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Enzymes hydrolysing wood polysaccharides

A progress curve study of oligosaccharide hydrolysis by two cellobiohydrolases and three β-mannanases

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Academic dissertation

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Abstract

Cellulose and hemicelluloses are major structural components of plant cell walls. Cellulases and hemicellulases are the enzymes which hydrolyse these biopolymers to small soluble oligosaccharides in nature. The enzymatic degradation processes are complex because of the need for different enzyme activities in the total degradation of the substrate. These degradation processes have been intensively studied since the 1970s, but there are still many unanswered questions concerning the degradation mechanisms.

In the present investigation only pure soluble oligosaccharides, both cello-oligosaccharides and manno-oligosaccharides, were used to study the hydrolysis of substrates catalysed by the cellobiohydrolases, CBHI and CBHII, and the β-mannanases, BMANI and BMANII, of *Trichoderma reesei* and one β-mannanase of *Aspergillus niger*. Hydrolysis experiments were performed using oligosaccharides with chain lengths of three to six sugar units. These reactions were monitored as a function of time and analysed by different independent analytical techniques, i.e. high performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). Experimental kinetic data were evaluated using progress-curve analysis, for which tailor-made computer programs were used.

NMR spectroscopy was used to study the stereochemical course of the enzyme reactions in order to determine the stereospecificities of the enzymes. The three β-mannanases used in the study behaved in a similar way and they belong to the same glycosyl hydrolase family. Both HPLC and NMR spectroscopy were used to study the cleavage patterns as well as to produce experimental progress curves for each substrate. According to the HPLC and NMR data, CBHI releases cellobiose from the reducing end of cellotriose, whereas CBHII releases cellobiose from the non-reducing end. Progress curves could be accurately fitted

by using tailor-made computer programs which can be applied to different enzymes. The rate and binding constants derived from the progress-curve analysis provide a reliable basis for the evaluation of enzyme kinetics. MS was used to screen the molecular mass composition in the reaction mixture during the hydrolysis. According to the MS analyses, no transglycosylation products were produced during the hydrolysis of cellotriose catalysed by CBHI or in the hydrolysis of mannotriose catalysed by BMANI. Interestingly, it was shown that at the early stage of the hydrolysis of cellotetraose, cellopentaose and cellohexaose, CBHI produced an intermediary product which was one glucose unit longer than the substrate. The β-mannanase BMANI produced a transglycosylation product two mannose units longer than the substrate when mannotetraose, mannopentaose and mannohexaose were used as substrates. Moreover, the rate of transglycosylation was determined for the β-mannanase and was found to be the highest rate of all the reactions studied. Interestingly, the transglycosylation rates of these two retaining enzymes of *T. reesei* showed that the more open active site of BMANI allows much faster transglycosylation than CBHI.

Preface

The present study was carried out in the laboratory of Chemical Technology of the Technical Research Centre of Finland (VTT) during the years 1995 - 1998. I wish to thank Reseacrh Directors Markku Auer, Veikko Komppa and Juha Sarlin for providing excellent and inspiring working facilities.

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Contents

Appendices of this publication are not included in the PDF-version. Please order the printed version to get the complete publication. (http://www.inf.vtt.fi/pdf/publications/1998/)

List of original publications

This thesis is based on the following original publications (Papers I - V in this publication), referred to in the text by the Roman numerals given below.

I Harjunpää, V., Teleman, A., Siika-aho, M. and Drakenberg, T. 1995. Kinetic and stereochemical studies of manno-oligosaccharide hydrolysis catalysed by β-mannanases from *T. reesei*. Eur. J. Biochem. 234, pp. 278 - 283.

II Ademark, P., Varga, A., Medve, J., Harjunpää, V., Drakenberg, T., Tjerneld, F. and Stålbrand, H. 1998. Softwood hemicellulose-degrading enzymes from *Aspergillus niger*: purification and properties of a β-mannanase. J. Biotechnol., in press.

III Harjunpää, V., Teleman, A., Koivula, A., Ruohonen, L., Teeri, T.T., Teleman, O. and Drakenberg, T. 1996. Cello-oligosaccharide hydrolysis by cellobiohydrolase II from *T. reesei*; association and rate constants derived from an analysis of progress curves. Eur. Biochem. 240, pp. 584 - 591.

IV Koivula, A., Kinnari, T., Harjunpää, V., Ruohonen, L., Teleman, A., Drakenberg, T., Rouvinen, J., Jones, A.T. and Teeri, T.T., 1998. Tryptophan 272: an essential determinant of crystalline cellulose degradation by *T. reesei* cellobiohydrolase Cel6A(CBHII). FEBS Lett. 429, pp. 341 - 346.

V Harjunpää, V., Helin, J., Koivula, A., Siika-aho, M.and Drakenberg, T. A comparative study of two retaining enzymes of *Trichoderma reesei*: transglycosylation of oligosaccharides catalysed by the cellobiohydrolase I, Cel7A, and β-mannanase, Man5A. FEBS Lett., submitted for publication.

Abbreviations

A	alanine
BMAN	β-mannanase
CBH	cellobiohydrolase
CBD	cellulose-binding domain
CMC	carboxymethyl cellulose
D	aspartic acid
DNS	3,5-dinitrosalisylic acid
DP	degree of polymerisation
E	glutamic acid
EC	Enzyme Commission
EG	endoglucanase
ESS	error square sum
Gal	galactose
Glc	glucose
HCA	hydrophobic cluster analysis
HEC	hydroxyethyl cellulose
HPLC	high performance liquid chromatography
NaAc	sodium acetate
MAN	mannanase
Man	mannose
MS	mass spectrometry
N	asparagine
NMR	nuclear magnetic resonance
pI	isoelectric point
RI	refractive index
SAXS	small-angle X-ray scattering
T	threonine
TEM	transmission electron microscopy
TLC	thin layer chromatography
XYN	xylanase
3D	three-dimensional
W	tryptophan
wt	wild type

Note: The old nomenclature of glycosyl hydrolases has been applied in the present manuscript.

CBHI = Cel7A
CBHII = Cel6A
BMANI = Man5A

1. Introduction

Enzymes are proteins which catalyse specific reactions in nature. The most striking characteristics of enzymes are their catalytic power and precise specificity. Proteins are active in water, where they adopt their natural three-dimensional structure needed for activity. Enzymes have also been found to retain their activity in organic solvents which has increased interest in the use of enzymes in industrial applications. Because enzymes are specific and the reactions are carried out in normal pressure and near to room temperature, energy consumption can be reduced and no harmful by-products are formed. By using enzymes the properties of end products can also be improved, which is not necessarily achievable by conventional methods. For example in the pulp and paper industry enzymatic pretreatment of pulp improves the mechanical properties of the wood fiber, leading eventually to better paper quality. Due to increasing demands for more environmentally friendly methods, the use of enzymes instead of polluting chemicals has significantly increased during the past two decades. Although various methods in isolation and purification or cloning and production have been improved in order to produce higher amounts of proteins, there are still many open questions. Before industrial enzymatic applications can be realised at high ecological and economical levels, more knowledge of the enzymes on the molecular level, both structural and functional, is required.

1.1 Chemical structure of wood components

Wood consists of elongated cells, most of which are oriented in the longitudinal direction of the stem and are connected to each other through openings, referred to as pits. These cells, varying in shape according to their function, provide the necessary mechanical strength to the tree and also permit liquid transport and provide storage of reserve food supplies. The tracheids in softwoods and libriform cells in hardwoods are generally called wood fibres (Fujita and Harada, 1991). These cell wall wood fibres can be divided into three main layers, i.e. the primary wall, secondary wall, and warty wall (Fig. 1). Cellulose, hemicellulose and lignin are the major constituents of wood material. Cellulose is the main component, accounting for approximately 40 - 45 % of the dry substance in most wood species and located mainly in the secondary cell wall.

Like cellulose, most hemicelluloses function as supporting material in the cell walls. Hemicelluloses appear in close association with cellulose, especially in lignified tissues. However, no covalent bonds have been found between cellulose and hemicelluloses, although marked mutual adhesion is provided by hydrogen bonding and van der Waals interactions. The location of different

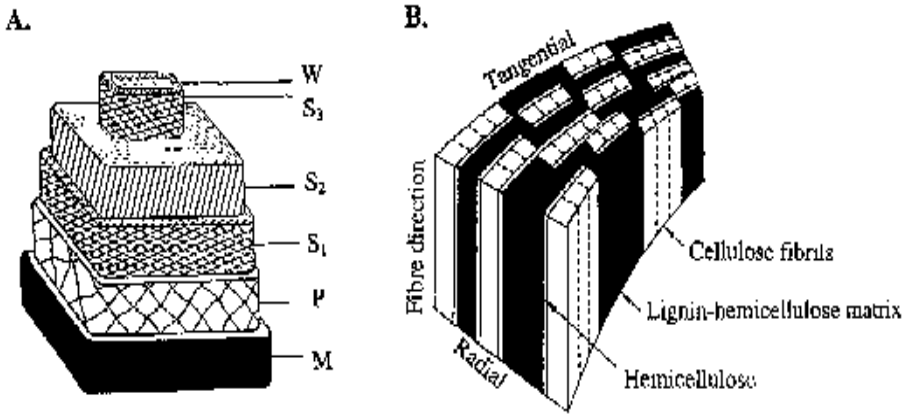


Fig. 1. A. Organisation of the structural layers in a wood fibre. W = warty layer, S_3 = inner laeyr of the secondary wall, S_2 = middle layer of the secondary wall, S_1 = outer layer of the secondary wall, P = primary wall and M = middle lamella. B. Hypothetical view of the arrangement of cellulose, hemicellulose and lignin in the wood cell wall (Kerr and Goring, 1975).

hemicelluloses in the fibre wall is dependent on the wood species and growth season, and the amount of hemicelluloses is usually 20 - 30 % of the wood dry weight. Lignin is located in primary and secondary cell walls and in the middle lamellae. It is closely associated with wood carbohydrates and covalently linked to hemicelluloses. The covalent cross-linking, lignification, acts as glue to cement cellulose microfibrils and produce rigid woody tissues able to withstand the compressive force of gravity.

1.1.1 Cellulose

Cellulose is a chemically rather simple molecule. It is a linear homo-polysaccharide composed of D-glucose units linked together by β-1,4-glycosidic linkages (Fig. 2). The cellulose molecule forms a linear, almost fully extended chain with a two-fold screw axis on which successive glucose residues are rotated 180° relative to each other and therefore the glycosidic oxygens point alternatively up and down. The structure of the cellulose chain is highly stabilised by intramolecular hydrogen bonds (Fig. 2). There are two intra-molecular hydrogen bonds between successive glucose residues, and the hydrogen bonding network consists of all polar functional groups except glycosidic oxygens (Gardner and Blackwell, 1974). These bonds affect the relative positions of repeating residues in the chain, which leads to a slight bending in the glycosidic linkages and a repeating distance of 1.03 nm along the chain axis. This corresponds to one cellobiose unit, which therefore should be regarded as the smallest repetitive unit rather than glucose. The number of glucose residues in plant polysaccharides varies extensively depending on the origin and it can be as high as 15 000, corresponding to a molecular mass of 2.4 MDa.

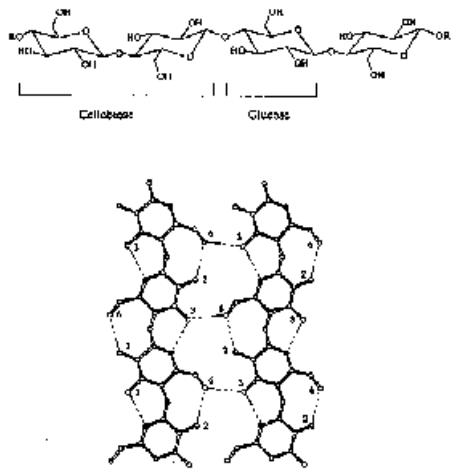


Fig. 2. The upper diagram represents a cellulose chain. The lower diagram represents the hydrogen bonding within and between the chains in a cellulose crystal. The hydrogen bonds are shown by dashed lines. The numbering of oxygen atoms is indicated. From Sjöström, 1981.

Cellulose chains have a strong tendency to interact through hydrogen bonding and hydrophobic interactions. This allows cellulose molecules to aggregate into the form of microfibrils, which in turn can pack tightly together to form even larger fibrils and finally cellulose fibres. X-ray diffraction and spectroscopic studies have been carried out to determine the crystal structure of microfibrils in different native and chemically treated celluloses. There are four distinct crystalline cellulose structures named cellulose I, II, III and IV (Sarko, 1987). The main differences between these structures are in the lateral shift of neighbouring chains and sheets. Cellulose I is sometimes called "native cellulose" since it is the predominant form in plant secondary cell walls. In cellulose I crystals the chains have a parallel orientation and are arranged side by side to form hydrogen bonded sheets which are held together by van der Waals interactions. Cellulose I exists in two different forms I_α and I_β which differ in their intermolecular hydrogen bonding patterns (Atalla and Vanderhart, 1984; Sugiyama et al., 1993). Most natural celluloses contain both of these crystal forms and their proportion depends on the cellulose source. Cellulose II is obtained from cellulose I by sodium hydroxide treatment. Its chains are oriented in an anti-parallel way with consecutive formation of extensive hydrogen bonding that makes cellulose II more stable than cellulose I. Cellulose III is obtained from either cellulose I or II by liquid ammonia treatment and spontaneously reverts to its parent type, slowly at room temperature and rapidly in boiling water. Cellulose IV is the predominant form in plant primary cell walls and is thought to be a poorly ordered form of cellulose I. It can also be obtained from cellulose III by heating in glycerol. Although cellulose has a very high regularity in its structure and packing, it also contains less ordered, so called "amorphous" regions which alternate with highly ordered crystalline regions. In the literature this has been described as a degree of crystallinity that is expressed as a percentage and can be measured by X-ray diffraction and solid state NMR. *Valonia* cellulose represents the most crystalline, and acid swollen cellulose the least crystalline cellulose structure (Kulshreshta and Dwelz, 1973).

1.1.2 Hemicelluloses

Hemicelluloses are low molecular weight linear or branched polysaccharides, normally having a degree of polymerisation (DP) less than 200. These polysaccharides have a heterogeneous composition of various sugar residues and substituted side chains, which also make hemicelluloses more soluble in

water. It is known that hemicelluloses are not involved in the biosynthesis of cellulose and thus form a distinct group of polysaccharides. Hemicelluloses are normally named according to the main sugar residues in the backbone, e.g. xylans (D-xylose units) and glucomannans (both D-glucose and D-mannose units). Branched polysaccharides contain neutral and/or acidic short side groups. The main hemicelluloses are divided into softwood and hardwood hemicelluloses according to the wood species and are summarised in Table 1 and Fig 3.

Table 1. The major hemicelluloses in softwoods and hardwoods.

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

Reference: 1. Sjöström, 1981. Abbreviations used: Xyl = xylose; Ara = arabinose; GlcA = 4-O-methylglucoronic acid; Man = mannose; Glc = glucose; Gal = galactose; Ac = acetic acid.

Xylans in hardwoods and softwoods have different side group patterns. Hardwood xylan is O-acetyl-4-O-methylglucuronoxylan, in which the xylan backbone is substituted at random intervals with acetyl and 4-O-methylglucuronic acid side groups. The ratio of xylose to α-1,2-linked 4-O-methylglucuronic acid is about 10:1, twice as high as in softwoods, and 60 - 70 % of the xylose units are acetylated at the C2 and/or C3 positions (Timell, 1967; Lindberg et al. 1973). Softwood xylan is mainly arabino-4-O-methylglucuronoxylan, in which L-arabinose units are α-1,3-linked to the xylan backbone. The ratio of xylose to 4-O-methylglucuronic acid is 5:1, and the xylose to arabinose ratio is 8:1 (Timell, 1967). There are no acetyl groups in softwood xylan, but L-rhamnose and galacturonic acid residues have been identified in the main chain linked to the reducing end, producing a more alkali-resistant end group. Mannans are also typical hemicelluloses in both softwoods and hardwoods. There are two main groups, namely galactoglucomannans and glucomannans, of which the former is mainly found in softwoods and the latter in hardwoods. Galactoglucomannans are composed of β-1,4-linked glucose and mannose units,

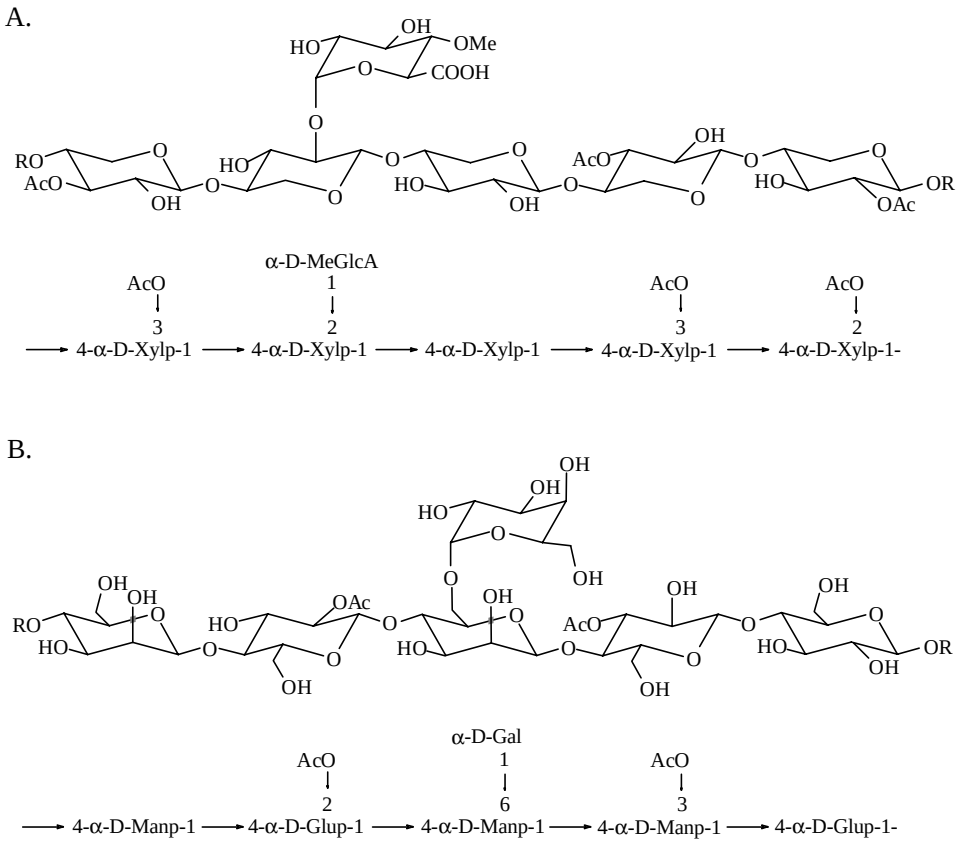


Fig. 3. Typical wood hemicelluloses. A) Hardwood O-acetyl-4-O-methyl-glucuronoxylan and B) softwood O-acetyl-galactoglucomannan.

which are randomly distributed in the backbone, and galactose side groups attached to glucose or mannose units through α-1,6-linkages. There are also partially acetylated hydroxyl groups at the positions C2 and C3, dividing acetylated galactoglucomannans into two fractions according to their galactose content (Gal:Glc:Man, 1:1:3 and 0.1:1:3). Hardwood glucomannan contains glucose and mannose units in the ratio of 1:1-2 and neither galactose nor acetyl groups are present (Timell, 1967).

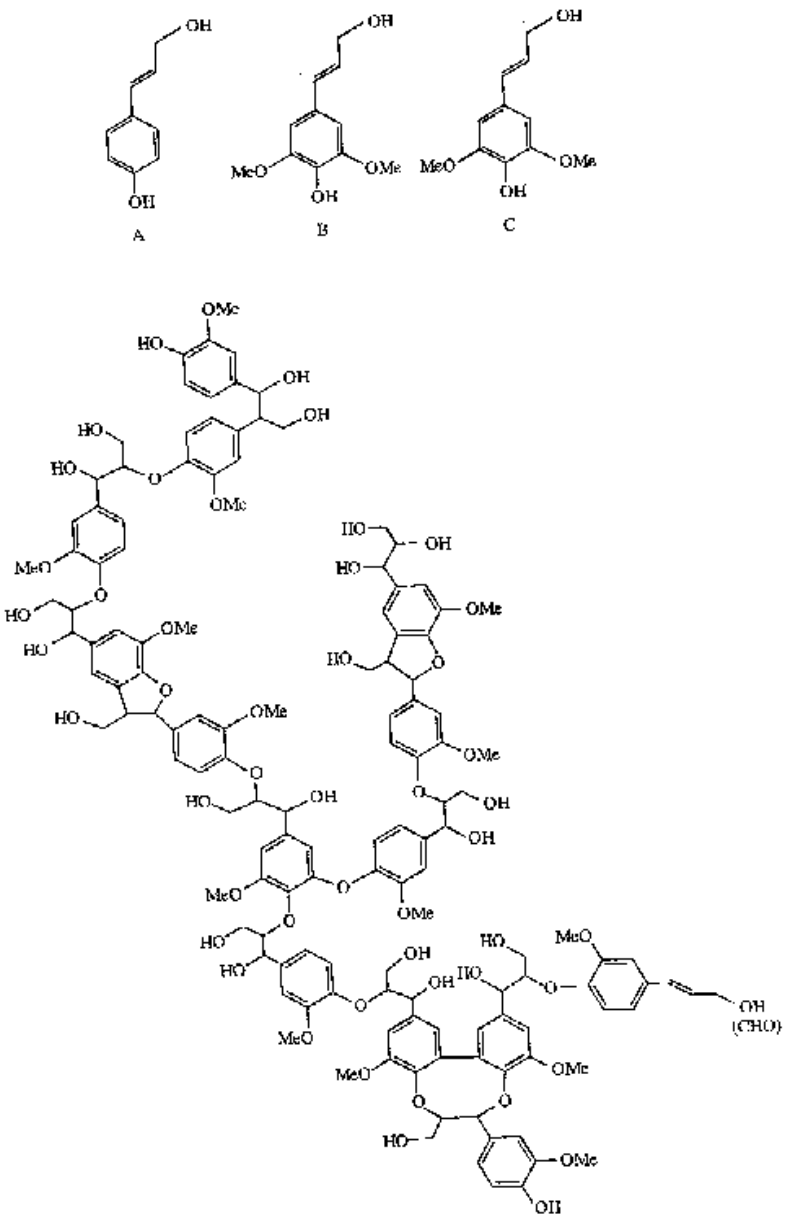


Fig. 4. A, B, and C (top) are the structural components of lignin: coniferyl-, sinapyl- and p-coumaryl alcohols. The lower part of the figure represents part of softwood lignin (redrawn from Brunow et al., 1998).

1.1.3 Lignin

Structurally, lignin is very distinct from the other major structural components of wood material. There are neither typical chains of repeating units nor easily hydrolysable bonds in lignin, as there are in wood carbohydrates. Lignin has a highly irregular three-dimensional structure (Fig. 4) and it is a water insoluble high-molecular mass compound (Brunow et al., 1998). The lignin molecule consists of phenylpropane subunits linked together with ether and/or carbon-carbon bonds. There are at least nine different ether and carbon-carbon bonds which are typically found between the phenylpropane subunits. The trans isomers of coniferyl-, sinapyl- and p-coumaryl alcohols, i.e. monolignols (Fig. 4), are the building blocks for the complex structure of lignin, which is formed in an enzyme-initiated, free radical-mediated, dehydrogenative polymerisation of the aromatic precursors. These random coupling reactions of resonance-stabilised phenoxy radicals are not catalysed by enzymes, which is evidently the reason for the wide variation in the bonds between phenylpropane units (Brown, 1985). Lignins are also divided into several classes according to their structural components. Guaiacyl lignins are mainly polymerised from coniferyl alcohol and are found predominantly in softwoods. Guaiacyl-syringyl lignins are copolymers of coniferyl and sinapyl alcohols and also contain small amounts of p-hydroxyphenyl units. Hardwood lignins are mainly composed of guaiacyl-syringyl units (Fengel and Wegener, 1984) and grass lignin contains all three monolignol structures.

1.2 Enzymatic degradation of cellulose and hemicellulose

In nature the vast majority of polysaccharides are degraded by cellulolytic and hemicellulolytic microorganisms. These organisms perform degradation mainly through the action of extracellular enzymes which hydrolyse glycosidic linkages in the polysaccharides. The degradation of cellulosic and hemicellulosic materials is not an isolated process, but integrated in the complete breakdown of plant material. Therefore the organisms produce a whole set of different enzymes specific for various components, and the enzyme production is carefully regulated so that the organism can adapt to the changing composition during the process (Cochet, 1991; Messner and Kubicek, 1991). Cellulolytic and hemicellulolytic enzymes are produced by many different microorganisms, e.g.

fungi, bacteria and yeasts (Biély, 1985; Biély and Tenkanen, 1998), only a few of which produce a complete set of enzymes capable of efficiently degrading their native substrates.

Cellulolytic enzymes, cellulases, are a group of enzymes which hydrolyse β-1,4-glycosidic linkages in cellulose. Since cellulose cannot penetrate into cells, cellulases are secreted outside the cells or bound to outer cell surfaces. Three different cellulolytic activities can be identified. Exoglucanases (1,4-β-D-glucan cellobiohydrolase, E.C. 3.2.1.91) hydrolyse cellulose from the free chain ends, producing mainly cellobiose as an end product. They are therefore called cellobiohydrolases. Without any cellobiohydrolase activity the cellulase system is unable to solubilise crystalline cellulose effectively. Endoglucanases (1,4-β-D-glucan-4-glucanohydrolase, E.C. 3.2.1.4) attack randomly internal linkages within the cellulose chain. β-Glucosidases (E.C. 3.2.1.21) are exoenzymes which are able to hydrolyse small oligomers, mainly trimers and dimers, to monomers. This exoactivity is important because the accumulation of cellobiose strongly inhibits cellobiohydrolases. The classification of cellulases as pure exo- and endoenzymes is normally used despite problems due to overlapping specificities and poorly defined substrates. There are still major differences in the activities of these enzymes, namely endoglucanases rapidly decrease the viscosity of a polymeric substrate and only they are able to make an “endotype” attack on substituted substrates such as HEC and CMC.

Total hydrolysis of crystalline cellulose by cellulases is possible only through the cooperative action of endo- and exoglucanases (Henrissat et al., 1985; Nidetsky et al., 1993). This cooperative action is called synergism, and several models have been proposed. After a prolonged time, cellulose degradation proceeds finally to glucose through the synergistic action of endo/exoglucanases and β-glucosidase (Fig. 5). It has been shown that there are two types of synergism: endo-exo- and exo-exo-synergism (Tomme et al., 1988; Irwin et al., 1993; Nidetsky et al., 1994a; Béguin and Aubert, 1994; Medve et al., 1994; Väljamäe et al., 1998). One hypothesis explains the former as a consequence of an “endotype” attack on less ordered amorphous regions of cellulose fibrils, which creates free chain ends available for the exotype action. Exo-exo-synergism is caused by the action of two types of exocellulases, of which one hydrolyses cellulose from the reducing end and the other from the non-reducing

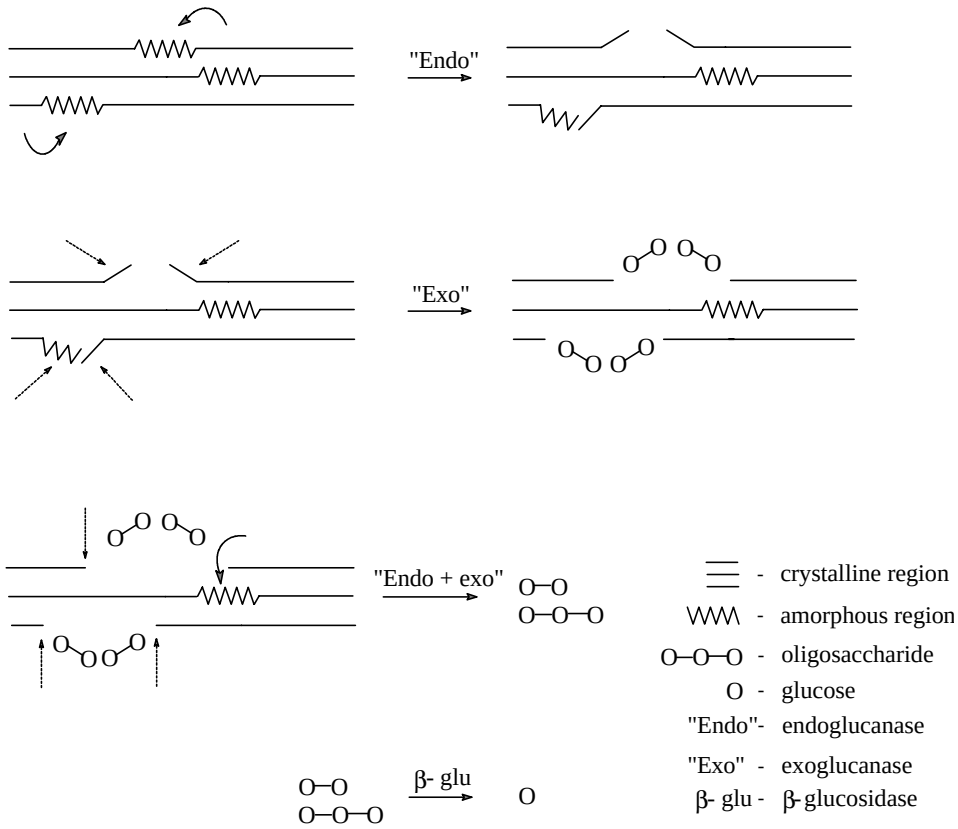


Fig. 5. Simplified figure of cellulose degradation. Curved arrows represent an "endotype" attack on amorphous regions whereas straight arrows represent an "exotype" attack on the free reducing ends. In the final stage of the process β-glucosidase hydrolyses the formed oligosaccharides to glucose.

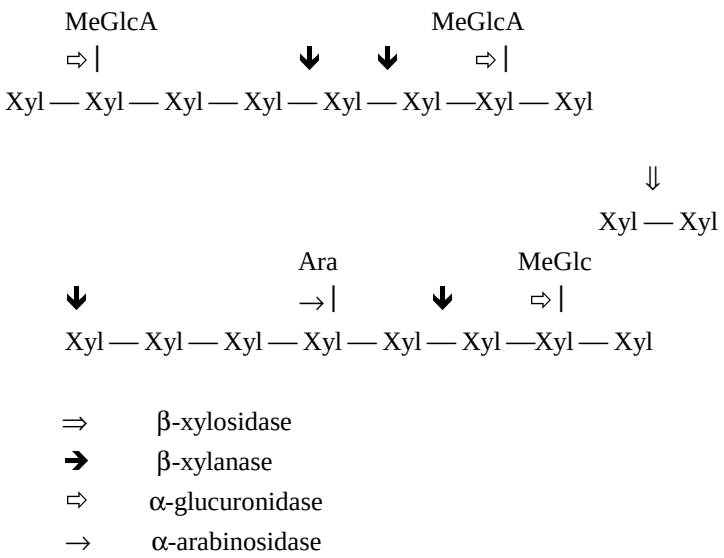
end (Divne et al., 1994; Shen et al., 1995; Barr et al., 1996). Although the hydrolysis reaction as such is a simple phenomenon, it is still poorly understood. However, it is well known that the degree of synergy is dependent on the relative amounts of enzymes and on the substrate used (Nidetsky et al., 1993).

Some microorganisms produce a complete set of enzymes capable of efficient degradation of native cellulose. Efficient cellulase systems are produced by white-rot and soft-rot fungi such as *Trichoderma, Fusarium, Humicola, Penicillium and Schizophyllum* (Wood and Garcia-Campayo, 1990; Nevalainen

and Penttilä, 1995; Clarke et al., 1997; Mackenzie et al., 1997; Schülein, 1997). These fungal cellulase systems usually contain various endo- and exoglucanases and at least one β-glucosidase. The number of enzyme components is dependent on the fungus and cultivation conditions. In addition, it may vary because the purification of cellulases is sometimes difficult and the enzymes also have different isoforms with slightly differing molecular masses and isoelectric points. Most bacterial cellulase systems cannot degrade crystalline cellulose effectively and they normally include only endoglucanases. The best studied bacterial cellulases are produced by *Cellulomonas fimi* and *Thermonospora fusca* (Johnson et al., 1996, Johnson et al., 1996; Gilkes et al., 1997; Sakon et al., 1997; Irwin et al., 1998; Stålbrand et al, 1998; Barr et al., 1998). In addition, a well characterised bacterial cellulase system is found in *Clostridium thermocellum* and in other *Clostridium* species (Lamed et al., 1983; Béguin and Aubert, 1994; Garcia-Campayo and Béguin, 1997; Bélaich et al., 1997; Zverlov et al., 1998). This special type of cellulase system is called a cellulosome, in which the enzymes are organised into a stable multienzyme complex. Cellulosomes have high molecular masses, of the order of MDa, and high activities against cellulose, even comparable to those of *Trichoderma* enzymes (Johnsson et al., 1982).

Hemicellulases hydrolyse glycosidic linkages in hemicelluloses and are named according to their substrate specificity. The degradation of hemicelluloses is relatively well known, despite their heterogeneous structure as compared to cellulose (Woodward, 1984; Biély, 1985; Biély and Tenkanen, 1998). Degradation studies have mainly been performed with isolated soluble substrates. Hydrolysis proceeds by the synergistic action of different hemicellulases. The main enzymes needed in the depolymerisation of hemicelluloses are endoenzymes, i.e. β-xylanases and β-mannanases. The endoxylanases and endomannanases attack randomly at the internal linkages of xylans and glucomannans, respectively, releasing substituted oligomeric products. In the subsequent steps these intermediary products are further hydrolysed by a set of exo-enzymes (β-xylosidase and β-mannosidase) and side-group cleaving enzymes (α-arabinosidase, α-galactosidase, α-glucuronidase and esterase), resulting in the final monomeric end products (Fig. 6).

A.



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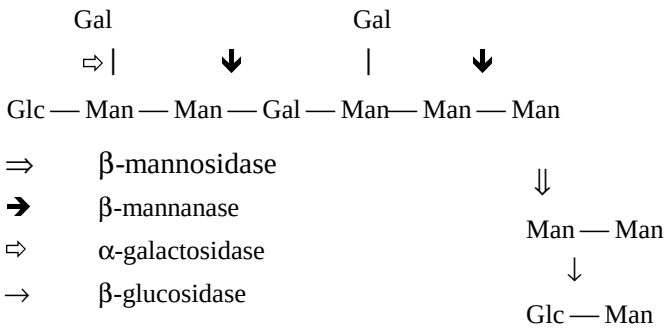


Fig. 6. Schematic presentation of the action of different hemicellulases on hardwood xylan (A) and on softwood galactoglucomannan (B).

Several species of fungi and bacteria produce a wide variety of hemicellulolytic activities needed in the total hydrolysis of hemicellulosic materials (Viikari et al., 1993; Sunna and Antranikian, 1997). The best characterised fungal hemicellulases are those produced by *Aspergillus, Penicillium* and *Trichoderma* (McCleary and Matheson, 1983; Coughlan and Hazlewood, 1993; Törrönen and Rouvinen, 1997; Biély et al., 1997; II) and bacterial hemicellulases produced by

Caldocellum, *Bacillus* and *Streptomyces* (Gibbs et al., 1992, 1996; Yosida et al., 1997; Breccia et al., 1998; Dupont et al., 1998). Furthermore, xylananes are the most thoroughly studied of all hemicellulolytic enzymes.

1.3 Major cellulases and hemicellulases of the fungus of *Trichoderma reesei*

Trichoderma reesei, a mesophilic soft rot fungus, is one of the best characterised fungi and the most efficient producer of cellulolytic and hemicellulolytic enzymes. This was experienced by american soldiers in Vietnam when their cotton tents were destroyed by an invisible enemy. The total amount of cellulases in the growth media of wt *T. reesei* can exceed few grams per liter; mutated strains of *T. reesei* can produce cellulases even up to 40 g/l under optimal conditions (Durand et al., 1988). The cellulases of *T. reesei* are the most extensively studied of all cellulolytic enzymes and the genes have been cloned and sequenced. The isolated genes encode seven different proteins: two cellobiohydrolases (CBHI and CBHII), four endoglucanases (EGI, EGII, EGIII, EGV) and one β-glucosidase (Shoemaker et al., 1983; Chen et al., 1987; Penttilä et al., 1986; Saloheimo et al., 1988; Ward et al., 1993; Saloheimo et al., 1994). Although hemicellulases of *T. reesei* have been studied less thoroughly than cellulases, some of these genes have also been cloned and sequenced, e.g. two xylanase genes and one mannanase gene (Törrönen et al., 1992; Saarelainen et al., 1993; Stålbrand et al., 1995). The observed multiplicity of the enzymes could be a result of heterogeneous glycosylation and/or post-secretional modification by proteolytic and glycanolytic enzymes (Hagspiel et al., 1989).

1.3.1 Cellobiohydrolases

The major constituent of the *T. reesei* cellulase system is cellobiohydrolase I (CBHI), which comprises 60 % of the cellulases secreted by the fungus. CBHI contains 497 amino acid residues and has a molecular mass of 59 - 68 kDa depending on the glycosylation, and an isoelectric point between 3.5 and 4.2. Hydrolysis of β-1,4-glycosidic linkages catalysed by CBHI proceeds with retention of the anomeric configuration and cellobiose is released from the reducing end of the cellulose chain (Knowles et al., 1988; Claeyssens et al., 1990). CBHI has a low catalytic rate on soluble cello-oligosaccharides and it

also exhibits transfer activity (V). The reported activities of CBHI on polymeric substrates are somewhat different mainly, because of minor impurities of other cellulolytic activities in the enzyme preparations studied. This is normally caused by differences in the purification methods used. When a carefully purified preparation is used, CBHI is active on crystalline cellulose but not on carboxymethylcellulose (CMC), hydroxyethylcellulose (HEC), or barley β-glucan (Penttilä et al., 1988). Although CBHI shows no exoglucanase activity on substituted substrates it has also been observed to behave like an endoglucanase under certain experimental conditions (Chanzy et al., 1984; van Tilbeurgh and Claeyssens, 1985; Claeyssens et al., 1989; Vrsanská and Biély, 1992). According to one electron microscopy study, CBHI caused significant lateral thinning of cellulose crystals (Chanzy and Henrissat, 1983).

Cellobiohydrolase II (CBHII) comprises 20 % of the cellulases secreted by the fungus. It contains 447 amino acid residues and has a molecular mass of 50 - 58 kDa and an isoelectric point between 5.1 and 6.3. The hydrolysis of β-1,4-glycosidic linkages catalysed by CBHII proceeds with inversion of the anomeric configuration and cellobiose is released from the non-reducing end of the cellulose chain. CBHII has higher activity than CBHI on soluble cello-oligosaccharides but is devoid of transfer activity (III). CBHII hydrolyses microcrystalline cellulose such as Avicel and *Valonia* cellulose as well as amorphous cellulose, but has no activity on carboxymethylcellulose (CMC) or hydroxyethylcellulose (HEC). Although protein purification causes severe problems in the case of CBHII, it still seems to be a more strict exoglucanase than CBHI on the basis of electron microscopy results (Chanzy and Henrissat, 1985).

1.3.2 Endoglucanases

T. reesei produces considerably lower amounts of endoglucanases than cellobiohydrolases. EGI, the major endoglucanase, comprises approximately 10 % of the cellulases secreted by the fungus. EGI contains 437 amino acid residues resulting in a molecular mass of 43 - 58 kDa, and has an isoelectric point between 4 and 6. The hydrolysis of β-1,4-glycosidic linkages catalysed by EGI proceeds with retention of the anomeric configuration and the enzyme also possesses transfer activity as well as considerable xylanase activity (Claeyssens et al., 1990; Biély et al., 1991; Bailey et al., 1993). Comparison with CBHI

revealed 45 % sequence identity and the two proteins belong to the same HCA-sequence family. EGI exhibits high activity on substituted celluloses (CMC and HEC) and thus it is thought to be a true endoglucanase. EGI is the most extensively studied endoglucanase of *T. reesei*, with a defined 3D structure as determined by X-ray diffraction (Kleywegt et al., 1997).

Endoglucanase II (EGII), previously known as EGIII, comprises 5 - 10 % of the cellulases secreted by the fungus. EGII contains 397 amino acid residues, resulting in a molecular mass of 48 kDa and has an isoelectric point of 5.5 (Saloheimo et al., 1988). It hydrolyses β-1,4-glycosidic linkages with retention of the anomeric configuration and it also possesses transfer activity (Macarron et al., 1993a). EGII is active on substituted celluloses (CMC and HEC), β-glucan, and Avicel but it is less active than EGI. The minor occurrence of two more endoglucanases in the culture filtrate of *T. reesei* has also been reported. These endoglucanases have lower molecular masses (20 - 25 kDa) and are called EGIII and EGV. EGIII, previously known as EGIV, is a nonglycosylated protein consisting of 218 amino acid residues. It has a molecular mass of 25 kDa and an isoelectric point of 7.5. EGIII is active on substituted celluloses (CMC and HEC) but not on filter paper or Avicel. EGV contains 225 amino acid residues, resulting in a molecular mass of 23 kDa (calculation based on gene sequence). The stereochemical course of oligosaccharide hydrolysis catalysed by EGIII and EGV has not been experimentally determined, but both enzymes have been proposed to work with retention of the anomeric configuration on the basis of their HCA-sequence family.

1.3.3 Mannanases

Five enzymes with mannanase activity have been isolated and purified from the culture filtrate of *T. reesei*. The crude enzymes have isoelectric points between 3.6 and 6.5 and two proteins with pI values of 4.6 and 5.4 comprise 60 - 70 % of the total mannanolytic proteins. These are the major mannanases of *T. reesei*, which have also been cloned, sequenced and characterised (Arisan-Atac et al., 1993; Stålbrand et al., 1993, 1995). They have slightly different molecular masses (51 and 53 kDa) and contain 410 amino acid residues each. Both mannanases hydrolyse β-1,4-mannosidic linkages with retention of the anomeric configuration and also possess high transfer activity (I). They are more active on homopolymannan than glucomannans or galactoglucomannans, resulting in

mannotriose, mannobiose and various mixed oligosaccharides as end products. The mode of acton of β-mannanase on galactoglucomannans has been determined and shown to be dependent on the degree of substitution as well as on the distribution of the substituents (Tenkanen et al., 1997). Furthermore, the hydrolysis is also affected by the glucose/mannose ratio of the substrate.

1.3.4 Xylanases

Two major xylanases have been isolated and characterised from the culture filtrate of *T. reesei* (Tenkanen et al., 1992). These enzymes, XYNI and XYNII, have low molecular masses of 19 and 20 kDa and isoelectric points of 5.1 and 9.0, respectively. The genes coding for these xylanases have also been cloned and sequenced (Törrönen et al., 1994). The hydrolysis of β-1,4-xylosidic linkages catalysed by the two enzymes proceeds with retention of the anomeric configuration, and both enzymes also exhibit transfer activity (Biély et al., 1993, 1994). XYNI and XYNII have equal activities on 4-O-methylglucoronoxylan and they hydrolyse the polysaccharide to the same degree, with only minor differences in the ratio of xylose to xylobiose produced. These two xylanases are also among the most thoroughly studied xylanase proteins and their 3D structures have been determined by X-ray diffraction (Törrönen et al., 1993, 1994; Törrönen and Rouvinen, 1995; Havukainen et al., 1996). Some general properties of cellulases and hemicellulases of *T. reesei* are summarised in Table 2.

Table 2. Physical properties of T. reesei cellulases and hemicellulases.

Enzyme	Number of residues	Molecular mass (kDa)	Isoelectric point (pI)	Position of CBD (Terminus)	HCA family	Ref
CBHI	497	59-68	3.5-4.2	C	7	1
CBHII	447	50-58	5.1-6.3	N	6	2
EGI	437	43-58	4.0-6.0	C	7	3
EGII	397	48	5.5	N	5	4
EGIII	218	25	7.5	-	12	5
EGV	225	23[a]	nd	C	45	6
β-gluco-sidase	713	75	8.7	-	3	7, 8
BMANI	410	51	4.6	C	5	9, 10
BMANII	410	53	5.4	C	5	9, 10
XYNI	178	19	5.1	-	11	11, 12
XYNII	190	20	9.0	-	11	11, 12

[a] Value is an approximation from the amino acid sequence without glycosylation.
nd Not determined.

References: 1. Shoemaker et al., 1983; 2. Chen et al., 1987; 3. Penttilä et al., 1986; 4. Saloheimo et al., 1988; 5. Ward et al., 1993; 6. Saloheimo et al., 1994; 7. Barnett et al., 1991; 8. Chen et al., 1992; 9. Stålbrand et al., 1995; 10. I; 11. Saarelainen et al., 1993 ; 12. Biély et al., 1994.

1.4 Structural organisation of the enzymes of *T. reesei*

The primary structures of glycosyl hydrolases have revealed striking similarities including a multidomain structure (Tomme et al., 1988). These enzymes share a common structural organisation with two individually functional domains, namely the catalytic and substrate-binding domains. The catalytic domain, the core, constitutes the major part of the enzyme and is connected to the highly conserved smaller terminal substrate-binding domain by a glycosylated linker sequence. High resolution small angle X-ray scattering (SAXS) and transmission electron microscopy (TEM) studies have shown that the overall

shapes of *T. reesei* CBHI and CBHII resemble a “tadpole-like” structure, having a large ellipsoidal head and an elongated cylindrical tail, Fig. 7 (Abuja et al., 1988a, 1988b; Lee and Brown, 1997).

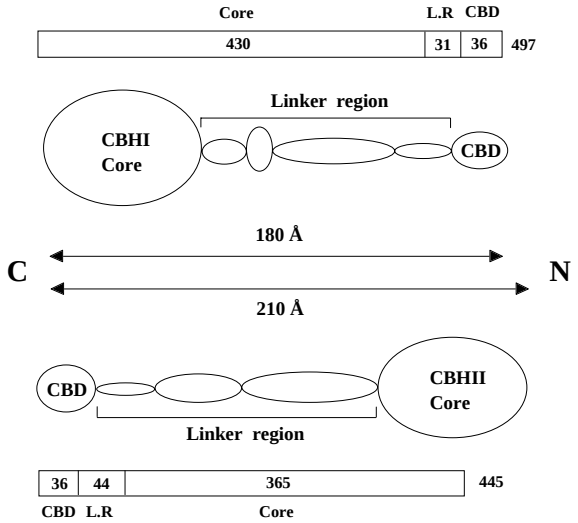


Fig. 7. Low resolution structure of T. reesei CBHI and CBHII as determined by SAXS measurements.

Furthermore, SAXS and TEM results revealed that conformational changes may also occur upon binding of the ligand (Abuja et al., 1989; Lee and Brown, 1997). The substrate-binding domain, called a cellulose-binding domain (CBD), is located either at the C- or the N-terminal end. All *T. reesei* cellulases except EGV contain a CBD but the only hemicellulase that has been shown to have a CBD is β-mannanase (Stålbrand et al., 1995). However, in some cases there is no experimental evidence to support the functionality of separate domains and the evidence for their existence relies solely on the amino acid sequence comparison. Activities of the enzymes on soluble oligosaccharides are unaffected by removal of the substrate-binding domain and therefore the roles of CBDs and linker sequences are ignored in the following discussions.

1.4.1 Catalytic domains

Despite considerable diversity, catalytic domains of glycosyl hydrolases do share significant sequence similarity. Sequence comparisons have been used to group the glycosyl hydrolases into different families. Henrissat et al. (Henrissat et al., 1989; Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Davies, 1997) used hydrophobic cluster analysis (HCA) to compare the amino acid sequences of the catalytic domains of glycosyl hydrolases and classified the enzymes into a restricted number of families. It has been found that all glycanases and glycosidases with a known 3D structure that belong to the same family, have similar folding properties, active site topology and exhibit the same stereoselectivity (Gebler et al., 1992). Therefore the stereochemical outcome of the reaction is a crucial point in the catalytic mechanism of the enzyme and it can, together with amino acid sequence similarities, provide additional support for the classification of polysaccharide hydrolysing enzymes.

High resolution three-dimensional structures provide detailed information about the protein. Combining structural and kinetic data, we can deduce information regarding enzyme-substrate interactions as well as the function of the enzyme-catalysed reaction. When all solved 3D structures are compared, three general activite site topologies can be identified regardless of the stereochemistry or the overall fold of the enzyme (Davies and Henrissat, 1995). The first category contains a pocket- or crater-like structure, normally found in glycosidases that hydrolase monosaccharide units from chain ends. The second category contains only two enzymes that have a tunnel-like active site, CBHI and CBHII from *T. reesei*. CBHI and CBHII are among the structurally best characterised cellulases with well defined high resolution 3D structures (Rouvinen et al., 1990; Divne et al., 1998). Both enzymes have a tunnel-like active site that spans through the core. CBHI has a 50 Å long tunnel that contains ten well defined binding subsites and an eleventh putative external site, which does not exhibit protein-sugar interactions. The tunnel is formed from four extensive loops having a flat shape. CBHII has a 20 Å long active site tunnel with four well defined binding subsites and two external subsites (Rouvinen et al, 1990; II). The third category is closely related to the second but the enclosing loops are shorter or turned away from the tunnel, producing an open cleft or groove-like active site. This kind of active site structure has been found in endoenzymes, such as endoglucanases and xylanases, for example EGI, XYNI and XYNII of *T. reesei*.

EGI and both xylanases, XYNI and XYNII, have well defined 3D structures but hitherto no 3D structures for mannanases are available. These three active site topologies are shown in Fig. 8.

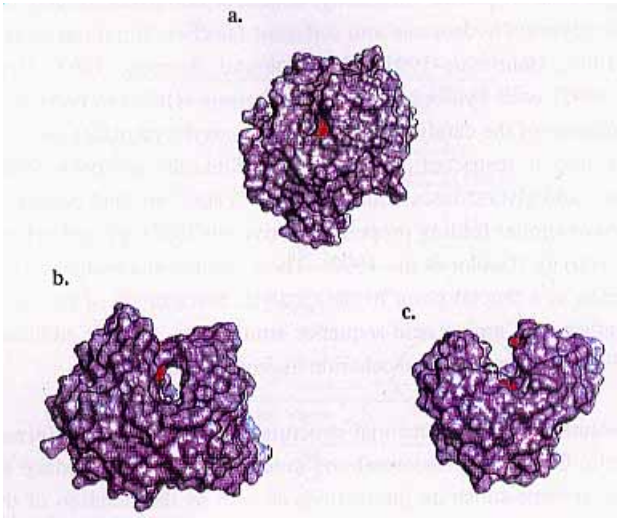


Fig. 8. Three active site topologies of glycosyl hydrolases described by Davies and Henrissat (1995). The pocket of A. awamori glucoamylase (a). The tunnel of CBHII of T. reesei (b). The cleft of T. fusca E2 (c). E2 as well as CBHII belong to family 6 of glycosyl hydrolases. The proposed catalytic amino acids are shown in red. From Davies and Henrissat, 1995.

1.4.2 Catalytic mechanisms

Hydrolysis of glycosidic linkages in polysaccharides, catalysed by glycosyl hydrolases, is formally a nucleophilic substitution at the saturated carbon of the anomeric center and can take place with either retention or inversion of the anomeric configuration. Accordingly, there are two types of glycosyl-transferring enzymes - ”retaining and inverting” - and the basic principles of the mechanism of the hydrolytic cleavage are well established (Koshland, 1953; Sinnott, 1990; McCarter and Withers, 1994). All of these enzymes contain a pair of essential carboxylic acid residues appropriately situated on opposite faces of the catalytic site and these two catalytically important amino acid residues have different roles in the reaction. When inversion of the anomeric

configuration occurs, the enzyme utilises the single displacement mechanism involving a general acid-base catalysis. Here, a general acid (AH) donates a proton to the leaving glycosidic oxygen and a general base (B^-) assists the nucleophilic attack of water (Fig. 9). Alternatively, retaining enzymes utilise the double-displacement mechanism in which a covalent glycosyl-enzyme intermediate is formed and further hydrolysed through an oxocarbenium ion-like transition state. The existence of the glycosyl-enzyme intermediate has been elegantly demonstrated by using competitive substrates allowing determination of the catalytic amino acid residue (Withers and Aebersold, 1995; McCarter and Withers, 1996). In the first step, glycosylation, a negatively charged carboxylate in the enzyme acts as a nucleophile by displacing the glycosidic oxygen (O4) in the leaving group with general acid-catalytic assistance from a protonated carboxylate. In the second step, deglycosylation, water attacks the anomeric center of the glycosyl-enzyme intermediate with general base-catalytic assistance from the currently deprotonated carboxylate, thereby displacing the nucleophile and releasing the product oligosaccharide (Fig. 9). Thus, the second inversion results in retention of the original configuration at the anomeric center. Furthermore, in the deglycosylation step the attacking nucleophile can also be another oligosaccharide instead of water, resulting in elongation of the oligosaccharide chain. This is known as transglycosylation and it can be applied for the enzymatic synthesis of different oligosaccharides. The distances between the general acid and base in the active sites of retaining and inverting enzymes have been reported to be about 5 and 10 Å, respectively (Wang et al., 1994; Kuroki et al., 1995). Thus in retaining enzymes these critical carboxyl groups are close together, resulting in the formation of a glycosyl-enzyme intermediate, whereas in inverting enzymes their greater separation allows the insertion of a water molecule for direct attack. It has also been reported that a retaining glycosidase has been changed into an inverting enzyme or vice versa by means of a single point mutation among the catalytic amino acid residues (McCarter and Withers, 1994). Catalytically important amino acid residues and stereospecificity of *T. reesei* cellulases and hemicellulases are shown in Table 3.

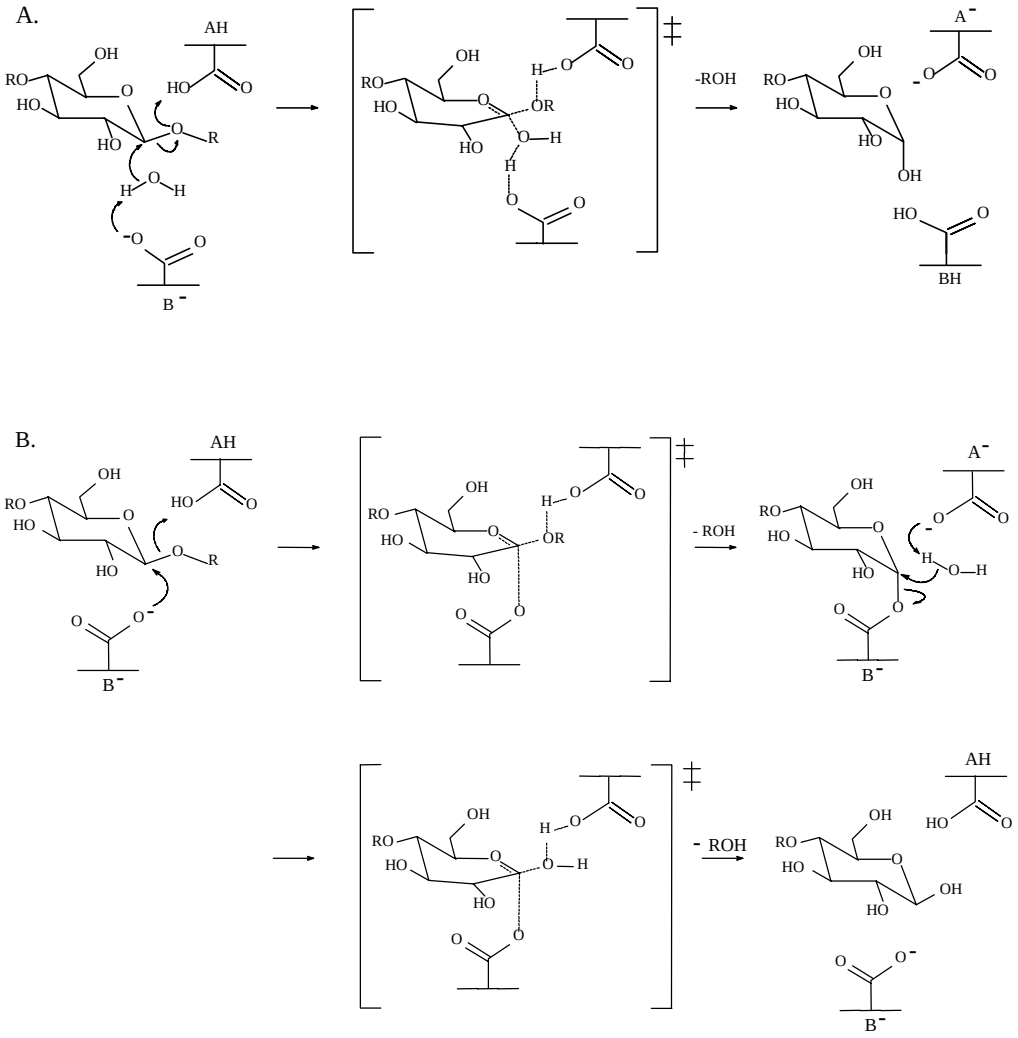


Fig. 9. Schematic presentation of the two reaction mechanisms of glycosyl hydrolases, i.e. (A, inverting) single-displacement and (B, retaining) double-displacement. For details see text.

Table 3. Experimentally identified amino acid residues involved in catalysis by T. reesei cellulases and hemicellulases.

Enzyme	Stereochemical course	Proton donor	Nucleophile/ assisting residue	Ref.
CBHI	retention	Glu217	Glu212	1-3
CBHII	inversion	Asp221	Asp175	3, 4
EGI	retention	Glu202	Glu197	5
EGII	retention	-	Glu329	6
XYNI	retention	Glu75	Glu164	7, 8
XYNII	retention	Glu177	Glu86	7, 8

Abbreviations used: Glu = Glutamic acid; Asp = Aspartic acid.
References: 1. Divne et al., 1994; 2. Divne et al., 1998; 3. Knowles et et al., 1988; 4. Rouvinen et al., 1990.; 5. Kleywegt et al., 1997; 6. Macarron et al., 1993b. 7. Törrönen and Rouvinen, 1995; 8. Biély et al., 1994.

1.5 Characterisation of the enzymes

Biochemical characterisation of enzymes is normally performed by following the change in concentration of substrate and/or product(s) with time. This may cause some problems since substrates can be either insoluble or hard to detect as in the case of cellulose and hemicelluloses. Thus there are difficulties in studying the properties of cellulases and hemicellulases when degrading their natural substrates. These reactions have normally been studied by following changes in the viscosity of the substrate solution, molecular size distribution of the reaction mixture, or production of soluble reducing sugars. Since the precise structure of cellulose and hemicelluloses is unknown, characterisation of the enzymatic activity of cellulases and hemicellulases is complicated and experimental data must be analysed with great care.

1.5.1 Detection techniques

Different soluble substrates, polymers and oligosaccharides, are easily available and are often used to study the biochemistry of cellulolytic and hemicellulolytic enzymes. Modified celluloses, HEC and CMC, are used to determine the endoglucanase activity of cellulolytic enzyme preparations by following the

change of viscosity of the substrate solution (Hulme, 1988). Numerous analytical methods have been used to follow the production of reducing oligosaccharides. The simple ones seem to be the most popular, e.g. the DNS-method (Sumner and Somers, 1949), Somogyi-Nelson method (Somogyi, 1952; Nelson, 1944), and enzymatic redox systems (Canevascini, 1985). The DNS-(3,5-dinitrosalisylic acid) and Somogyi-Nelson methods are based on colour formation, which is detected by spectrophotometry. Nevertheless, there are some problems because colour reagents such as 3,5-dinitrosalisylic acid partly hydrolyse cellulosic materials, and some compounds such as metal and phosphate ions may disturb the analysis of soluble oligosaccharides, which is already rather insensitive. Enzyme-coupled methods are based on oxidation of the produced oligosaccharides, normally cellobiose, e.g. by cellobiose oxidase or cellobiose dehydrogenase in the presence of an electron acceptor. The problem is that these enzymes are not specific for cellobiose but also act on the longer cello-oligosaccharides. Because of the many practical and analytical problems involved in these methods, a more reliable liquid chromatographic techinique should preferably be used. Small soluble oligosaccharides and their derivatives have been used to study the biochemical behaviour of cellulases and hemicellulases in detail. Pure soluble oligosaccharides have been used to study the kinetics of the enzymes and these substrates are also the best mimics of natural substrates since there are no structural changes, the only difference being the shorter chain length. Nevertheless, the number of studies in which these pure oligosaccharides have been used is surprisingly small, possibly because of the need for advanced high performance liquid chromatography (HPLC), which is the most reliable method for analysing soluble oligosaccharides (Braun et al., 1993; Reinikainen et al., 1995). Instead of pure oligosaccharides, derivatised analogs of dimers to pentamers have often been used. The most commonly used derivatives are those which contain either a chromophore or a fluorophore, e.g. a p-nitrophenyl or 4-methylumbelliferyl group, and are detected by spectrophotometric and fluorometric methods, respectively (van Tilbeurgh et al., 1982; van Tilbeurgh and Claeyssens, 1985; van Tilbeurgh et al., 1988; Bhat et al., 1990; Macarron et al., 1993a). These analogs are very useful since they can be detected at low concentrations. However, it must be borne in mind that these compounds are modified substrates, and the results must be interpreted with care. The simplest derivatives are those which utilise isotopes such as ^{3}H, ^{14}C and ^{18}O. Labelled compounds are easily detected either by counting the radioactivity of the sample

or by mass spectrometry (MS), and are useful when analysing the cleavage pattern, i.e. from which end the cleaved product originated (Chirico et al., 1985; Biély et al., 1993; Barr et al., 1996). Thin layer chromatography (TLC) has also been used both in the separation and the analysis of different reaction products (Chirico et al., 1985; Biély et al., 1993). The stereochemical course of the reaction can elegantly be followed by ^{1}H NMR spectroscopy as demonstrated by Withers et al. (Withers et al., 1986), and the technique can also be applied to cleavage pattern determination (Teleman et al., 1995; I, III). Furthermore, NMR spectra are recorded in real time, which is very practical since no sample preparation is needed, unlike all the other methods mentioned here. Protein difference spectroscopy, microcalorymetry, fluoresence titration and fluoresence energy transfer methods have successfully been used to study the binding of substrate analogs or inhibitors to these enzymes (van Tilbeurgh and Claeyssens, 1985; van Tilbeurgh and Clayssens 1989; Armand et al., 1997).

1.5.2 Analysis of enzyme kinetic data

Methods of analysing experimental kinetic data of enzymatic reactions are briefly discussed according to the main principles of the data evaluation. As is well known, in enzyme kinetics either depletion of substrate and/or formation of product(s) are followed as a function of time. Two methods used in the data analysis utilise initial rates or the full time-course of the reaction.

In initial rate determination only the initial part of the reaction, usually as short as a few percent of the total reaction, is taken into account. Unfortunately, in many cases even 1% of the complete reaction is nonlinear. If a series of initial rates is measured at different substrate concentrations, it is desirable to present the results graphically so that the values of the kinetic parameters can be estimated and the precison of the experiment assessed. The most obvious way of doing so is to plot v (velocity) against s (substrate concentration) as shown below in Fig. 10.

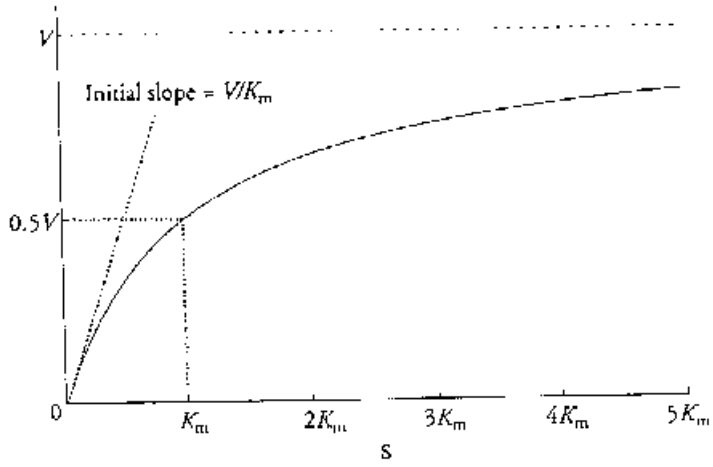


Fig. 10. Dependence of initial rate v on the substrate concentration s for a reaction obeying the Michaelis-Menten equation.

In practice, however, this is an unsatisfactory plot because it is difficult to draw a rectangular hyberbola accurately, locate the asymptotes correctly, perceive the relationship between a family of hyperbolas, and detect deviations from the expected curves if they occur. Actually Michaelis and Menten noticed these difficulties and instead plotted v against log s (Michaelis and Menten, 1913). Most scientists since Lineweaver and Burk (1934) have preferred to rewrite the Michaelis-Menten equation so that it allows the results to be plotted as a straight line. This plot is known as the Lineweaver-Burk plot or double-reciprocal plot and it is by far the most commonly used in enzyme kinetics even if it is not necessarily recommended. It tends to give an incorrect impression of the experimental error, i.e. for small values of v small errors in v lead to a large errors in 1/v; but for large values of v small errors in v lead to barely noticeable errors in 1/v, as seen in Fig. 11. The inventors of this plot were well aware of the need for weights and the methods to be used for determining them (Lineweaver and Burk, 1934; Lineweaver et al., 1934). There are also other plots which are either simple variations of the double-reciprocal plot or a rearrangement of the Michaleis-Menten equation, such as Hanes (see Fig. 11), Eadie-Hofstee and direct linear plots. These are considered more accurate and should be preferred when analysing experimental data graphically (Eadie, 1942; Hofstee, 1959; Dowd and Riggs, 1965; Eisenthal and Cornish-Bowden, 1974; Atkins and Nimmo, 1975; Cornish-Bowden and Eisenthal, 1978).

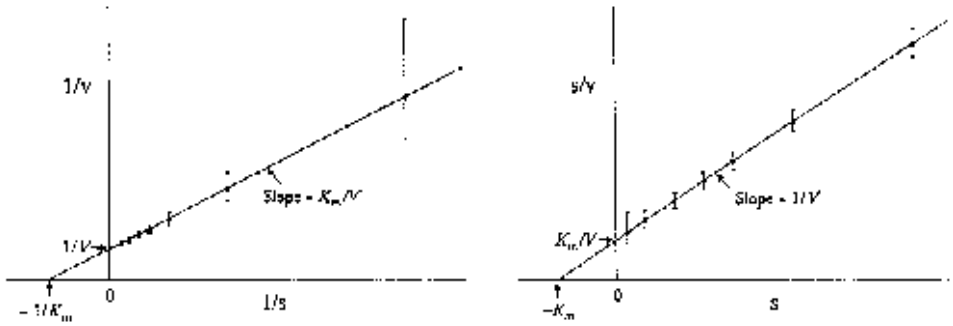


Fig. 11. At left, Lineweaver-Burk plot and at right, Hanes plot, both showing the identically calculated error bars for v, each of which represents ±0.05V. From Cornish-Bowden, 1995.

The graphical methods were developed at a time when no computers were available or were difficult to access and therefore graphical methods were used. However, computers are now avaible in every laboratory and thus they should be used in the data analysis. In addition, almost all enzymatic reactions exhibit nonlinear behaviour and simple mathematical relationships between data and transformation are incorrect and no longer valid when attempting to transform nonlinear models into linear ones. Therefore a nonlinear data analysis should be used, bearing in mind when and how linear transformations can be used.

When the full time-course of a reaction has been taken into account in data analysis it is often called as progress-curve analysis. For a simple reaction it is possible to derive integrated forms. Progress-curve analysis is based on a combination of numerical integration and nonlinear least-squares regression to fit rate equations to the experimentally determined progress curves. It is critically important to realise that the collection of experimental data and their subsequent analysis is not a process of two independent successive steps. The inherent random and systematic experimental errors in any type of experimental

data have a profound influence on the method of choice for the data analysis. Thus, the experimental procedure must be chosen carefully so that the data with their concomitant errors are compatible with the method of analysis. It is necessary to be aware of the assumptions inherent within different analysis procedures as well as the problems that may arise in matching experimental data to an analysis method.

Least-squares is a numerical method for finding an optimal set of parameters, α, of an equation, $G(\alpha,X)$, so that this equation will describe a set of data points X_i and Y_i. This numerical method is simply an algorithm which, when given an initial estimate for vector α, will find a better estimate for α. The procedure is then applied in an iterative fashion until the vector α does not change within some specified tolerance. There are a number of published algorithms (Nelder-Mead, Newton-Raphson, Gauss-Newton, Steepest descendent, and Marquardt-Levenberg) for performing this procedure, which are discussed elsewhere (Marquardt, 1963; Nelder and Mead, 1965; Bevington, 1969; Magar, 1972; Johnson and Faunt, 1992; Johnson, 1995). For most problems any of the algorithms will yield essentially the same parameter values within realistic confidence limits. However, each algorithm has different properties, such as the rate of convergence or sensitivity to experimental error, which may dictate the choice of the algorithm.

The methodology of least-squares parameter estimation methods is briefly discussed here and there are many references in the literature dealing with more thorough discussion of the least-squares parameter estimation procedure (for reviews see, Johnson and Faunt, 1992; Johnson, 1995). The least-squares parameter estimation method makes several assumptions, which are partially interrelated. First, all of the experimental errors in the data can be attributed to the Y-axis as graphically depicted in Fig. 12.

The least-squares method selects values for the various parameters of the function so that the sum of the squares of the vertical distances, r_i, is a minimum. It should be noted that it is only the vertical distances which are minimised, not the horizontal or perpendicular distances. Therefore, the problem must be formulated in such a way that the precision of determination of the values along the X-axis is significantly greater than the precision along the Y-

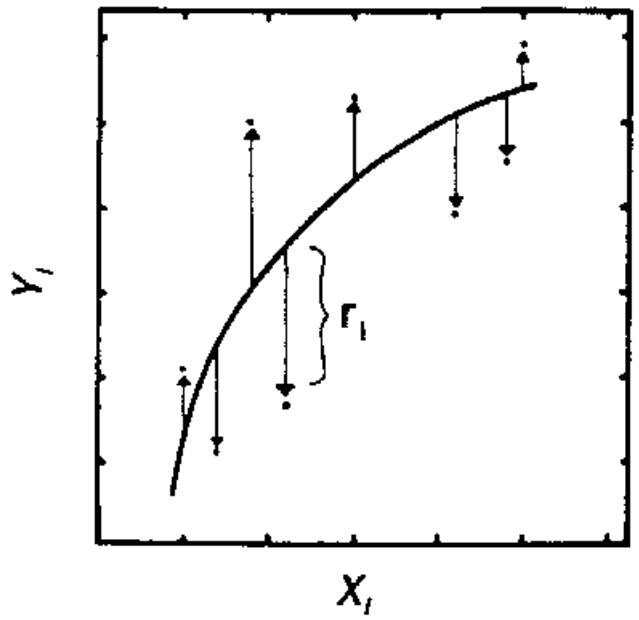


Fig. 12. Graphical representation of the residuals, r_i, of a least-squares parameter estimation procedure. From Johnson and Faunt, 1992.

axis. Secondly, random experimental errors in the data can be described by a Gaussian distribution. This means that if a given data point on Fig. 12 was experimentally measured an infinite number of times, the resulting distribution of error values (r_i's) could be described by a Gaussian distribution. This is a reasonable assumption for many, but not all, experimental procedures. It also implies that the number of data points is sufficient to ensure a good statistical sampling of the random error. The optimal number of data points required to meet this assumption is difficult to specify precisely but the theoretical minimum number of data points is equal to the number of parameters being simultaneously estimated. Because all data points contain experimental uncertainty, significantly more data points than the minimum should be used. The system is "overdetermined" when more than the minimum number of data points are used. Another assumption is that no systematic error exists in the data. The only way to control for this requirement is to include a series of terms in the function, G, to describe the systematic errors in the data. It is of course

necessary that the functional form of the fitting function, $G(\alpha,X)$, is correct and is capable of predicting the experimental data. In order for the determined parameters to have any physical meaning, $G(\alpha,X)$ must be the correct mathematical description of the phenomenon studied, including any systematic errors which might be present in the data. In other words, the dependent variable, Y_i, can be expressed as a function, G, of an optimal set of parameter values and the independent variable, X_i:

$$Y_i = G\left(\alpha, X_i\right) + \text{noise}_i \qquad (1)$$

In this equation noise_i represents the experimental uncertainty, or noise in the measurements. With these assumptions it is possible to define the probability, P_i, of making the observed measurement, Y_i, at the independent variable, X_i, and any particular value of α:

$$P_i(\alpha) = \frac{1}{\sigma_i\sqrt{2\pi}} \exp\left\{-\frac{1}{2}\left[\frac{Y_i - G(\alpha, X_i)}{\sigma_i}\right]^2\right\} \qquad (2)$$

where σ_i represents standard deviation of the random experimental error at a particular data point. The probability of making a series of these measurements at n distinct, independent data points is then

$$\begin{aligned} P(\alpha) &= \prod_{i=1}^{n} P_i(\alpha) \\ &= \left\{\prod_{i=1}^{n} \left(\frac{1}{\sigma_i\sqrt{2\pi}}\right)\right\} \exp\left\{-\frac{1}{2}\sum_{i=1}^{n}\left[\frac{Y_i - G(\alpha, X_i)}{\sigma_i}\right]^2\right\} \end{aligned} \qquad (3)$$

where the product $\prod$ and summation $\sum$ apply to each of the n data points with subscript *i*. The best estimates for the parameters, α, given these stated assumptions, are the values which maximize the probability in Eq. (3). Since the first term of Eq. (3) is constant and independent of the values of the coefficients, maximizing the probability of $P(\alpha)$ is the same as minimizing the sum in the exponential term in Eq. (3); that is minimizing:

$$\chi^2 = \sum \left[\frac{Y_i - G(\alpha, X_i)}{\sigma_i} \right]^2 \qquad (4)$$

Thus, the method dictated by these assumptions for finding the optimal values of the parameters from the data is to minimize the weighted sum of squares of the deviations, χ^2. This is commonly referred to as the method of least-squares.

All parameter estimation procedures minimize some norm of the data. For the assumptions presented above the appropriate norm is the sum of the squares of the deviations, the χ^2 in Eq. (4). However, if the assumptions are not strictly followed then the appropriate norm to minimize will not necessarily be χ^2. Consequently, use of the least-squares procedure when the assumptions are not satisfied will yield values for the parameters which are not the maximum likelihood estimates of their true values. In brief, graphical methods should only be used for displaying the results of experiments but not for producing the kinetic constants. Rather, a nonlinear least-squares technique is the method of choice when analysing data in order to determine kinetic constants.

2. Aims of the present study

The focus of this work was to study the kinetic behaviour of two cellobiohydrolases, CBHI and CBHII and two β-mannanases of *T. reesei* and one β-mannanase of *A. niger*. Pure soluble cello- and manno-oligosaccharides were used as model substrates.

The specific aims were:

1) To study the stereospecificities of the enzymes by ^{1}H NMR.

2) To describe models for enzymatic reactions reproducible by computer simulations, and to determine both the binding and the rate constants.

3) To study the transglycosylation pathway of the retaining enzymes of *T. reesei*.

3. Materials and methods

3.1 Materials

Two β-mannanases, BMANI and BMANII, of *T. reesei* were purified and the protein contents of the enzyme preparations were estimated by UV absorption at 280 nm as described by Stålbrand et al. or in paper V (Stålbrand et al., 1993). Purification of the *A. niger* β-mannanase is described in paper II. Purification of the *T. reesei* wt CBHI is described in detail in paper V. Wild type CBHII of *T. reesei*, free from contaminating endoglucanases (≤ 0.15 %), were purified as reported earlier (Reinikainen et al., 1995). Protein concentrations were estimated by UV absorption at 280 nm and the following molar absorption coefficients were used; CBHI, 73 000 M^{-1} cm^{-3} and CBHII, 80 500 M^{-1} cm^{-3} (van Tilbeurgh et al., 1986; Tomme, 1991). The production and purification of the two mutants of *T. reesei* CBHII, W272D and W272A, are described in detail in paper IV. The protein contents of the mutated enzymes were estimated by UV absorption at 280 nm and the following, tryptophan subtracted molar absorption coefficents were used: 74 950 M^{-1} cm^{-3} for the intact proteins and 69 450 M^{-1} cm^{-3} for the catalytic domains.

Deuterium oxide (D_2O, 99.8 % isotopic purity), acetic acid (CD_3CO_2D, 99.5 % isotopic purity), Dextran 1000 and α-D-Mannose were obtained from Fluka AG (Switzerland), manno-oligosaccharides (Man_2-Man_6) from Megazyme (Australia), sodium 3-trimethylsilyl-(2,2,3,3-D_4)-propionate from Ciba-Geigy (Switzerland), glucose and cello-oligosaccharides (Glc_2-Glc_6) were purchased from Merck (Darmstadt, Germany) and Seikagaku, Sigmacote from Sigma (St. Louis, USA), and 2,5-dihydroxybenzoic acid from Aldrich (Switzerland).

3.2 Analytical methods

Enzymatic reactions were followed as a function of time and analysed by HPLC, NMR and MS techniques.

3.2.1 HPLC assays (III - V)

The hydrolysis experiments with cello-oligosaccharides were carried out in 10 mM sodium acetate buffer, pH 5.0 at 27°C. In all experiments samples were taken at different time points and analysed by HPLC equipped with an RI detector. The separation was achieved by means of an HC-40 cation-exchange column (8×300 mm, Hamilton, Ca^{2+} form). Deashing cartridges (1 cation and 1 anion exchanger, 4.6×30 mm each, BIO-RAD) before the column in the eluent line removed buffer ions from the samples. Injected sample volumes varied between 20 and 100 µl and the column oven temperature was 80°C.

3.2.2 NMR assays (I - V)

For the proton NMR experiments deuterium-exchanged enzyme preparations were used. The buffer exchange for CBHI, CBHII, BMANI and BMANII of *T. reesei* and β-mannanase of *A. niger* was performed by repeated dilution in 10 mM deuterated sodium acetate buffer, pH 5.0 and ultrafiltration (Ultrafree-MC, cut-off 10 kDa, Millipore). The substrates, both manno- and cello-oligosaccharides, were then dissolved in the same buffer and reactions were carried out directly in the NMR tube at 5, 21, 27 and 50°C depending on the enzyme preparation and the purpose of the reaction. ^{1}H NMR spectra were recorded at 599.86 MHz on a Varian Unity 600-MHz Spectrometer using 5-mm NMR tubes usually containing 0.7 - 0.75 ml solution. Typical acquisition parameters were an 11 - 13 µs pulse (90°), a spectral width of 3000 Hz, an acquisition time of 2 s and a repetition time of 22 s. Normally 30 - 40 consecutive spectra, each based on 20 - 26 transients, were recorded for the time courses of the reactions.

3.2.3 MS assays (V)

The hydrolysis experiments with cello-oligosaccharides were carried out in 10 mM sodium acetate buffer, pH 5.0 at 27°C. In all experiments samples were taken at different time points and analysed by MS. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed with a BIFLEX™ mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany), using a 337 nm nitrogen laser. 0.5 - 1 µl of sample (5 - 20 pmol) and 2 µl of 2,5-dihydroxybenzoic acid matrix (DHB, 10 mg/ml in water)

were mixed on the target plate and dried with a gentle stream of air. Dextran 1000 was used as an external calibrant.

3.3 Data evaluation

The full kinetic equations, as described in detail in appendices 6 and 7, were solved by direct forward numerical integration with a time step of 0.1 - 1 s to produce complete progress curves. All the possible equilibrium stages and reaction steps were included in the model in each case when needed. The calculated progress curves were then fitted to the experimental data points by varying the relevant kinetic and equilibrium constants, in order to minimize the error square sum. The goodness of the fit was judged by the error square sum and the uncertainty in reported parameters was obtained by fixing a given parameter to various values while varying all other parameters. A doubling of the error square sum was taken as the permitted range of this parameter. The error analysis for the reported parameters was treated in the same way in each individual paper.

4. Results and discussion

4.1 Stereochemistry and kinetics of manno-oligosaccharide hydrolysis catalysed by two β-mannanases from *T. reesei* and one from *A. niger* (I, II).

Well resolved α- and β-anomeric proton resonances can be used to follow the stereochemistry of the oligosaccharide hydrolysis, and the stereospecificity of the β-mannanases was determined by ^{1}H NMR spectroscopy (Fig. 1, I). At first, the mutarotation rate of α-D-mannose was determined at 5°C (pH 4.5). Curve fitting resulted in a mutarotation rate of 1.2×10^{-5} s^{-1} and an anomeric equilibrium with 70/30 for α- and β-anomers. The stereochemical course of the hydrolysis of mannopentaose catalysed by the two β-mannanases from *T. reesei* and one from *A. niger* was determined at 5°C. These three β-mannanases hydrolyse β-1,4-mannosidic linkages with retention of the anomeric configuration (Figs. 2, I; 8b, II). Degradation pattern experiments were carried out with mannotriose and mannopentaose at 5 or 21°C by following the intensity of the anomeric proton resonance of the formed mannose or mannotriose, respectively (Figs. 4 and 3A, I; Fig. 8a, II). No mannose was detected during the first phase of the mannopentaose hydrolysis catalysed by BMANI and BMANII, showing that only the two middle mannosidic linkages are cleaved by the two β-mannanases of *T. reesei*. Curve fitting of the mannopentaose hydrolysis catalysed by BMANI resulted in a 70 % cleavage at the third mannosidic linkage and 30 % at the second linkage (Fig. 3B and Scheme 1, I). Because of the similar behaviour of BMANI and BMANII, the major β-mannanase of *T. reesei*, BMANI, was chosen for further studies. The hydrolysis of mannotriose catalysed by BMANI showed a minor preference for the first mannosidic linkage over the second linkage but there was no preference for the α- or β-mannotrioses as a substrate (Fig. 4, I). *A. niger* β-mannanase exhibited a degradation pattern similar to that of BMANI derived from the model calculation of mannopentaose hydrolysis (data not shown). No mannose was detected in the first phase of the mannopentaose hydrolysis catalysed by *A. niger* β-mannanase. Degradation patterns of these three β-mannanases are summarised in Fig. 13.

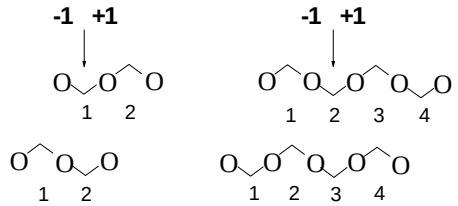
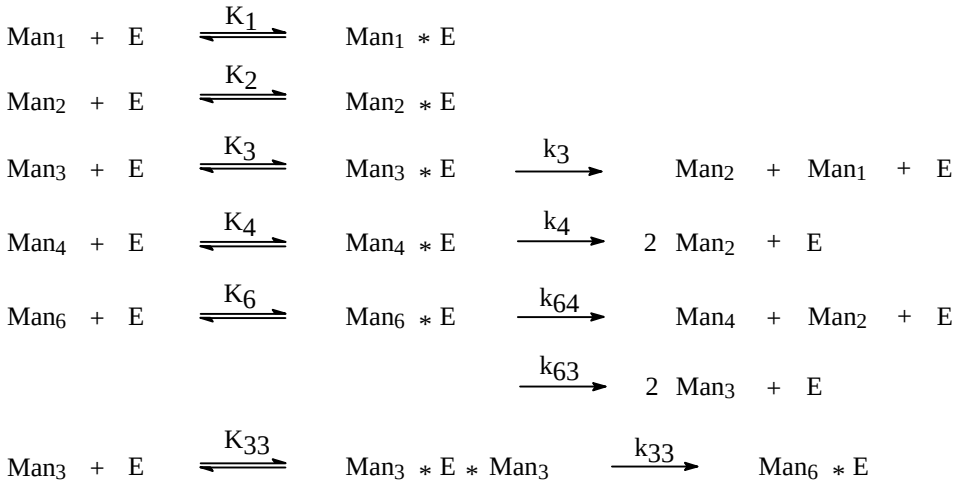


Fig. 13. Degradation patterns of the β-mannanases from T. reesei and A. niger for mannotriose and mannopentaose. An open circle denotes a mannose unit and the mannosidic linkages are numbered from the non-reducing end. Subsites –1 and +1 represent the cleavage site in the active site of the enzyme.

The kinetic behaviour of BMANI was studied using mannotriose as a model substrate at 50°C and the progress of the reaction was followed by ^{1}H NMR spectroscopy. All four anomeric proton resonances, i.e. reducing end α and β, internal, and terminal, were used in the simulations when fitting the experimental progress curves (Fig. 5, I). The possible transfer activity of the retaining BMANI was immediately seen as a rapid increase in the intensity of the terminal anomeric proton resonance (Fig. 5, I) and thus the transglycosylation was included in the model. This was also the case with *A. niger* β-mannanase, although no detailed kinetic analysis of the hydrolysis was performed (Fig. 8b, II). Mannotriose concentrations varied between 1 and 20 mM in these experiments and five different substrate concentrations were used in the simulations when determining the rate and the binding constants of BMANI (Fig. 6, I). Three different models for transglycosylation were tested in order to reproduce the experimental progress curves and only the model of two mannotrioses to mannohexaose (see below, Scheme 1) was able to fit the calculated progress curves with the experimentally determined data points (Scheme 2, I).



Scheme 1. A model for the transglycosylation between two mannotrioses. $Man_1 \rightarrow Man_6$ are the corresponding oligosaccharides and E is BMANI. K_n are binding constants for the equilibrium stages and k_n are rate constants for the bound enzyme complexes.

At first, only one concentration at a time was used in the simulation in order to reproduce the experimental progress curves. Even if a good fit was generated, it was still not enough for that set of parameters to fit the other concentrations (Fig. 5, I). Therefore five different concentrations (1, 2, 3, 10 and 20 mM) were used in the simulations when calculating the final values of the rate and binding constants. Model calculations showed that the rate of hydrolysis of longer manno-oligosaccharides was almost two orders of magnitude (2 s^{-1}) greater than the rate of hydrolysis of mannotriose (0.03 s^{-1}). Interestingly, the highest rate of all was the rate of transglycosylation of two mannotrioses to mannohexaose (15 s^{-1}). Furthermore, indirectly observed mannotetraose and mannohexaose had a major effect on the quality of the fit (Fig. 6, I). This was checked using 1 mM mannotriose as a test experiment and no longer manno-oligosaccharides were detected by MS (see 4.3, V). The result of the final calculation is given in Table 4 (Table 2, I). It should be borne in mind that the constants derived from the simple black box model do not necessarily agree with the constants produced

using the full degradation mechanism of mannotriose as discussed later in 4.3 (paper V).

Table 4. The calculated binding and rate constants of the mannotriose hydrolysis catalysed by BMANI at 50°C (pH 4.5).

Mannoside	Binding constant M^{-1}	M^{-2}	Rate constant s^{-1}
Man_1	30		-
Man_2	2×10^3		-
Man_3	5×10^3		$(3\pm1)\times10^{-2}$
Man_4	$>1\times10^6$		2 ± 1
Man_6	$>1\times10^6$		2 ± 1
$2\times Man_3$		$7x10^4$	15 ± 1

In brief, these β-mannanases from *T. reesei* and *A. niger* have similar biochemical properties. The enzymes have slightly different molecular masses, 51 and 53 kDa for *T. reesei* β-mannanases and 40 kDa for the *A. niger* β-mannanase and only the former contain a cellulose binding domain. They are active over a wide pH range (pH 3-7) as well as at high temperatures (< 70°C). According to the hydrophobic cluster analysis they all belong to the same glycosyl hydrolase family, i.e. family 5, which also contains a conserved catalytic machinery having two glutamates as the catalytic amino acid residues (E169 and E275 for *T. reesei* β-mannanase, Stålbrand et al., 1995). They catalyse the hydrolysis of β-1,4-mannosidic linkages with retention of the anomeric configuration and also show transient synthesis of longer manno-oligosaccharides, i.e. transglycosylation (I, II, and McCleary and Matheson, 1983). Furthermore, these β-mannanases are able to hydrolyse mannotriose, although four mannose units are essential for the efficient hydrolysis, supporting the hypothesis that the active site of the enzyme should consist of at least four sugar-binding subsites.

4.2 Degradation patterns and progress-curve analysis of cello-oligosaccharide hydrolysis catalysed by wt CBHII and two mutants, W272A AND W272D, of *T. reesei* (III - IV).

A series of hydrolysis experiments was carried out with cellotetraose, cellopentaose and cellohexaose at 27°C to determine both the cleavage patterns and the kinetics of the hydrolysis catalysed by the wt CBHII. Time-courses for the wt CBHII-catalysed hydrolysis of these cello-oligosaccharides were followed by HPLC and NMR spectroscopy. Both techniques showed that no glucose was produced in the initial phase of hydrolysis of cellotetraose, cellopentaose, or cellohexaose. Accordingly, the terminal linkages of these cello-oligosaccharides are not hydrolysed. Thus cellotetraose is exclusively cleaved in the middle, producing two cellobioses (Fig. 1, III), and cellopentaose is cleaved at the second and third glycosidic linkages into equal amounts of cellobiose and cellotriose. Cellopentaose hydrolysis was carried out in such a way that there was a rapid hydrolysis of cellopentaose to cellobiose and cellotriose, whereafter the mutarotation of the formed cellotriose was monitored. Using this secondary phase of the hydrolysis, it was possible to back-calculate which of the two middle linkages is the preferred one. Curve fitting resulted in a slight preference for the second glycosidic linkage (55 %) over the third linkage (45 %), thus releasing slightly more cellobiose from the non-reducing end of the substrate. Cellohexaose was cleaved to cellobiose and cellotriose with a product ratio close to 3/2. Therefore the second (or fourth) and third glycosidic linkages are cleaved with almost equal probability. Using the product formation rates from the HPLC data, there was a minor but reproducible preference for cleavage of the third glycosidic linkage (52 %). This almost 50/50 cleavage probablity at the two middle glycosidic linkages in cellopentaose and cellohexaose suggests that the orientation of the entering substrate is random. In addition, only traces of cellotetraose were detected during cellohexaose hydrolysis and thus the bound cellotetraose does not leave the enzyme but is further cleaved to two cellobioses (Fig. 4A, III). These experiments were also performed for the two mutants of *T. reesei* CBHII, W272A and W272D, to determine the cleavage patterns of the mutated enzymes (unpublished results). Both of the mutants exhibited the same cleavage pattern as the wt CBHII. Furthermore, no glucose was detected during the first phase of the hydrolysis of cellotetraose, cellopentaose, or cellohexaose and no cellotetraose was produced during the hydrolysis of cellopentaose or

cellohexaose. These cleavage pattern results are consistent with other results reported in the literature using both tritium labelled and chromophoric groups containing oligosaccharides as well as with those in which CBHII has been isolated from other sources, i.e. *Penicillium pinophilum* and *Humicola insolens* (Claeyssens et al. 1989; Schou et al, 1993). The cleavage patterns of these three enzymes of *T. reesei* are summarized in Fig. 14.

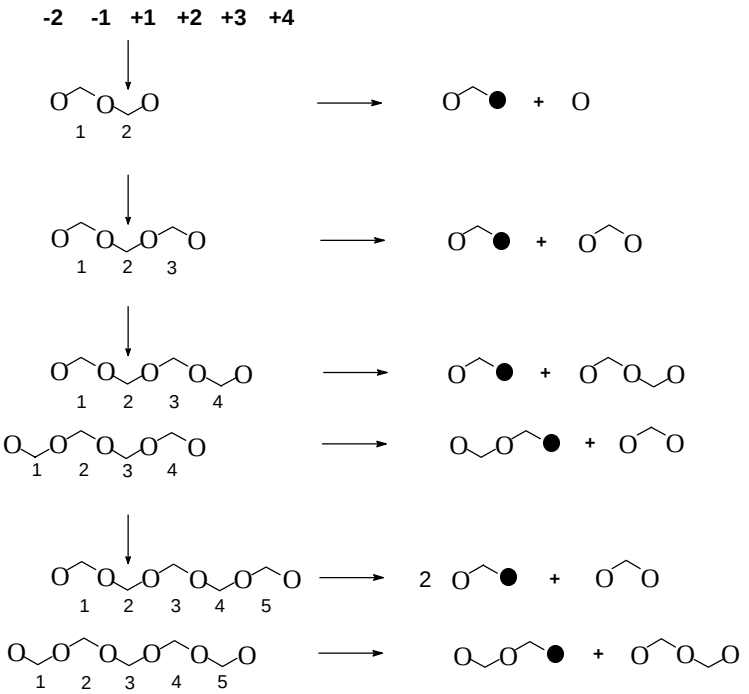


Fig. 14. Cleavage patterns of cello-oligosaccharides catalysed by the wild type, W272A, and W272D of T. reesei CBHII. Open circles represent glucose units and filled circles the new reducing ends with an α-anomeric form. The possible orientations for cleavage of the cello-oligosaccharides in the active site of the enzyme (-2, -1, +1, +2, +3, and +4) are shown. The glycosidic linkages are numbered starting from the non-reducing end, and arrows represent the cleaved linkages between the subsites –1 and +1. The up and down orientations of the glycosidic linkage are denoted by ∧ and ∨, respectively.

Kinetic constants of the hydrolysis catalysed by wt CBHII were obtained from the progress-curve analysis and are given in Table 5. These experiments were carried out both at lower cello-oligosaccharide concentrations (≤ 0.3 mM) and at higher concentrations (≥ 1 mM), and were followed by HPLC (Fig. 6A, III) and NMR (Fig. 6B, III), respectively. The initial parts of the progress curves of cellotetraose and cellopentaose at higher concentrations were nonlinear, indicating product inhibition, and thus no initial slope approximation was used. The cellopentaose hydrolysis consisted of two different phases having a rapid hydrolysis of cellopentaose to cellobiose and cellotriose, and a concomitant slow hydrolysis of the formed cellotriose to glucose and cellobiose (Fig. 7, III). Cellohexaose hydrolysis exhibited an unexpected feature. With a low cellohexaose to enzyme ratio (≈1000) the expected biphasic behaviour was obtained, i.e. the rapid hydrolysis of cellohexaose to cellobiose and cellotriose. When the cellohexaose to enzyme ratio was higher than 1000, the first phase of the hydrolysis was no longer completed as quickly as expected and it had a nonlinear curvature. This was not caused by adsorption of the enzyme to the glass walls of the container, since a control experiment with Sigmacote produced identical progress curves. The simple kinetic model that correctly reproduces the progress curves for cellohexaose is shown below:

$$E + Glc_6 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E * Glc_6$$

$$E * Glc_6 \overset{k_{inact.}}{\rightleftharpoons} E_I * Glc_6$$

$$E * Glc_6 \xrightarrow{k'_6} E * Glc_4 + Glc_2^{\alpha}$$

$$E * Glc_6 \xrightarrow{k_6} E * Glc_3 + Glc_3^{\alpha}$$

By using a binding constant of 10^4 M^{-1} for cellobiose, the experimental and calculated progress curves could be accurately fitted and produced a binding constant of $> 3\times10^5$ M^{-1} for cellotetraose and cellopentaose. The rate constants derived from the progress-curve analysis of cellotetraose, cellopentaose and cellohexaose were clearly higher, 1-12 s^{-1}, than for cellotriose (0.06 s^{-1}). The active site of CBHII is a 20 Å long tunnel, consisting of four individual sugar binding sites, +2 to -2 (formerly A-D). The cleavage site is situated between the

subsites -1 and +1, having two suitably positioned aspartates D221 and D175 in close proximity (Rouvinen et al., 1990; Davies and Henrissat, 1995). Furthermore, Rouvinen et al. suggested from a modelling study that there could be an extra binding subsite at the entrance of the tunnel, i.e. tryptophan W272, that would be able to interact with longer chains (Rouvinen et al., 1990). Accordingly, the rates derived from progress-curve analysis of the cello-oligosaccharides, Glc_{4-6}, prove the existence of at least four binding subsites in the active site of the wt CBHII. Furthermore, the interaction at the subsite +2 is evidently needed for the efficient hydrolysis of soluble cello-oligosaccharides. The contribution of the subsite +2 is not as important as the subsite -2 that is essential for the hydrolysis. Moreover, the rate of hydrolysis of cellopentaose is slower than that of cellotetraose, whereas cellohexaose is hydrolysed faster than either of these. Thus an extra interaction between the enzyme and substrate other than -2 to +2 must be taken into account (Rouvinen et al., 1990; III; see Fig. 15). For cellopentaose several binding modes can be drawn which support the idea that the catalytic step could be rate-limiting and hence two important binding modes for cellopentaose could be differentiated, namely -2 to +3 and -1 to +4. On the basis of this, the stronger binding mode -1 to +4 explains the slower hydrolysis rate of cellopentaose if the interaction at the subsite +4 is as strong as at -2 and the same binding avoids the distortion of the ring 2 as is the case for the productive binding mode. For cellohexaose hydrolysis two important features can be seen. First, there is a barely detectable amount of cellotetraose and thus cellotetraose is immediately hydrolysed to two cellobioses without leaving the active site of the enzyme. This is consistent with earlier results of Nidetsky showing that only Glc_1, Glc_2 and Glc_3 are detected (Nidetsky et al., 1994b). Second, progress curves of the hydrolysis are too curved to be explained by product inhibiton. Hydrolysis seems to be a time-dependent phenomenon because high enzyme to cellohexaose ratios produce linear progress curves. Progress-curve analysis resulted in an inactivation rate of 10^{-3} s^{-1} using the kinetic model as shown earlier. This could be the result of an unsuitable fitting of cellohexaose to the active site, which could cause some strain leading to minor conformational changes.

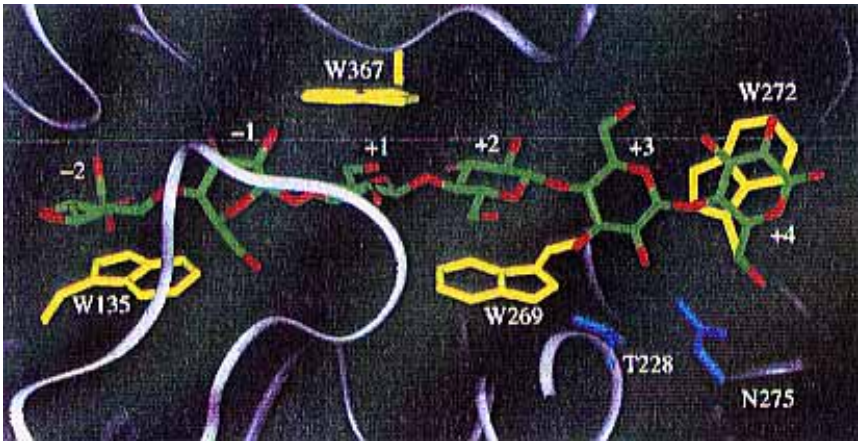


Fig. 15. A model of cellohexaose binding to the active site of CBHII. The subsites and protein side chains are numbered by the white labels and the tryptophan residues are shown in yellow. The two turquoise residues,T228 and N275, have been proposed to form hydrogen bonds at subsites +3 and +4.

The kinetics of the hydrolysis of soluble cello-oligosaccharides catalysed by the two mutants, W272A and W272D, were studied by using HPLC and NMR spectroscopy. The kinetic constants were determined for cellotriose, cellotetraose, cellopentaose and cellohexaose. The same set of kinetic equations, i.e. the program, was used both for the mutants and for the intact enzyme, wt CBHII. Progress-curve analysis resulted in a clear rate enhancement for cellotriose and cellopentaose but not for cellotetraose or cellohexaose, showing a gradual increase in the rate constants as a function of chain length, unlike in the case of wt CBHII. This also provides evidence for the competitive binding mode for cellopentaose and the results observed with cellulosic substrates indicate that the single binding subsite could be important in disrupting and directing long chains into the active site of the enzyme for hydrolysis. The rate and binding constants derived from the progress-curve analysis of Glc_{3-6}, catalysed by the wt CBHII and the two mutants, W272A and W272D, are given below in Table 5.

Table 5. The rate and binding constants for the hydrolysis of wild type Cel6A, W272A, and W272D on cello-oligosaccharides, $Glc_3 \rightarrow Glc_6$, in 10 mM NaAc buffer at 27°C (pH 5.0).

Substrate	wild type		W272A		W272D	
	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m
Glc_3	$(6\pm1)\times10^{-2}$	17±5	0.4±0.1	67±5	0.4±0.1	67±5
Glc_4	4.1±0.5	2.6±0.5	5±1	Nd	4±1	Nd
Glc_5	1.1±0.2	1.3±0.4	8±2	(~20)	8±2	16±5
Glc_6	14±2	14±6	≥15	Nd	≥15	Nd

4.3 Degradation patterns and transglycosylation of cello- and manno-oligosaccharide hydrolysis catalysed by CBHI and BMANI of *T. reesei* (V).

A number of hydrolysis experiments were carried out in order to determine both the cleavage patterns and the transglycosylation products of the hydrolysis of cello- (Glc_{3-6}) and manno-oligosaccharides (Man_{3-6}) catalysed by CBHI and BMANI, respectively. Both HPLC and NMR data showed that glucose was produced in the initial phase of the hydrolysis of cellotriose, cellotetraose, cellopentaose and cellohexaose (data not shown). For cellotriose, cleavage took place almost exclusively at the first glycosidic linkage, resulting in released cellobiose from the reducing end and β-glucose, see Fig. 16.

In the case of cellotetraose the major cleavage (75 %) took place at the second glycosidic linkage, producing two molecules of cellobiose. In addition, a minor (25 %) cleavage took place at the first glycosidic linkage, releasing cellotriose from the reducing end and β-glucose. Cellopentaose and cellohexaose were mainly hydrolysed to Glc_1, Glc_2 and Glc_3, and only trace amounts of Glc_4 and Glc_5 were detected during the hydrolysis of cellopentaose and cellohexaose, respectively. This indicates that the reducing end of the substrate enters the active site tunnel from its -7 site end and it also shows that the bound Glc_4 and Glc_5 in the active site of CBHI are further hydrolysed to Glc_1, Glc_2, and

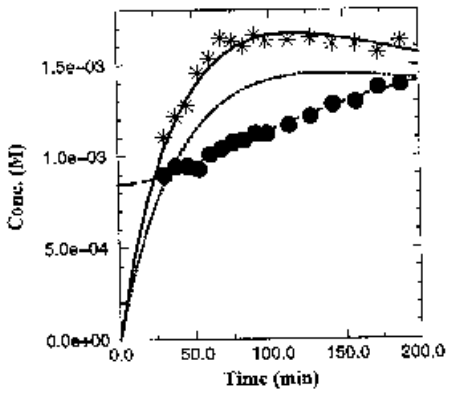


Fig. 16. Cellotriose (2 mM) hydrolysis by CBHI (36 μM). The changes in the concentrations of α-anomeric (•) and β-anomeric (+) protons of glucose are shown as determined by ^{1}H NMR spectroscopy. The solid and dotted lines show the calculated concentrations of β-glucose assuming (–) cleavage exclusively at the non-reducing end or (...) random cleavage. The dashed line shows the calculated concentrations of α-anomeric protons.

Glc_3 before leaving the active site. This behaviour is referred to as processivity and agrees well with the results of Nidetsky et al., showing that no intermediate products longer than cellotriose are released during the hydrolysis of Glc_{4-8} (Nidetsky et al., 1994b). The cleavage pattern of cellopentaose can be obtained from the progress curve of the initially formed cellotriose as described earlier for CBHII (III) but for CBHI this approach cannot be utilised. Because of the high enzyme concentration used, a prerequisite for this experiment, some of the proton resonances of cellotriose are selectively broadened and hence it is impossible to resolve the internal anomeric proton resonances for α- and β-cellotrioses. The cleavage patterns agree well with the results obtained using either tritium-labelled or 4-methylumbelliferyl substituted cello-oligo-saccharides (Vrsanska and Biély, 1992; Biély et al. 1993; Claeyssens et al., 1989). The only difference is that in our hands the glycosidic linkage at the non-reducing end is preferred over the reducing end. An explanation for this

discrepancy could be based on the purity of the enzyme preparations used. Reinikainen et al. showed that a minor endoglucanase contamination of CBHII caused considerable variation in the hydrolysis of β-glucan. Even as little as 0.4 % endoglucanase of the total protein content was found to have a marked effect (Reinikainen et al., 1995) and thus there may have been some interfering endoglucanase activity in the enzyme preparations used in earlier studies. The cleavage pattern of CBHI is summarised in Fig. 17.

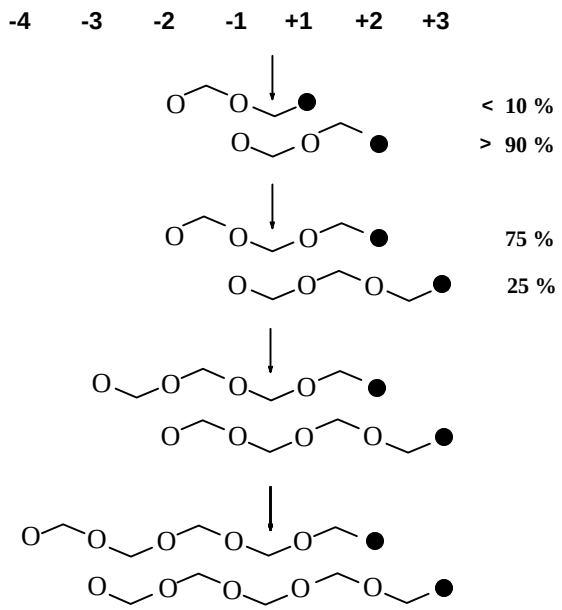


Fig. 17. CBHI-catalysed cleavage pattern of cello-oligosaccharides. (O) Glucose unit; (●) reducing end. The possible orientations for cleavage of the cello-oligoasaccharides in the active site of CBHI (only -4→+3) are shown. The glycosidic linkages are numbered starting from the non-reducing end and arrows represent the cleaved linkages at cleavage site between subsites –1 and +1. The up and down orientations of the glycosidic linkage are denoted by ∧ and ∨, respectively. Cleavage probabilities for cellotriose and cellotetraose are also indicated.

During the hydrolysis of mannotetraose, mannopentaose and mannohexaose catalysed by BMANI no mannose was detected in the first phase of the hydrolysis. Only trace amounts of mannotetraose were detected in the hydrolysis of mannopentaose, whereas in the case of mannohexaose as substrate the tetramer was detected in relatively high amounts. This shows that BMANI behaves like an endoglycanase without processivity, unlike CBHI and CBHII. Accordingly, mannotetraose is cleaved in the middle and mannopentaose at the second (30 %) and third (70 %) mannosidic linkages as reported (I). Thus the cleavage patterns of BMANI agree well with the results reported for the β-mannanases of *Pseudomonas fluorescens spp. cellulosa, Aspergillus niger, Sclerotium rolfsii* and *Streptomyces* (Bolam et al., 1996; II; Coulombel et al., 1981; Gübitz et al., 1996).

MALDI-TOF MS was used to study the possible transglycosylation products in the hydrolysis of cello- and manno-oligosaccharides catalysed by CBHI and BMANI. In the case of cellotriose and mannotriose, no transglycosylation products were detected. In the case of cellotetraose, cellopentaose and cellohexaose, n+1 cello-oligosaccharides were detected (n denotes the number of glucose units in the substrate). In the case of mannotetraose, mannopentaose and mannohexaose, n+2 manno-oligosaccharides were initially detected and somewhat later n+1 manno-oligosaccharides, as is shown in Fig. 18.

Although no transglycosylation products were detected in the hydrolysis of mannotriose by MS, NMR results clearly showed that transglycosylation must be involved. The increase in the intensity of the terminal anomeric proton resonance can not be explained without transglycosylation. However, the degradation of $Man_{4\text{-}6}$ was much more rapid than the rate of transglycosylation and thus the longer manno-oligosaccharides did not accumulate in detectable amounts. The NMR progress curves obtained from the hydrolysis of mannotriose could be accurately fitted in two different ways using both mannosyl-enzyme and mannobiosyl-enzyme intermediates which are transglycosylated into Man_4 and Man_5, respectively. The mannopentaose formed can also be hydrolysed in two different ways, producing either mannobiosyl-enzyme intermediate or mannotriosyl-enzyme intermediate and the latter can be transglycosylated into Man_6. Mannohexaose can be hydrolysed in three different

ways, producing Man_2+Man_4, Man_3+Man_3, and Man_4+Man_2. The Man_3+Man_3 reaction is actually the reverse reaction of transglycosylation and therefore cannot be detected, whereas the other two reactions will ultimately result in the formation of three molecules of mannobiose.

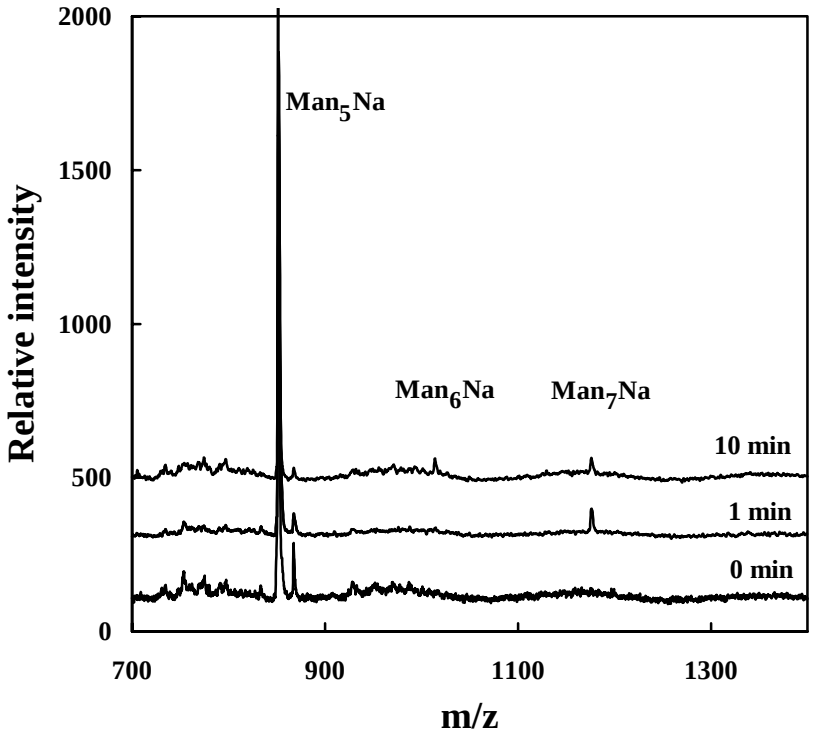
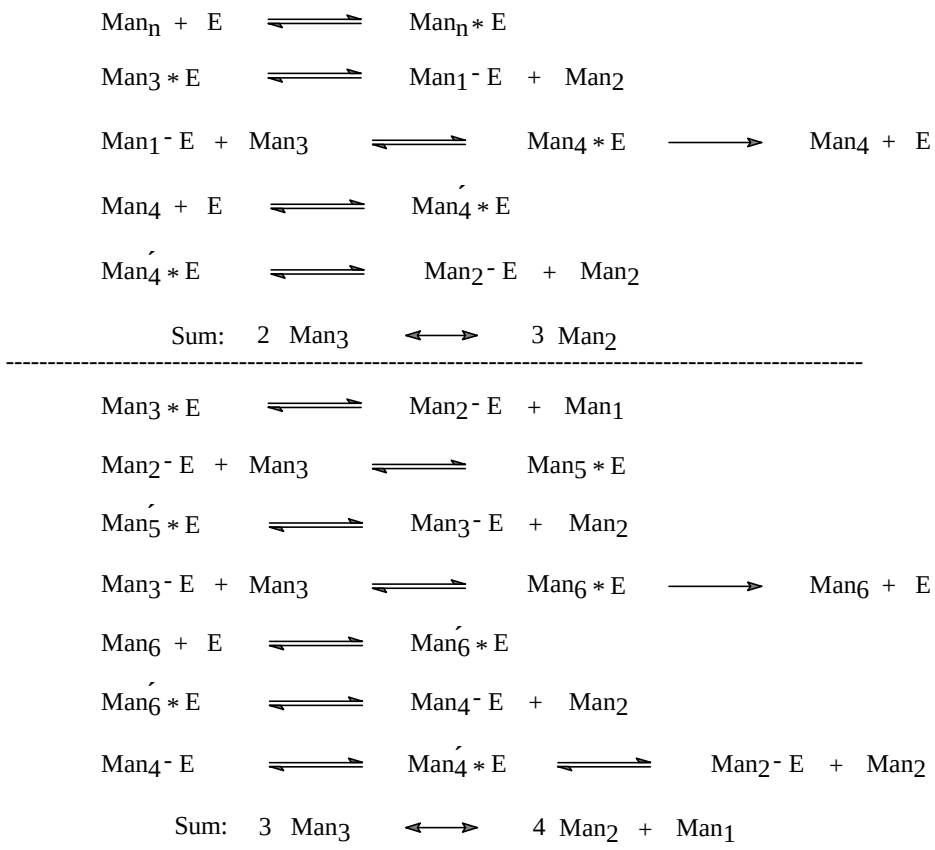


Fig. 18. Mass spectra of mannopentaose (1 mM) hydrolysis by BMANI (0.035 μM) before enzyme addition and 1 and 10 minutes after the addition. Manno-oligosaccharides were detected as sodium adducts.



Scheme 2. The full degradation model of mannotriose hydrolysis by BMANI. Man$_{1-6}$ are the corresponding manno-oligosaccharides and E is BMANI. Man$_n$-E is the covalent saccharide-enzyme intermediate and ‘ represents hydrolysis in such a way that Man$_2$ is hydrolysed from the reducing end.

As reported earlier for the mannotriose hydrolysis, both mannosidic linkages are cleaved almost equally rapidly by BMANI, with only a slight preference for the second mannosidic linkage (I). This indicates that the full degradation mechanism of mannotriose must include both mannosyl-enzyme and mannobiosyl-enzyme intermediates in the transglycosylation pathways (Scheme 2). The relative weights of these two pathways strongly depend on the hydrolysis rates of mannosyl-enzyme and mannobiosyl-enzyme intermediates as well as on the two intial rates of transglycosylation.

The time-courses of the hydrolysis of cellotriose, cellotetraose, cellopentaose and cellohexaose catalysed by CBHI were also followed by both HPLC and NMR spectroscopy. Results of the progress-curve analysis are shown in Table 6.

Table 6. Kinetic constants for the hydrolysis of cello-oligosaccharides (Glc_{3-6}) by CBHI in 10 mM NaAc buffer at 27°C (pH 5.0).

Glucoside	Rate of hydrolysis	Rate of transglycosylation	Dissociation constant
Glc_3	0.04	-	160
Glc_4	2	0.4	50
Glc_5	2	0.2	50
Glc_6	≥2	0.1	50

The program used to calculate the kinetic constants of CBHI-catalysed hydrolysis is presented in Appendix 7. Interestingly, the hydrolysis rates for the longer cello-oligosaccharides, i.e. Glc_{4-6}, were all at the same level, about 2 s^{-1}, and there was only a minor increase for cellohexaose. Dissociation constants estimated for Glc_{4-6} also appeared to be at the same level, 50 μM, showing no major difference between Glc_3 and Glc_{4-6}. Progress-curve analysis showed that experimental progress curves could be reproduced when transglycosylation was involved, as shown below in Fig. 19.

The results also show that the transglycosylation rate is dependent on the chain length of the substrate, i.e. the longer the chain the slower the rate (G6<G5<G4). Furthermore, the hydrolysis of trisaccharides by CBHI and BMANI is slow, whereas for the longer oligosaccharides BMANI exhibits much faster hydrolysis (>100). The most obvious reason for this is in the active site structures of the enzymes, since the active site tunnel of CBHI is much more difficult to access than the active site of BMANI.

In brief, both of the enzymes studied are retaining ones, and therefore able to effect transient synthesis of longer oligosaccharides in addition to the hydrolysis. Using trisaccharides as subtrates it was clear that BMANI showed efficient transglycosylation. However, no longer manno-oligosaccharides were detected, whereas in the case of CBHI tranglycosylation was too slow to be

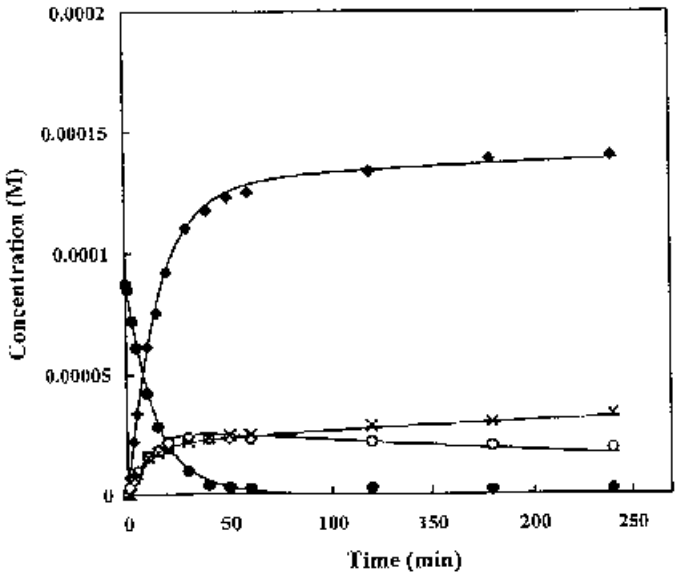


Fig. 19. Progress curves for the CBHI-catalysed hydrolysis of cellotetraose. Experimental and calculated cello-oligosaccharide concentrations are shown as a function of time. The initial cellotetraose and CBHI concentrations were 0.1 mM and 0.2 μM. HPLC was used to monitor the concentrations of cellotetraose (•), cellotriose (o), cellobiose (♦) and glucose (x). Solid lines represent the calculated progress curves.

detected even if it existed at all. For the longer oligosaccharides, $Glc_{4\text{-}6}$, and $Man_{4\text{-}6}$, both enzymes exhibited transfer activity and intermediates longer than the substrate were detected. Interestingly, CBHI produced an intermediate molecule one unit longer than the substrate, whereas BMANI produced an intermediate two units longer than the substrate. Since BMANI is an efficient transglycosylating agent this property could be utilised in the synthesis of longer oligosaccharides and if the enzyme retains activity in organic solvents, it could possibly be used in the production of different carbohydrate derivatives with industrial applications comparable to the use of alkylated oligosaccharides as detergents.

5. Conclusions

The present investigation was focused on the hydrolysis of cello-oligosaccharides by two cellobiohydrolases and on the hydrolysis of manno-oligosaccharides by three β-mannanases. Time courses of the enzyme-catalysed hydrolyses were monitored and three different analytical techniques were applied, i.e. HPLC, MALDI-TOF MS and NMR spectroscopy. Enzyme kinetic data were evaluated using tailor-made programs with user defined equations which were executed by a computer. The program was constructed in such a way that it described all the stages for binding equilibria and hydrolysis steps of each enzyme so that all the steps involved could be treated uniquely in order to produce kinetic constants for the enzyme in question.

NMR spectroscopy was applied to determine the stereospecificity of the three β-mannanases because of its efficiency in detecting the two possible configurations of the newly formed anomeric center of the reducing end in real time. NMR data showed that the three β-mannanases studied are retaining glycosyl hydrolases which are able to transglycosylate manno-oligosaccharides having at least three mannose units. NMR data also confirmed that these enzymes have active sites which contain at least four mannose binding subsites to enhance the rate of hydrolysis when comparing Man_3 to Man_{4-6}.

Both HPLC and NMR data demonstrated that CBHI and CBHII hydrolyse soluble cello-oligosaccharides selectively. CBHI immediately produced glucose as an end product, unlike CBHII, when Glc_{4-6} were used as substrates, and thus only the internal glycosidic linkages are uniquely cleaved by CBHII. Interestingly, it was shown for the first time that hydrolysis of cellotriose can proceed in different ways: CBHI releases cellobiose from the reducing end whereas CBHII releases cellobiose from the non-reducing end. Furthermore, the progress-curve analysis produced new valuable data for the CBHII-catalysed hydrolysis of cello-oligosaccharides, indicating that time-dependent inactivation of CBHII for cellohexaose hydrolysis results in a rate constant of 10^3 without losing the activity. The progress-curve analysis also provided evidence for the competing binding modes of cellopentaose, as well as for the existence of two extra binding subsites in the near vicinity of the active site tunnel entrance. The importance of the two extra binding subsites was also demonstrated using mutated CBHII preparations with cellulosic substrates, showing that a single

binding subsite could have a marked contribution in disrupting and directing long chains into the active site for hydrolysis.

The possible existence of intermediate reaction products longer than the substrate was studied using MALDI-TOF MS. Different transglycosylation pathways were observed for CBHI and BMANI and the rate constants for the transglycosylations were determined. CBHI produced one unit longer transient products than the corresponding substrate, whereas BMANI produced two units longer transient products than the substrate. The rate of transglycosylation was two orders of magnitude higher for BMANI than for CBHI. CBHI also showed chain length dependent transglycosylation.

Finally, using the methods applied in the present investigation for producing kinetic data, and combining our results with structural data when available, we were able to add another piece towards the understanding of the hydrolysis of complex substrates at the molecular level.

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