *P. chrysosporium* was fully sequenced (Martinez *et al.*, 2004) (Teeri, 2004). Other completely sequenced fungal genomes are species from the ascomycetes such as *Saccharomyces cerevisiae*, However, the *P. chrysosporium* genome represents the first sequence from a basidiomycete as well as the first from a cellulose degrading fungus. It is possible that this achievement will draw the focus back to fungal cellulases especially since the cellulolytical system of *P. chryspsporium* in many respects is similar to that of the more studied and used *T. reesei* (Pettersson, Johansson & Ståhlberg, 1990; Uzcategui *et al.*, 1991a; Uzcategui *et al.*, 1991c).

3.2.2 A nomenclature suggestion

The nomenclature of cellulases as well as for CBMs has seen a lot of changes over the last decade. A lot of important work has been done by Bernard Henrissat who currently is one of the persons maintaining the CAZy-server (http://afmb.cnrs-mrs.fr/CAZY/; 30-Aug-2004). The new nomenclature is based on amino acid sequence similarities that indicate common folding patterns. However, there is a

problem built into this nomenclature. It can be unhandy when discussing several enzymes in parallel since as for example the *P. chrysosporium* cellobiohydrolase formerly known as CBH58 and the *T. reesei* endoglucanase formerly known as EGI, are today denoted Cel7D and Cel7B respectively. This is because they are closely related but have small but important deviations in their active site surroundings Loops surrounding the Cel7D active site are more tightly closed than in Cel7B, restricting Cel7D to processive degradation of cellulose (Munoz *et al.*, 2001b). To revert to the old nomenclature can not be recommended, but in paper II an amendment is suggested. The above mentioned enzymes would with this nomenclature be denoted as Cel7Dc (c for cellobiohydrolase) and Cel7Be (e for endoglucanase) respectively.

3.2.3 Topics with few or no publications

Another interesting observation when scanning the cellulase literature is that the allergic properties of cellulases have over the last three years only rendered a few articles. However, if cellulases are to be used in the pulp and paper industry it is important to consider that the enzymes (especially if dry) have been questioned (as a occupational health problem) in other commercial applications such as in the detergent and baking industry (Schweigert, MacKenzie & Sarlo, 2000) (Elms *et al.*, 2003). From practical experience in my laboratory I can also add that even a rather limited exposure to cellulases can cause allergy. This may also be worth considering if pulp should be modified for use in items such as diapers.

Another area where publications are lacking is the successful degradation of native substrates with mutated enzymes. A lot of mutants exist (see paper II and references cited therein) but so far, to my knowledge, no studies describing increased degradation of "native" substrates such as bagass or waste paper based on genetically improved enzymes have been published. This may be only a matter of time but it may also reflect other aspects of enzymatic degradation of cellulose. Why has the evolution of cellulases not been driven towards quick degradation? Is it perhaps impossible? David Wilson stated in a recent paper that he believes, based on studies of mutant enzymes, that the binding of cellulose to the active site rather than the cleavage of the cellulose chain is the rate limiting step (Wilson, 2004). The question may be taken one step further - is the release of the cellulose chain from adjacent chains the rate limiting step, at least in some cases?

3.3 Enzymes as quantitative tools - degradation of pulp with endoglucanases correlate to certain paper properties

3.3.1 Background to the results

Paper is composed of fibres connected to each other by non-covalent bonds. The strength of paper is determined by the strength and intrinsic properties e.g., flexibility of the fibres themselves and of the strength of the bonds between the fibres (Duchesne, 2001). Other components, i.e. carbohydrates, lignin etc, present at the surface of the fibres affect the latter. Bearing this in mind, paper III was designed to investigate one aspect of the fibre surface - the distribution and character of non-crystalline cellulose.

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The surface ultrastructure of pulp fibres have been thoroughly investigated especially with different types of electron microscopy (Duchesne & Daniel, 1999). These investigations have rendered important information, for example we now know, from FE-SEM studies that the apparently smooth surface observed on fully bleached pulp fibres with light microscopy as well as with SEM is actually not smooth. It is perforated with pores giving an appearance of a rolled up fishing net (Duchesne & Daniel, 2000). The "net" has been interpreted as single macrofibrils attached to each other. The size of most of the pores are in the range of 10-100 nm. Noteworthy is that the size of the pores is in the same order as the size of cellulase (10-20 nm).

The problem with many microscopic techniques is the lack of quantitative information. In this context the use of cellulases represent a good complementary technique to render information about the pulp fibre surface. Enzymes have been used been used previously for studying pulp fibres i various ways, e.g. (Yang, Pettersson & Eriksson, 1988; Ander, 2002). In paper III endoglucanases were chosen for the study of chemistry of the fibre surface. However, if one is looking for a correlation between substances on pulp fibre surfaces and paper strength the amount of released sugars alone will have little meaning. Therefore at an early stage it was decided to incorporate information about the mechanical behaviour of the pulps studied.

3.3.2 Choice of enzymes - endoglucanases

From earlier unpublished SEM studies it was evident that a system including both endoglucanases and exoglucanases would not have been able to render reliable information about the surface of pulp fibres. Such a full system leads quite rapidly to an extensive breakdown of bleached pulp fibres whereas the endoglucanase Cel5A from *T. reesei* gave no visible changes to the fibre surface although an increase in the number of broken fibres was noted (see fig. 15 and 16). Cel5A was therefore chosen for establishing the practical aspects of the analysis. It was found that within a certain range of pulp concentration the release of products by Cel5A is linearly dependant on pulp concentration. This made incubation in syringes equipped with a filter possible since the increase in substrate concentration upon withdrawal of sample containing only product and enzyme could be compensated for during the analysis. Incubation in syringes was desired partly because fibres do not form stable pellets upon centrifugation and partly due to the obvious complication in weighing in small samples of a moist, comparatively inhomogeneous substrate. The fines fraction of the pulp released more sugar upon endoglucanase treatment than the fibre fraction. However, since the proportion of the fines fraction represents only a few percent of the total pulp concentration its contribution was found to be of little importance for the total analysis. This was also confirmed in later experiments (see below). A statistical difference in sugar release was noted between two similar, fully bleached pulps degraded with *T. reesei* Cel5A. The degradation pattern was bi-phasic with a rapid burst of sugar release at the beginning (see fig 17).
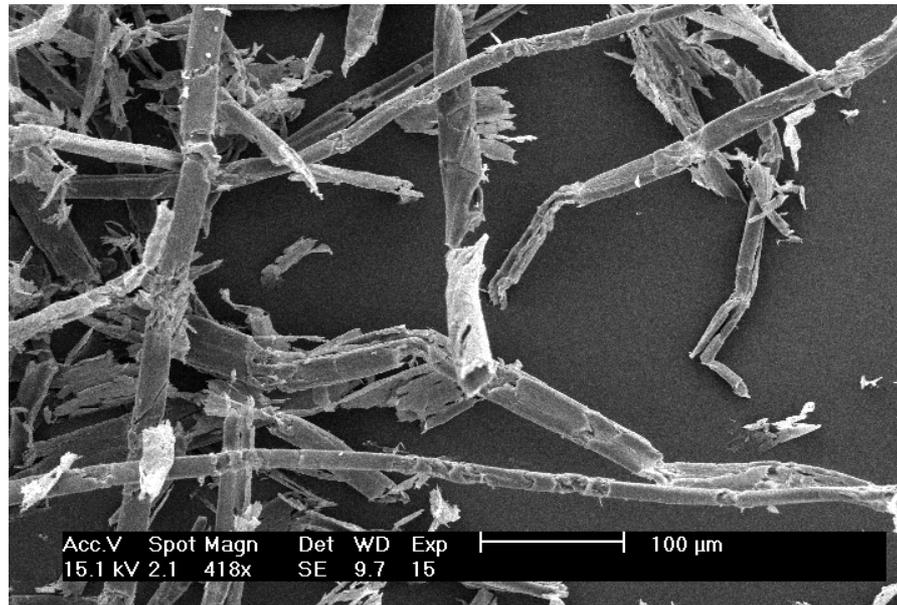
Figure 15. SEM-picture of leached pulp fibres degraded with both endo- and exoglucanases. Visible degradation is extensive.
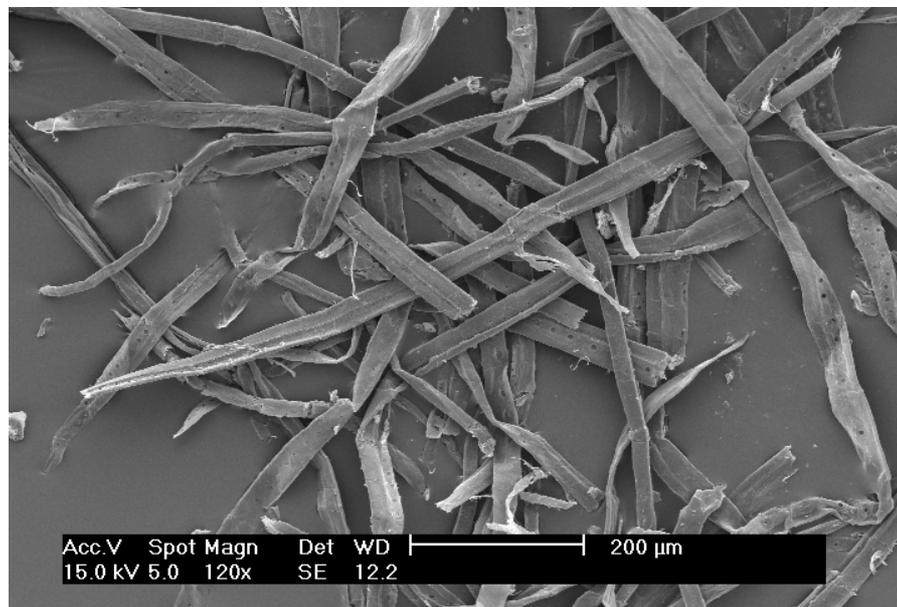

Figure 16. SEM-picture of bleached pulp fibres degraded with endoglucanases only. Visible degradation is limited to breakage of a few fibres
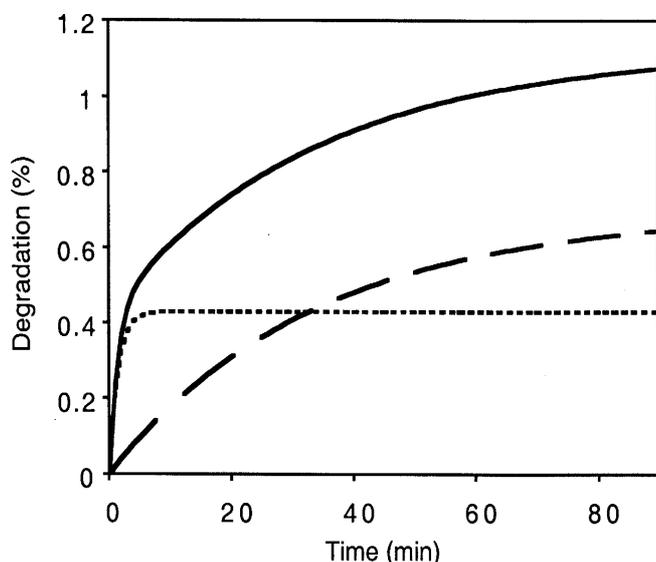
Figure 17. An actual degradation curve (solid line) used to show the two participating parts ("fast" (points) and "slow" (dashed) substrate) in the double exponential equation 5.

Having established the method we changed the endoglucanase from *T. reesei* Cel5A to Novozym 476 (N476), a commercially available, recombinant endoglucanase from an *Aspergillus* species. The change was done partly to verify the method with another endoglucanase but foremost to allow an increased number of larger (50 ml instead of 10 ml) incubations with a new set of - with respect to mechanical properties - well characterised pulps. Of the pulps (all were fully bleached) two were from birch and three from spruce. N476 proved to function well and the degradation pattern was reproducible and similar for all of the pulps, although the relative degradation varied (ca 0.8 - 1.4 % degradation at 90 min). For N476, like *T. reesei* Cel5A the degradation pattern was bi-phasic.

3.3.3 A biphasic degradation pattern and its correlation with paper properties

The idea of different subclasses of substrates of native lignocellulose and cellulose is not new (for reviews see, (Mansfield, Mooney & Saddler, 1999; Lynd *et al.*, 2002)) and the three reasons generally discussed are cellulase inactivation, product inhibition or substrate heterogeneity. Both for *T. reesei* Cel5A and N476, cellulase inhibition could be ruled out since an excess of the enzyme was used in both cases. Product inhibition could also be ruled out since the maximum level of sugar (ca 200 µM) is well below inhibition concentrations for Cel5A (in the range of 10 mM) (Gruno *et al.*, 2004). The degradation pattern was therefore interpreted that

the pulp fibres may present a heterogeneous substrate composed of at least two main types of substrate. One easily degraded and/or highly exposed and one more resistant to degradation and/or less exposed.

Since our aim was to couple the surface character of the fibres to the mechanical properties of paper, we had to convert the degradation curves to empirical parameters. To perform this we tried three different models (Equation 3-5) with non-linear regression to the data including; i) a pseudo first order reaction, ii) a fractal-like kinetics analogue of a pseudo first order reaction (used in synergy studies (Väljamäe *et al.*, 2003)) and iii) the sum of two pseudo first order reactions.

$$[\text{P}] = a \times \left(1 - e^{-b \times t}\right)$$

Equation 3.

$$[\text{P}] = s \times \left(1 - e^{-a \times t^{n}}\right), \text{n=1-h}$$

Equation 4.

$$[\text{P}] = a \times \left(1 - e^{-b \times t}\right) + c \times \left(1 - e^{-d \times t}\right)$$

Equation 5.

Equations 3-5. In all of the equations [P] stands for the extent of degradation (% of total pulp) and t is the hydrolysis time. All other parameters are empirical. In equation 5 one can consider a as being amount of "fast" substrate disappearing with a speed described by b. The same is valid for the "slow" substrate and parameters c and d.

The pseudo first order model gave a poor fit. The two latter models both turned out to describe the degradation well but since the empirical parameters (a is amount of "fast" substrate and b describes the speed with which it is degraded, the same is true for the "slow" substrate and parameters c and d) derived from the sum of two pseudo first order reactions are simpler to attach a physical meaning, we choose to use this model although it contains one parameter more than the fractal-like kinetics analogue of a pseudo first order reaction. The quote a/c in equation 5 gave correlation to mechanical parameters depending on the strength of fibre-fibre interactions but not to the same extent with, e.g. zero-span, which is a parameter describing fibre strength (Fig. 18). These results are in good correlation with the observation that endoglucanase pre-treatment of pulp decreases strength with respect to fibre-fibre interactions (Oksanen *et al.*, 1997).
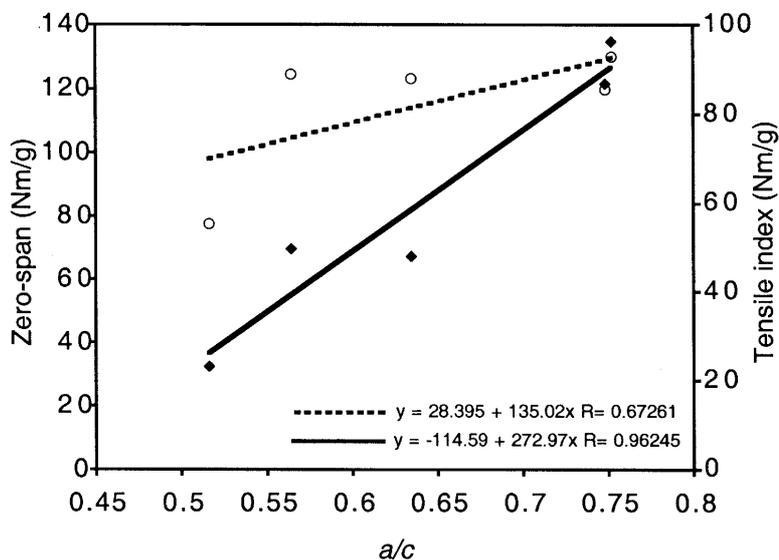
Figure 18. a/c from a degradation of one pulp determined with equation 5 vs zero-span (○) and tensile index (♦). Correlation to the fibre-fibre-interaction dependent tensile index is better than the one to zero-span which is not fibre-fibre-interaction dependent.

3.3.4 Further analysis of the degradation pattern of pulp fibres by labelling reducing ends

In order to obtain a better understanding of the substrate character in the first phase we labelled the reducing ends of the carbohydrates present in one hardwood pulp. We choose a technique originally used for HPLC- and capillary electrophoresis analysis of reducing sugars involving labelling of reducing ends with anthranilic acid (AA) as described in (Sato *et al.*, 1998). Such labelling does not hinder degradation of the cellulose but gives reduced activity (Kipper, Väljamae & Johansson, 2004).

The degradation of AA-labelled pulps gave a first phase of rapidly released AA-labelled sugar and a second phase of slower release. Thus, N476 has a preference for polysaccharide chains with loose ends (cellulose chains only connected to the pulp fibre in one end). From these results, the conclusion was drawn that the first phase of degradation consists to a greater degree of loose, or highly accessible (reducing) ends than does the second phase.

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3.3.5 Conclusions - a useful tool for studying pulp fibre surface ultrastructure

As originally considered, it may seem appealing to determine the length of the loose ends but this would probably be complicated, if at all possible. Oligosaccharides longer than six glucose units are poorly soluble, so cutting off longer loose ends is most likely difficult. Actually, it is hard to imagine loose ends with a length much longer that six glucose units. Another possibility is that the first phase of degradation is actually composed of small highly accessible bundles of non-crystalline cellulose. A way to study this aspect would be to pack a column of AA-labelled pulp (as done with pectin in paper IV) and allow a small amount of endoglucanase to pass through the column. A domination of cellobiose and shorter oligosaccharides over the longer oligosaccharides in the outflow would indicate very short loose ends. Important in this case is of course to have a separation of enzyme and product that prevents degradation of the primary product.

   It is not absolute that loose ends on the fibre surface are more important for paper properties than highly accessible loops. For analysis of exposed non-crystalline cellulose on fibre surfaces it is probably not important to distinguish between the two of them but just consider the amount of substrate in the first phase. An interesting continuation of the study would be to follow the sulphate pulping process and reveal new information on when and why the non-crystalline cellulose, the roughness, appears. A difficult but interesting task could also involve a search for changes in character of the non-crystalline part of cellulose in pulp fibres. A further continuation would include the use of different enzymes in order to investigate the correlation between paper properties and the exposure of some other pulp fibre constituent (e.g xylan).

3.4 Enzymes as logical tools - characterization of a substance making the tori of water-sprinkled spruce fluorescent

3.4.1 Background to the experiments

When wood is stored (e.g. outside a paper mill) it is often water-sprinkled to avoid drying out, insect attack and fungal degradation. During this process the outermost annual rings of the logs are often discolored brown (Dahm, 1963). One suggested reason for such discolouring is the migration of bark substances into the wood. This phenomenon is often referred to as "tannin-stain" or "bark-stain" and it has a negative impact on the wood value in e.g., mechanical pulping (Lorås, 1974) (Persson & Elowson, 2001). When studying samples of the outermost year rings of water-sprinkled spruce (as well as heartwood of fresh spruce) in a fluorescence microscope the most striking difference from the sapwood of fresh spruce was a very intense fluorescence in the tori of the pits (Fig 19). The pits are the water-permeable channels that connect wood cells to each other (see introduction) so an accumulation of a discolouring substance migrating with the sprinkled water did not seem odd. Some questions were however, raised. What is the substance and why does it fasten only (or at least very predominantly) to the tori of the pits? Can it somehow be removed? In paper IV an attempt was made to solve those questions by using enzymes with different substrate specificity as logical tools. With "logical

tools" implies that little or no qualitative and quantitative information is derived by using the enzymes - the answer is more of the type "either degradation or no degradation".
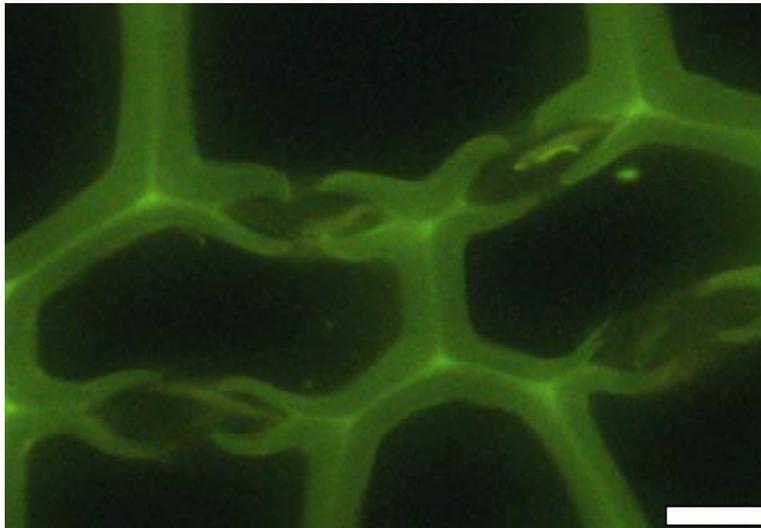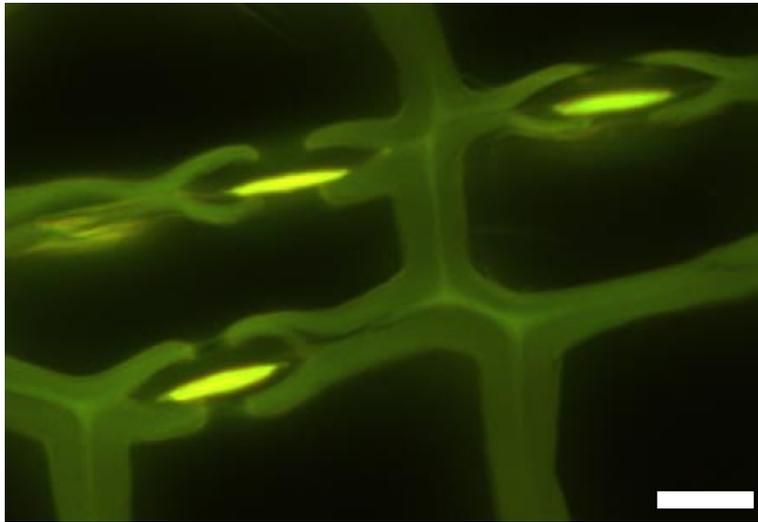

Figure 19. Fluorescent tori in water-sprinkled spruce (above) and tori in fresh spruce (below). Scale bar is 10 μm.

Bark is rich in phenolic substances, among those tannins. Tannic acid is one such substance (Sjöström, 1981). It is water soluble and seemed a good candidate

substance for explaining discolouration of the fluorescent tori. The tori are rich in pectin (Thomas, 1975; Hafren & Westermark, 2001) as well as metal ions (Thomas, 1975; Saka & Goring, 1983) and it was therefor natural to start the experiments by investigating the interactions between tannic acid and pectin as well metal ions (Ca^{2+}, known to be an important structural element in pectin, reviewed in (Thakur, Singh & Handa, 1997)).

3.4.2 Model experiments with a tannic acid/pectin system

Initially, micrographs of fresh spruce sapwood and heartwood as well a sapwood from water-sprinkled spruce were taken as reference . When comparing tannic acid binding to commercial pectin (originating from oranges) and pectin from the white part of orange peel, we found that tannic acid bound better to "white part" orange peel pectin. However it was not possible to determine whether this was due to the higher content of Ca^{2+} or due to the higher content of protein in orange peel pectin. Tannic acid is known to bind to both proteins and metal ions (Budavari, 1989) (Taiz & Zeiger, 1991). We also tried washing with 20 mM H_2SO_4, a method believed to remove Ca^{2+} from pectin (Zhang *et al.*, 2003), but this had only a minor effect on tannic acid B_{max}. We choose to use orange peel pectin as our model substance since any processing steps that the commercial pectin may had been subjected were unknown.

Since the tori are rich in pectin we studied the efficiency of pectinase under different conditions. It was found that the presence of tannic acid on commercial pectin had no inhibitory effect on pectinase while the presence of tannic acid on orange peel pectin had an inhibitory effect. Thereafter we studied whether tannin acyl hydrolase (an enzyme degrading tannic acid) was able to degrade tannic acid complexed with pectin. To investigate this aspect we first sought the isobestic point of the tannin acyl hydrolase causing release of gallic- and m-digallic acid from tannic acid. This was found as 267 nm (see inset in Fig. 20), which means that at 267 nm there is no difference between substrate and product in absorption of light, and an increase of absorption must, in this case, be due to an increased amount of released product. Following this, a column was packed with orange peel pectin grains (0.1 - 0.5 mm in diameter), connected to an HPLC-system and equilibrated in flow with 10 μM tannic acid. Injection of buffer into this system rendered a small decrease in A_{267} while the injection of tannin acyl hydrolase gave a sharp A_{267}-peak. This could not be explained by A_{267} of the injected protein itself. Nor could it be explained by simple dissociation due to the plug itself (buffer gave no release). It must thus be caused by a specific release of products or possibly tannic acid with the loss of one or more gallic acids previously responsible for its coupling to pectin. This was interpreted that tannin acyl hydrolase had the ability to degrade tannic acid complexed to orange peel pectin (Fig. 20).
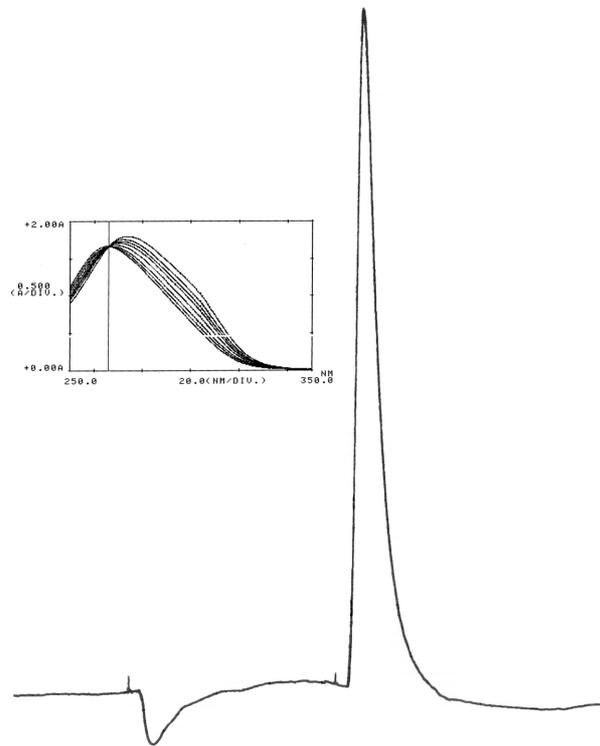
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Figure 20. Elution pattern from HPLC-column equilibrated whit 10 μM tannic acid. Injections: buffer (to the left) and tannin acyl hydrolase (to the right). The peak is due to degradation of tannic acid. Inset shows the determination of the isosbestic point

3.4.3 Studies on native wood - tannic acid becomes a less likely candidate for explaining fluorescent tori

At his point we directed our studies to intact spruce. It was found that pectinase, as expected (Ohkoshi, Tokuda & Sadoh, 1987), had the ability to degrade tori of fresh spruce sapwood. It was also, together with tannin acyl hydrolase, able to degrade fluorescent tori created by incubation in tannic acid. However, the tori of water-sprinkled wood were unaffected. Attempts to promote pectinase activity by pre-treating water-sprinkled spruce with H_2SO_4 or tannin acyl hydrolase failed. The fluorescent tori were unaffected. Noteworthy the tori created by tannic acid impregnation were rather less fluorescent than the water-sprinkling induced tori.

At this stage we were convinced that soluble tannic acid could be ruled out as responsible for tori fluorescence. We began to lean towards condensed tannins as being at least part of the explanation. Attempts to dissolve the fluorescent substance with ethanol and acetone also indicated a very inert, or well entangled,

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substance. Application of the lignin degrading enzyme manganese peroxidase from *P. chrysosporium* to the fluorescent tori samples strengthened this belief. *P. chrysosporium* manganese peroxidase normally participates in the degradation of phenolic lignin components and is also known to degrade condensed tannins in solution (Gnanamani, Sekaran & Babu, 2001). In some pit samples the margo, a thin structure holding the torus, was fluorescent. The number of fluorescent margos decreased from ca 50 % to ca 14 % in the samples treated with most manganese peroxidase. Finally we also performed a staining test (incubation in vanillin and HCl) normally used for detecting condensed tannins in solution. The tori were stained red. This indicated the possible presence of condensed tannins. Tori of fresh spruce were unaffected.

3.4.4 Conclusions - the fluorescent tori may be due to a stress respons

In conclusion, the explanation for the fluorescent tori is probably not due to tannic acid migrating from the bark upon water-sprinkling. Enzyme systems well equipped to degrade tannic acid situated on a torus had no visible effect on the fluorescent tori. It seems more likely that the fluorescent substance is, at least partly, composed of condensed tannins. We also found a striking similarity between the tori of water-sprinkled wood and heartwood. Heartwood formation is a naturally occurring saturation of the inner part of a tree with phenolic, poisonous, substances (Panshin & de Zeeuw, 1980). It is caused/induced by dying cells (Sjöström, 1981). A Finnish study recently put focus on synthesis of condensed tannins in the inner part of the stem upon conventional drying of birch. The authors noted differences in discoloration patterns upon different drying conditions (Luostarinen & Möttönen, 2004). Synthesis of precursors for condensed tannins as a protective response to stress (damage and insect attack) has been shown in *Populus tremuloides* (Peters & Constable, 2002).

It is tempting to speculate that there may be similarities between the processes and that the fluorescent tori are caused by local synthesis of condensed tannins by dying phloem cells. Possibly this may represent a way to close the tori, or at lest protect them from degradation. To further investigate this process it is necessary to compare the substances present in the two types of tori by isolating them for a more detailed chemical analysis. I would also like to create limited damage to growing spruces and take samples for tori analysis at different times. Finally it is obviously of great interest to counteract/limit the problem with brown wood upon water-sprinkling. However it seems unlikely that enzyme treatment is the solution. If the hypothesis of stress induced synthesis of condensed tannins is proven, the solution will likely involve shutting the synthesis off in some way. In general I feel that the continuation of this project is rather a question of wood biology and chemistry rather than using enzymes although they were very useful in putting the project on the right track.

Conclusions and future perspectives

The FITC conjugated CBM proved to be a useful tool for studying the distribution and, perhaps more importantly the exposure of cellulose in fresh wood and in pulp samples. In radially cut spruce sections a pattern indicating a possible lamellation of the S2-layer was noted. The CBM is rather small and it is possible to synthesize using chemical methods. Such a synthetic domain could be modified further by inserting a lysine residue to facilitate biotinylation on the "back" of the domain, i.e. on the side not facing the substrate. Adding cystein can not be recommended since the domain sequence already contains four cystein residues. Misfolding of the CBM would be the most likely result. Another possibility is to use the FITC-derivatized domain for a quantitative evaluation of the amount of cellulose exposed per gram of a pulp. Biotinylation provides a possibility to attach larger molecules for studies on accessibility of a surface but, bearing in mind that the domain binds comparatively weakly, such an experiment may give less reliable data. Thus in this case a stronger binding bacterial CBM would be preferable.

In the review article we suggested an amendment to the present nomenclature, at least for cellulases. The nomenclature used today is far better than the old system but it provides only immediate information about the main action and folding family. It provides no information about the dominating mode of enzyme action. This could be overcome with an addition of a lower-case letter at the end of the enzyme designation so that the *Trichoderma reesei* cellobiohydrolase Cel7A would read Cel7Ac and the *Trichoderma reesei* endoglucanase Cel7B would read Cel7Be. The system amendment could also be applied to other glycohydrolases.

The use of endoglucanses to study the character of non-crystalline cellulose on fibre surfaces seems promising. A number of correlations to parameters reflecting fibre interactions were found. Furthermore, a new methodology for studying release of reducing ends from BMCC was successfully adapted to pulp fibres. It showed that non-crystalline cellulose chains attached at only one end are released predominantly in the initial phase of enzymatic degradation. However, pulp fibres contain pores and a natural continuation of the project would be to connect large particles to the enzymes in order to restrict the degradation of the fibre surface. The sugar analysis could possibly be made easier with HPLC or biosensor in-line detection of released sugars. Another intriguing aspect to solve is how to control or eliminate contributions from the fibre interior which are accessible to the enzymes but most likely have little or no impact on paper strength properties. In conclusion we believe that this methodology can render new information about the character of pulp fibre surfaces and possibly on their impact on paper strength.

The experiments with water-sprinkled spruce indicated that the fluorescent substance found associated with tori from the outermost annual rings of logs is most likely composed of condensed tannins. The presence of tannic acid, which was the original hypothesis could successively be ruled out. Model experiments with orange peel pectin packed into a HPLC column showed that if present on the pectin of tori tannic acid would have been degraded by tannase. However, no degradation was detected with tannase. Degradation, although weak, was however

detected with *P.chrysosporium.* manganese peroxidase. This in combination with vanillin/HCl analysis indicated that condensed tannins dominate over hydrolysable tannins in the tori of water-sprinkled wood. Since tori of heartwood appear similar (fluorescent) and stress to wood is known to induce synthesis of condensed tannins, a natural continuation would be to study the colouration of spruce tori in a time resolved manner. The HPLC-experiment mentioned could also be used for studies of other similar systems, such as pulp, where studies on the interactions between a solid substrate and a degrading protein is a t hand.

In this work a number of aspects of wood and wood fibre ultrastructure have been investigated and clarified. Mostly this has been done with the aid of enzymes or part of enzymes such as a CBM. It seems that the use of enzymes as probes and analytical tools is limited by the imagination only. The fact that the enzymesand their analogues are commercially available, and are often well characterised and generally very resistant are also important.

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