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# Mannan-hydrolysis by hemicellulases

Enzyme-polysaccharide interaction of a modular  $\beta$ -mannanase

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Department of Biochemistry  
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2002



**LUND**  
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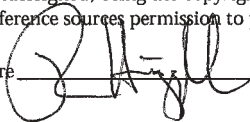
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Title and subtitle Mannan-hydrolysis by hemicellulases: enzyme-polysaccharide interaction of a modular $\beta$ -mannanase			
<p>Abstract</p> <p>The enzymatic degradation of plant polysaccharides is a process of fundamental importance in nature which involves a wide range of enzymes. In this work, the structure and function of hemicellulose-degrading enzymes was investigated. The focus was on a <math>\beta</math>-mannanase (TrMan5A) produced by the filamentous fungus <i>Trichoderma reesei</i>. This enzyme is composed of a catalytic module and a carbohydrate-binding module (CBM). In this thesis, the enzyme-polysaccharide interaction in both these modules was investigated.</p> <p>The results demonstrate that the CBM of TrMan5A is important for hydrolysis of complex mannan substrates containing cellulose. Furthermore, the increase in activity could be linked to binding of the CBM to the complex substrate. Binding studies revealed that the CBM binds to cellulose, but not to mannan. Studies of the enzyme/polysaccharide interaction in the active site cleft of the catalytic module of TrMan5A showed that a mutant of Arg 171 displayed activity in the same range as the wild-type enzyme toward polymeric substrates. However, the Arg171 mutant was impaired in hydrolysis of small substrates. Interestingly, this mutant also appears to have a more alkaline activity pH-optimum than the wild-type. The low or abolished activity observed with mutants of the predicted catalytic glutamates (Glu169 and Glu276) support their importance in hydrolysis. In addition to TrMan5A, the properties of a <math>\beta</math>-mannanase (MeMan5A) from blue mussel and a <math>\beta</math>-mannosidase (AnMan2A) from <i>Aspergillus niger</i>, were studied in this work. Investigations on the catalytic properties of the enzymes showed that all three enzymes are capable of degrading polymeric mannan. Furthermore, analysis by transmission electron microscopy revealed that TrMan5A and AnMan2A degrade highly crystalline mannan. Degradation of glucomannan and galactoglucomannan by several polysaccharide-degrading enzymes shows that these substrates can be hydrolysed by both mannoside- and glucoside-hydrolases. Furthermore, the results presented show that cellulases potentially are able to hydrolyse other components in the plant cell wall. Altogether, the results presented demonstrates the need to use complex substrates in order to reveal the mechanisms of plant polysaccharide degradation.</p> <p>In conclusion, this work has shown that the enzyme/polysaccharide interaction in the two modules of TrMan5A is important in determining the overall enzymatic activity and specificity.</p>			
Key words: $\beta$ -mannanase, carbohydrate-binding modules, hemicellulase, <i>Trichoderma reesei</i> , $\beta$ -mannosidase, galactoglucomannan, modular enzymes, enzyme-polysaccharide interaction, hemicellulose			
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# Mannan-hydrolysis by hemicellulases

Enzyme-polysaccharide interaction of a modular  $\beta$ -mannanase

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Department of Biochemistry  
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2002



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# Publication List

This thesis is based on the following papers, referred to in the text by their Roman numerals.

- I. Ademark, P., Lundqvist, J., Hägglund, P., Tenkanen, M., Torto, N., Tjerneld, F. and Stålbrand, H. (1999) Hydrolytic properties of a  $\beta$ -mannosidase purified from *Aspergillus niger*. *J. Biotechnol.* **75**, 281-289. <sup>1</sup>
- II. Ademark, P., de Vries, R. P., Hägglund, P., Stålbrand, H. and Visser, J. (2001) Cloning and characterization of *Aspergillus niger* genes encoding an  $\alpha$ -galactosidase and a  $\beta$ -mannosidase involved in galactomannan degradation. *Eur. J. Biochem.* **268**, 2982-2990. <sup>2</sup>
- III. Hägglund, P., Sabini, E., Boisset, C., Wilson, K., Chanzy, H. and Stålbrand, H. (2001) Degradation of mannan I and II crystals by fungal endo- $\beta$ -1,4-mannanases and a  $\beta$ -1,4-mannosidase studied with transmission electron microscopy. *Biomacromolecules* **2**, 694-699. <sup>3</sup>
- IV. Hägglund, P., Eriksson, T., Collén, A., Nerinckx, W., Claeyssens, M. and Stålbrand, H. (2002) A cellulose-binding module of the *Trichoderma reesei*  $\beta$ -mannanase Man5A increases the mannan-hydrolysis of complex substrates. Submitted for publication in *J. Biotechnol.*
- V. Xu, B., Hägglund, P., Stålbrand, H. and Janson, J. C. (2002) endo- $\beta$ -1,4-Mannanases from blue mussel, *Mytilus edulis*: purification, characterization, and mode of action. *J. Biotechnol.* **92**, 267-277. <sup>1</sup>
- VI. Lundqvist, J., Hägglund, P., Eriksson, T., Persson, P., Stoll, D., Siika-aho, M., Gorton, L. and Stålbrand, H. Degradation of glucomannan and *O*-acetyl-galactoglucomannan by mannoside- and glucoside-hydrolases. Manuscript.
- VII. Hägglund, P., Anderson, L. and Stålbrand, H. The active site cleft of *Trichoderma reesei*  $\beta$ -mannanase Man5A: analysis of mutants of the catalytic glutamates and arginine 171 positioned in the +2 subsite. Manuscript.

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# Abbreviations

3D	Three-dimensional
<i>A. niger</i>	<i>Aspergillus niger</i>
AnMan2A	<i>A. niger</i> $\beta$ -mannosidase
Arg	Arginine
<i>C. fimi</i>	<i>Cellulomonas fimi</i>
CBM	Carbohydrate-binding module
CfMan26A	<i>C. fimi</i> $\beta$ -mannanase
DP	Degree of Polymerisation
<i>E. coli</i>	<i>Escherichia coli</i>
EC	Enzyme Commission
GH-A	Glycoside Hydrolase clan A
Gln	Glutamine
Glu	Glutamate
<i>gpdA</i>	glyceraldehyde-3-phosphate dehydrogenase
LBG	Locust Bean Gum
Lys	Lysine
<i>M. edulis</i>	<i>Mytilus edulis</i>
MeMan5A	<i>M. edulis</i> $\beta$ -mannanase
O-acetyl-GGM	O-acetyl-galactoglucomannan
<i>P. cellulosa</i>	<i>Pseudomonas cellulosa</i>
<i>P. pastoris</i>	<i>Pichia pastoris</i>
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SLH	S-Layer Homology
sp.	Species
<i>T. reesei</i>	<i>Trichoderma reesei</i>
TrMan5A	<i>T. reesei</i> $\beta$ -mannanase
TrMan5A $\Delta$ CBM	Catalytic module of TrMan5A
Trp	Tryptophan
Tyr	Tyrosine

# Summary

The enzymatic degradation of plant polysaccharides is a process of fundamental importance in nature. Furthermore, polysaccharide-degrading enzymes are very important in many industrial processes. Therefore, the study of these enzymes is an important field of research. The degradation of the plant cell wall is a complex process that involves a wide range of enzymes, mainly produced by microorganisms. These enzymes include those which degrade cellulose and hemicellulose – two of the main components in plant cell walls. Such enzymes are often composed of two or several separated modules which perform different functions. Carbohydrate-binding modules (CBMs) are frequently present and are known to be important for efficient hydrolysis of cellulose. However, the role of CBMs in hemicellulose degradation is less clear.

In this work, the structure and function of hemicellulose-degrading enzymes was investigated. The focus was on enzymes which degrade mannan and heteromannans such as galactoglucomannan, the major softwood hemicellulose. The main enzyme studied was a  $\beta$ -mannanase (TrMan5A) produced by the filamentous fungus *Trichoderma reesei*. This enzyme is composed of a catalytic module and a CBM. In order to study the function of the two modules, a mutant lacking the C-terminal CBM was constructed (Paper IV). By use of this mutant and the full-length enzyme, the binding properties of the CBM and its effect on hydrolysis of different substrates was investigated. The results of this study demonstrate that the CBM of TrMan5A has a positive influence on the hydrolysis of complex mannan substrates containing cellulose. Furthermore, this increase in hydrolysis could be linked to binding of the CBM to the substrate. Binding studies revealed that the CBM binds to cellulose, but not to mannan.

The enzyme-polysaccharide interaction in the active site cleft of the catalytic module of TrMan5A was also studied in this work (Paper VII). Several mutants of specific amino acids were designed, based on the previously solved structure of the catalytic module. The enzymatic activity of the catalytic residue mutants was very low or abolished. In contrast, a mutant of Arg171 (Arg171Lys) displayed activity in the same range as the wild-type enzyme. Interestingly, this mutant also appears to have a more alkaline pH-optimum than the wild-type. However, the Arg171Lys mutant was impaired in hydrolysis of small substrates.

In addition to TrMan5A, the properties of a  $\beta$ -mannanase (MeMan5A) from blue mussel (*Mytilus edulis*) and a  $\beta$ -mannosidase (AnMan2A) from the fungus *Aspergillus niger*, were studied in this work (Papers I, II and V). MeMan5A belongs to the same enzyme family as TrMan5A. Also, the  $\beta$ -mannosidase is related to the  $\beta$ -mannanases since they are members of the same enzyme clan (GH-A). Studies on the catalytic properties of the enzymes showed that all three enzymes are capable of degrading polymeric mannan

(Papers I, V and VI). Furthermore, TrMan5A and AnMan2A also degraded highly crystalline mannan, which was visualised by transmission electron microscopy (Paper III).

Also included in the present study is a comparative investigation of the enzymatic degradation of heteromannans (Paper VI). Here, glucomannan and galactoglucomannan were degraded by several polysaccharide-degrading enzymes involved in the breakdown of cellulose and hemicellulose. The results show that these substrates can be hydrolysed by both mannoside- and glucoside-hydrolases.

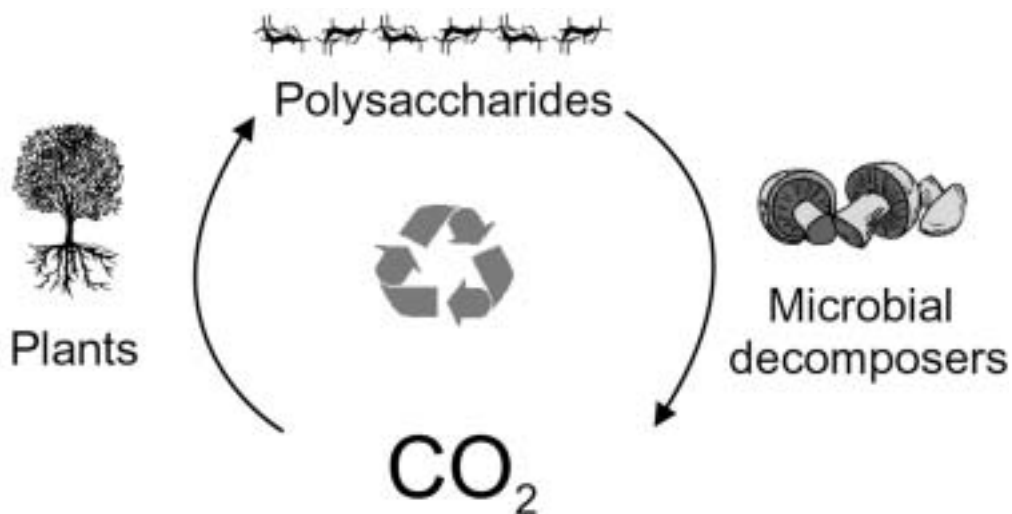
In conclusion, this work showed that the enzyme-polysaccharide interaction in the two modules of TrMan5A is important in determining overall enzymatic efficiency and specificity in the hydrolysis of complex substrates. Altogether, the results presented demonstrate the need to use complex substrates in order to reveal the mechanisms of plant polysaccharide degradation.

# Introduction

Carbohydrates are essential for life on earth.

They function as long-term storage depots of the energy captured in photosynthesis, and as integral parts of genetic information carried in DNA. Furthermore, carbohydrates play many other important roles in nature: for example, they are involved in intercellular communication and host/pathogen recognition. Moreover, carbohydrates in the form of polysaccharides are the main structural elements of plants.

In terms of biomass, cellulose and hemicellulose are the most abundant polysaccharides on earth and are synthesised in huge amounts: it has been estimated that  $10^{12}$  tonnes of cellulose are produced per annum [60]. Thus, these polymers are powerful renewable resources. However, in order to maintain a balance in the ecosystem, these polysaccharides must eventually be degraded. Even though the spontaneous degradation of polysaccharides under physiological conditions is thermodynamically favourable, it is exceedingly slow; it has been estimated that the half-life of cellulose is almost 5 million years [282]. In nature, the rate of turnover of plant polysaccharides is enhanced by polysaccharide-degrading enzymes, produced mainly by various soil-living decomposers which degrade decaying plant material (Figure 1).



**Figure 1.** Synthesis and degradation of plant polysaccharides.

The plant cell wall is mainly composed of tightly associated cellulose, hemicellulose and lignin. Due to this complex structural composition, the degradation of the plant cell wall is a difficult task. Accordingly, a complex mixture of enzymes is required in order to degrade the cell wall components. Many of these enzymes have modular structures with separate carbohydrate-binding modules (CBMs) which anchor the enzymes to different components in the cell wall [278].

In several cases, these CBMs are very important for efficient substrate hydrolysis. For example, generally cellulose-binding CBMs linked to cellulases mediate an increase in rate of cellulose degradation [268]. However, cellulose-binding CBMs are also found in many hemicellulose-degrading enzymes and their function in these enzymes is more elusive [95].

Hemicelluloses comprise a family of diverse polysaccharides. Generally, hemicelluloses have a complex chemical structure and are often referred to as mannans, xylans and galactans on the basis of the predominant sugar type in the main chain. One of the most common mannans is *O*-acetyl-galactoglucomannan which comprises up to 25 % of the dry weight in softwood [263]. However, a range of other mannan-type polysaccharides are synthesised by a wide variety of plants, and are found in different types of plant tissue [185]. Their main role is often to function as structural polysaccharides and/or as reserve energy.

Due to the complex structure of hemicellulose, several different hemicellulose-degrading enzymes (usually referred to as an enzyme system) are produced for the complete degradation of these polymers into their monomeric components [26, 61]. Such a system often includes a combination of endo- and exo-acting enzymes. Two of the major endo-acting enzymes involved in degradation of hemicellulose are  $\beta$ -mannanase and  $\beta$ -xylanase. In the case of *O*-acetyl-galactoglucomannan,  $\beta$ -mannanase is the major depolymerising enzyme. In addition, the exo-acting enzymes  $\beta$ -mannosidase,  $\alpha$ -galactosidase and  $\beta$ -glucosidase are needed for a complete degradation of galactoglucomannan.

In addition to their importance in nature, hemicellulases are important in many industrial applications [183, 280, 284]. Polysaccharide-degrading enzymes in general are the second largest group of commercially produced enzymes [100]. Furthermore, cellulases and hemicellulases account for approximately 20 % of the world enzyme market [193]. In particular, hemicellulases have several existing and potential uses in the pulp and paper industry [280]. Environmental concerns have been raised against the use of large quantities of chlorinated compounds in pulp bleaching processes. Treatment of pulp with  $\beta$ -xylanases prior to bleaching reduces the amount of bleaching chemicals needed in the process, thus providing an environment-friendly alternative [280]. In addition,  $\beta$ -mannanases also have potential uses in pulp-bleaching [45, 249].

From prehistoric time, wood have been used by humans as building material and as an energy source. The more refined industrial uses of wood have been focused on the cellulose component. However, in recent years it has been realised that hemicelluloses also have many potential industrial applications [89]. As environmental problems increase, it is likely that recyclable polysaccharides will be attractive for industrial applications in the future [16]. In order to study the structure of hemicelluloses, and to modify their properties, hemicellulose-degrading enzymes are important tools [158]. Thus, research in these enzymes is of vital interest.

*The general aim* of the current work was to increase the understanding of the molecular mechanisms in mannan-degrading hemicellulases. In particular the enzyme-polysaccharide interaction in a number of mannan-degrading enzymes was investigated. The main enzyme studied was a modular  $\beta$ -mannanase (TrMan5A) from *Trichoderma reesei*, which contains a catalytic module and a CBM. In this work, the binding properties of the CBM and its effect on the overall activity of this enzyme was studied (Paper IV). Moreover, the substrate interaction in the active site cleft of the catalytic module of TrMan5A was investigated (Paper VII).

In addition, the molecular properties of a  $\beta$ -mannosidase (Papers I and II) from the fungus *Aspergillus niger* and a  $\beta$ -mannanase (Paper V) from the blue mussel (*Mytilus edulis*) were studied. Furthermore, the activity of these enzymes and TrMan5A were investigated, with an emphasis on their ability to degrade mannan polymers (Papers I, III and VI).

This thesis is divided into two major parts (see Table of contents, page 3)

In the first part of this thesis (**General background**), the mannan-degrading enzymes are described in the context of plant cell-wall degradation. First, the mannan-containing polysaccharides are presented. Then, an overview of polysaccharide-degrading enzymes is given. Finally, the mannan-degrading enzymes are introduced, with an emphasis on  $\beta$ -mannanases.

In the second part (**Present investigation**), the results described in Papers I-VII (listed on page 5) are presented and discussed.





# General background

## Mannan-based polysaccharides

### Mannans in wood: hemicelluloses

Wood comprises the bulk of the tree trunk and is essentially composed of the cell walls of xylem cells. The major constituents of these cell walls are cellulose, hemicellulose and lignin [83]. The relative amounts of cellulose, hemicellulose and lignin can vary to some degree, depending on the species (Table 1). Furthermore, the chemical composition of wood depends on the tissue, cell type and growth rate [226].

**Table 1.** Composition of woods from some representative species. Data from Timell [263]

Species	Cellulose (%)	Hemicellulose (%)	Lignin (%)
<i>Acer rubrum</i>	45	29	24
<i>Betula papyrifera</i>	42	38	19
<i>Fagus grandifora</i>	45	29	22
<i>Pinus strobus</i>	41	27	29
<i>Picea glauca</i>	41	31	27

The concept of hemicellulose was introduced by Schulze in 1891, to define alkali-extractable plant polysaccharides [83]. Hemicelluloses are a group of heteropolysaccharides with a degree of polymerisation (DP) around 100-200 [263]. They are built up by a main chain composed of one or several types of sugar monomers. In addition, different types of side-groups are frequently attached to the main chain [234].

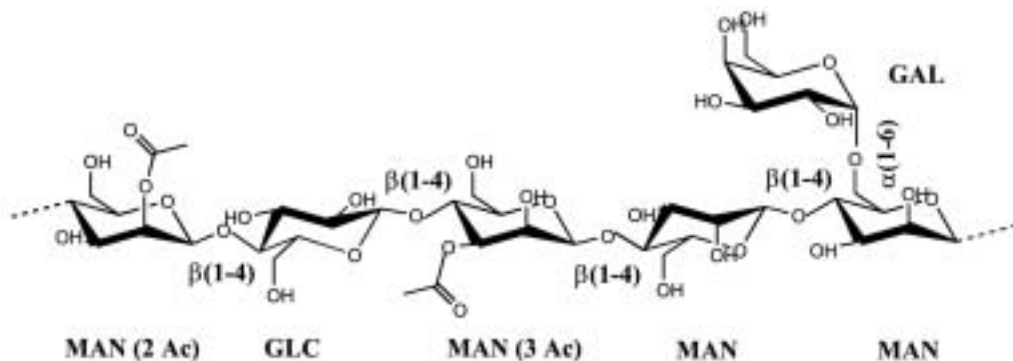
As seen in Table 2, different hemicelluloses are found in wood derived from gymnosperms (softwoods) and angiosperms (hardwoods) [263]. The amounts of the different hemicelluloses can also vary considerably depending on the cell type and the stage in development. In softwoods, the major hemicelluloses are *O*-acetyl-galactoglucomannan and arabino-4-*O*-methylglucuronoxylan, an exception being larchwood where arabinogalactan is the predominant hemicellulose [263]. In hardwoods, the major hemicellulose component is *O*-acetyl-4-*O*-methylglucuronoxylan, but a smaller amount of glucomannan is also found (Table 2).

**Table 2.** Hemicelluloses in hardwoods and softwoods. Data from Timell [263].

Hemicellulose	Amount (% of wood )	Degree of polymerisation
Hardwood		
Glucomannan	3-5	>70
<i>O</i> -acetyl-4- <i>O</i> -methylglucuronoxylan	10-35	~200
Softwood		
Arabino-4- <i>O</i> -methylglucuronoxylan	10-15	>120
<i>O</i> -acetyl-galactoglucomannan	15-25	>100
Arabinogalactan <sup>a</sup>	10-20	~220

<sup>a</sup> In larchwood only

Hardwood glucomannan is built up by a linear chain of  $\beta$ -(1 $\rightarrow$ 4)-linked mannose and glucose residues. The mannose/glucose ratio in glucomannans is typically in the range 1/1-2/1 [263]. Galactoglucomannan is built up by a glucomannan main chain, but also includes  $\alpha$ -(1 $\rightarrow$ 6)-linked galactosyl side-groups attached at some mannose residues (Figure 2). In softwood galactoglucomannan, acetyl groups have been reported to be attached at the C-2 and C-3 positions of some mannose residues [150, 158], in an apparently random fashion [139]. *O*-acetyl galactoglucomannan can be divided in two fractions: one soluble in water, which has a galactose/glucose/mannose ratio of 1/1/3 and one soluble in aqueous alkali, which has a galactose/glucose/mannose ratio of 0.1/1/3 [263].



**Figure 2.** A schematic view of *O*-acetyl-galactoglucomannan.

### Algal mannans

A range of different polysaccharides are produced by algae [208], some of which have industrial importance [79]. *In vivo*, two of the main roles of these polysaccharides in the algae are to function as reserve energy and as structural material. Linear  $\beta$ -(1 $\rightarrow$ 4) mannan is present in the cell walls of several siphonaceous green algae in the families *Acetabularia*, *Codium* and *Halicoryne* [88, 126, 157]. Furthermore, mannan is also found in some red algae, such as *Porphyra umbilicalis*

[135]. In some of these algae, mannan is the main structural polymer and displays a microfibrillar morphology [51, 166]. Some algal mannans display a high degree of polydispersity: the mannan from *Codium fragile* has a degree of polymerisation between 20 and 10 000 [167].

## Mannan-based storage polysaccharides

Besides amylose and amylopectin, which are the most widespread storage polysaccharides in plants, there is a diverse group of mannan-based storage polysaccharides found in the seeds, roots, bulbs and tubers of various plants [185]. These include the mannans, galactomannans and glucomannans which are discussed below.

**Mannan:** Linear chains of  $\beta$ -(1 $\rightarrow$ 4) mannan are found in the plant seed endosperms of certain plant species [18, 184, 283]. Mannan has been isolated from ivory nut (*Phytalephas macrocarpa*), date (*Phoenix dactylifera*) and green coffee bean (*Coffea arabica*). In most cases, these polysaccharides are highly insoluble in water and very dense. Accordingly, it has been suggested that the mannan forms the molecular basis for the hardness which is characteristic for palm kernels, such as the ivory nut. In the cell wall of the seed endosperm of ivory nut, mannan is the major component and it has been characterised in some detail. Based on their solubility in alkali, two different fractions of mannan have been isolated from the ivory nut [162]. These fractions differ mainly in their DP [18, 184] and morphology [51, 184].

**Galactomannan:** Galactomannans are reserve polysaccharides in the seed endosperm of leguminous plants (*Leguminosae*) [217]. In contrast to unsubstituted mannans, the galactomannans are water soluble and can imbibe water, thus providing a water-holding function for the seed [217]. They are composed of  $\beta$ -(1 $\rightarrow$ 4)-linked mannan chains with  $\alpha$ -(1 $\rightarrow$ 6)-linked galactosyl side groups [179]. Both the solubility and the viscosity of the galactomannans are influenced by the mannose/galactose ratio, which can vary from 1 to 5 [217]. Furthermore, the distribution of the substituents can vary considerably [67], which also affects the physical properties of galactomannans [73]. Two of the most well characterised galactomannans are those found in locust bean gum and guar gum, isolated from the seeds of *Ceratonia siliqua* and *Cyanopsis tetragonolobus*, respectively [101, 220]. Locust bean gum galactomannan has a mannose/galactose ratio of approximately 5/1 and a molecular weight of 310 000 [220]; guar gum galactomannan has a mannose/galactose ratio of 2/1 and a molecular weight of 220 000 [101]. In combination with other polysaccharides, these galactomannans have strong gelling properties and are thus used as thickeners in the food and feed industries. Galactomannans and their derivatives are also used in paper making, mining and in the textile industry [101, 220].

**Glucomannan:** Some glucomannans are found as storage polysaccharides in the seeds of certain annual plants, for example some lilies (*Liliaceae*) and irises (*Iridaceae*) [185]. Furthermore, glucomannans are found in the bulbs, roots and tubers of several other types of plants [185]. Many of these glucomannans are water soluble and have the same general structure as glucomannans found in wood: they are composed of a  $\beta$ -(1 $\rightarrow$ 4)-linked mannan chain with interspersed glucose residues in the main chain and are often acetylated [185]. The mannose/glucose ratio ranges from 4/1 to below 1/1 [185]. One of the most thoroughly characterised of these glucomannans is the so-called konjac mannan – isolated from the tubers of *Amorphophallus konjac* [198]. This polysaccharide has a mannose/glucose ratio of 1.6/1 and a degree of polymerisation above 6000. [198].

## Crystalline mannans

Polysaccharides in nature can be organised in more or less regular structures; ranging from irregular or amorphous structures to highly organised and crystalline structures. The most abundant polysaccharide in nature, cellulose, is partially crystalline. In addition to cellulose, X-ray analysis has yielded information about the structures of a number of other polysaccharides such as chitin, xylan, amylose and mannan [169].

Crystalline linear  $\beta$ -(1 $\rightarrow$ 4) D-mannan has been found in the cell walls of ivory nuts and in the algae *Acetabularia crenulata* and *Codium fragile* [51, 88, 184]. Two polymorphs – mannans I and II – have been observed in these cell walls. The morphologies of mannan I and mannan II are granular and fibrillar, respectively [51]. Crystalline glucomannan from wood can be obtained, but only after partial degradation or modification [262]. After dissolution of glucomannan in alkali it can be recrystallised as mannan I or mannan II, depending on the crystallisation conditions [50]. Furthermore both mannan and glucomannan can be recrystallised onto cellulose fibers, yielding the so-called shish/kebab (cellulose/mannan) morphology [50]. Native glucomannan and *O*-acetyl-galactoglucomannan appear to be mostly non-crystalline in nature, probably due to their more complex structures [169].

## Polysaccharide-degrading enzymes: an introduction

### Polysaccharide-degrading microorganisms

Bacteria and fungi thriving on decaying plant material constitute an important part of the ecosystem. These microorganisms decompose polysaccharides and other plant materials, thus recycling organic and inorganic material in the atmosphere and biosphere. Many of these polysaccharide-degrading microorganisms are soil- or water-living. However, some polysaccharide degrading anaerobes degrade plant polysaccharides in the stomach of ruminants [55]. For several decomposers, plant cell wall polysaccharides are the principal carbon and energy source. Accordingly, these organisms usually produce secreted enzymes which degrade the polysaccharides into mono- and oligosaccharides which can be further metabolised in the cell.

### Industrially important fungi

The ability of some soil-living fungi to produce large amounts of polysaccharide-degrading enzymes and other groups of extracellular enzymes (*e.g.* proteinases and lipases) makes them attractive for use in industrial enzyme production. Fungi from the genera *Aspergillus* and *Trichoderma* are examples of two families of industrially important fungi. Notably, *Trichoderma reesei* is a potent producer of several cellulases and hemicellulases which are widely used in industrial applications [279, 280]. Besides its high enzyme production capacity, *T. reesei* has the advantage of being non-toxic and non-pathogenic which is important in large scale fermentation processes [196]. In addition to endogenous enzymes, strains of both *Aspergillus* and *Trichoderma* have also

been used as hosts for expression of foreign eukaryotic proteins [142, 206]. For example, *T. reesei* has been used for the production of single chain antibodies and mammalian interleukin [206].

## **Modularity of polysaccharide-degrading enzymes**

Polysaccharide-degrading enzymes often have a modular structure. By definition, modular enzymes are composed of two or more independently folded modules – each formed from a contiguous sequence [37]. Furthermore, each module has a distinct structure which is related to its function. The overall structures and the functionally important residues are often conserved amongst similar modules. A modular architecture has been found amongst lipases, endonucleases, peptide synthases and several other classes of enzymes [141]. Modular polysaccharide-degrading enzymes are commonly composed of a catalytic module connected to one or more additional catalytic or non-catalytic module [278]. In many cases, these modules are connected by linker sequences [96]. The most common modules are the catalytic *O*-glycoside hydrolases and the non-catalytic carbohydrate-binding modules (CBM), which are discussed below. However, several additional non-catalytic modules have also been described [268]. Thermostabilizing modules have been found in some xylanases [114], and S-layer homology (SLH) modules, involved in cell-surface attachment, have been described in several types of polysaccharide-degrading enzymes [268]. Dockerin modules, found in polysaccharide-degrading enzymes from anaerobic bacteria and fungi, attach the enzymes to the “cellulosome” – a large multi-enzyme complex composed of several different enzymatic activities [230]. This cellulosome is composed of a core structure called a scaffold, onto which several types of enzymes can be attached.

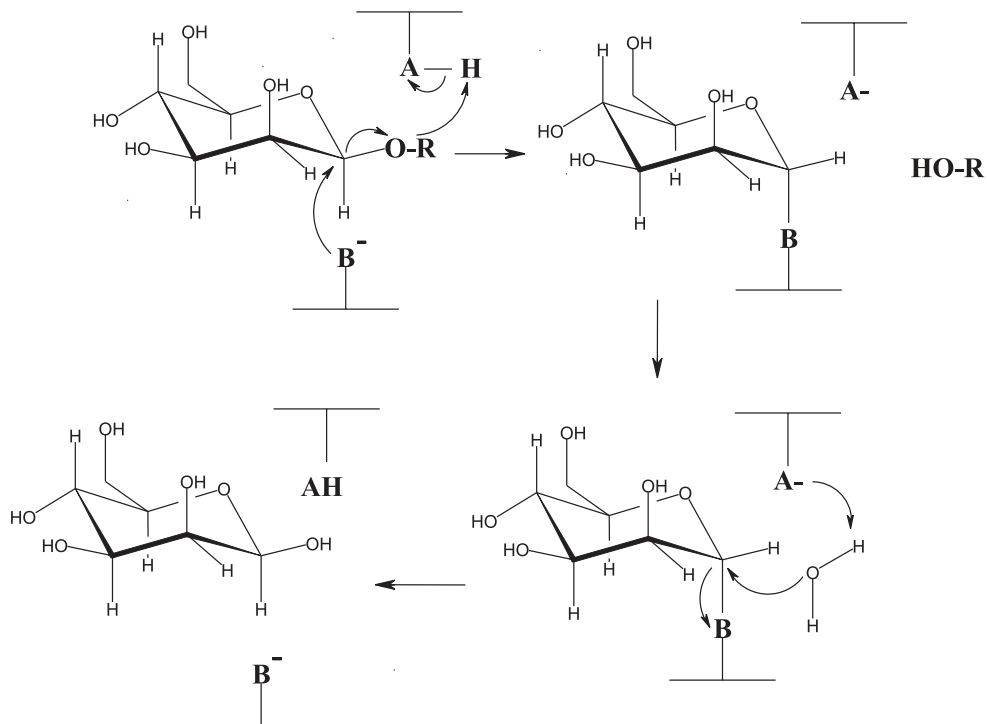
# **Glycoside hydrolases**

## **General catalytic features**

The *O*-glycoside hydrolases constitute the main group of enzymes which participate in the degradation of plant polysaccharides. The diversity of carbohydrate structures is reflected in the wide range of substrate specificity found amongst glycoside hydrolases. The structure and function of glycoside hydrolases has been intensively studied: the first enzyme structure solved by X-ray diffraction was a glycoside hydrolase (hen egg-white lysozyme), solved in 1965 [30]. Based on kinetic data and later on structural information, a general mechanism for glycoside hydrolases has been proposed [70, 144, 233]. According to this theory, two main mechanisms for glycoside hydrolases exist. These are called retaining and inverting mechanisms, referring to the stereochemical outcome at the anomeric carbon in the product.

In both mechanisms, the hydrolysis of the glycosidic bond proceeds through general acid/base catalysis involving two carboxylates (glutamates or aspartates) positioned in the active site [281]. The inverting mechanism proceeds through a single step reaction involving the direct attack by a nucleophilic water on the anomeric carbon, and the simultaneous protonation of the glycosidic

oxygen and aglycone departure. The mechanism of retaining enzymes (double displacement) includes a first step which involves the attack by a nucleophilic carboxylate on the anomeric carbon and the concomitant release of the aglycone, resulting in a covalent enzyme-glycoside intermediate. In the second step the covalent intermediate is attacked by a nucleophilic water which releases the glycoside from the enzyme (Figure 3).



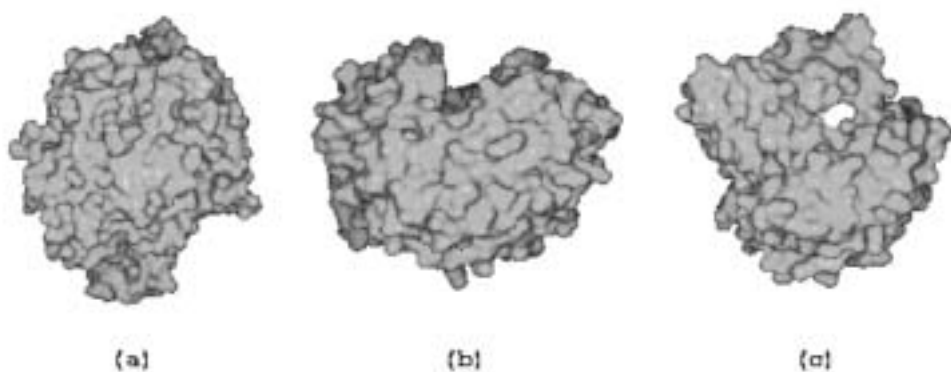
**Figure 3.** General mechanism for retaining glycoside hydrolases. The nucleophile (B) and acid/base (A) catalytic residues are shown. The aglycone (R) is also indicated.

## Family classification

A little more than a decade ago, Henrissat *et al.* presented a family classification of glycoside hydrolases, based on sequence alignment and hydrophobic cluster analysis [115, 149]. As new sequences have been reported, they have been annotated into different families and new families have been added [116, 117]. A continuously updated database is available on the internet at: <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html> [63]. At this stage 87 families have been reported. The relevance of this family classification has also been supported by the increasing amount of structural information, which has accumulated in recent years [39, 119]. Based on structural similarities, several families have been organised into clans. One of the major clans is the GH-A clan comprising at least 16 families [118, 131, 137]. This clan shares the  $(\beta/\alpha)_8$ -barrel (TIM-barrel) fold, which is one of the most common protein folds [264]. In addition, the positions of the catalytic residues are conserved in clan GH-A: the catalytic acid/base and nucleophile are positioned at the C-termini of  $\beta$ -strands 4 and 7, respectively [131].

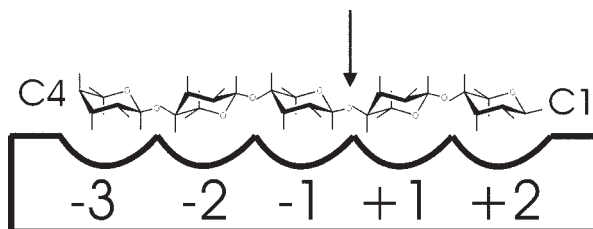
## Structural features

Glycoside hydrolases have been found to have one of two major cleavage preferences: exo-acting enzymes hydrolyse residues at the chain ends and endo-acting enzymes hydrolyse glycosidic bonds internally in the polysaccharide chain [233]. For several structurally determined enzymes the cleavage preference is reflected by the active site architecture (Figure 4) [69]. Endo-acting enzymes, such as endoglucanase and  $\beta$ -mannanase, often have cleft shaped active site surfaces [76, 223]. On the other hand, pocket-shaped active site structures are found amongst several exo-acting enzymes which release monosaccharides from non-reducing ends, such as  $\beta$ -galactosidase and glucoamylase [8, 136]. A tunnel-shaped active surface is found in cellobiohydrolases, which release cellobiose from the reducing or non-reducing ends of cellulose [75]. However, it should be pointed out that the architectures of the active sites of endo- and exo-acting enzymes does not necessarily reflect the observed cleavage preference – discrepancies may exist. Furthermore, the boundaries between exo- and endo-acting enzymes are not absolute: enzymes which are mainly endo-active may show exo-activity and *vice versa* [241, 266].



**Figure 4.** Examples of active site topologies in glycoside hydrolases: (a) the pocket (glucoamylase from *Aspergillus awamori*). (b) The cleft (endoglucanase from *Clostridium thermocellum*). (c) The tunnel (cellobiohydrolase Cel6A from *Trichoderma reesei*). Adapted from [222].

In glycoside hydrolases, binding of several glycoside residues in the active site region is often required. Accordingly the substrate binding surface can be divided into several subsites. According to the recommended nomenclature [72], these are numbered +4, +3, +2, +1, -1, -2 *etc.*, from the non-reducing end to the reducing end, with the *O*-glycosidic bond to be cleaved being positioned between subsite +1 and -1 (Figure 5).



**Figure 5.** A schematic overview of the sugar residue binding subsites in the active site region in glycoside hydrolases. The arrow indicates the point of cleavage.



# Carbohydrate-binding modules

## Family classification

Carbohydrate-binding sites in general are common and are found in several types of proteins, such as toxins, sugar transporters, lectins, antibodies and glycolytic enzymes [214]. Carbohydrate-binding modules (CBMs) however, are found mostly in polysaccharide-degrading enzymes. CBMs have been widely studied in cellulose-degrading enzymes which bind tightly to cellulose [257, 267, 269, 276]. These binding entities were originally referred to as “cellulose-binding domains (CBDs)” [269]. However, more recently CBMs which bind to chitin, starch, xylan, mannan and other polysaccharides have been described, not only in cellulases but also in other polysaccharide-degrading enzymes [278]. In order to encompass a broader binding specificity, the concept of the carbohydrate-binding module (CBM) was introduced [36]. By analogy to the glycoside hydrolases, CBMs are classified into families based on sequence similarities and when possible, 3D-structures [36, 39, 95, 269]. This classification is continuously updated and is available at: <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html> [63]. Presently, 30 families of CBMs have been reported, some of which are listed in Table 3. A number of these families are closely related and consequently were recently grouped into a superfamily [66, 247].

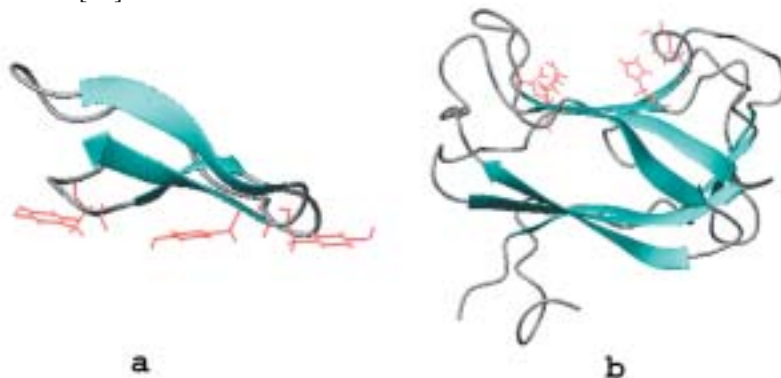
## Structure and function

Depending on their binding properties, three types of CBMs have been identified [36]. Type A CBMs bind to insoluble polysaccharides, such as cellulose and chitin with high affinity [155]. These types of CBMs, are found in *e.g.* family I, II, III and V. Type B binding modules, found in *e.g.* family II and IV, bind soluble polysaccharides and oligosaccharides, and in some cases also amorphous, insoluble polysaccharides [265]. In addition to poly- and oligosaccharides, type C CBMs also have relatively high affinity for monosaccharides [34]. These CBMs are found in *e.g.* family IX and XIII.

The structure of CBMs from several families has been solved by X-ray crystallography and NMR spectroscopy [39, 95]. In most cases, the structures are built up mainly of  $\beta$ -sheets, often with an anti-parallel  $\beta$ -strand topology [39]. In general, CBMs which bind crystalline polysaccharides (type A) display a flat ligand-binding surface with exposed aromatic residues [145, 288] (Figure 6). On the other hand, several modules which bind amorphous polysaccharides and oligosaccharides (Type B) display a groove-shaped binding surface lined with polar residues [132, 143]. Recently, the structure of a Type C CBM from family IX was solved and it was found to have a slot-shaped binding site, “large enough to accommodate a disaccharide” [34, 202].

Analyses of mutational and chemical modifications have demonstrated the importance of the aromatic residues found on the flat surfaces of type A CBMs in ligand binding [41, 152, 218]. In at least some type B CBMs, both aromatic and polar residues have been shown to contribute significantly to ligand binding [143, 285]. The significance of aromatic and polar residues in CBMs is not surprising; the presence of these types of residues is a recurring theme in protein-carbohydrate interactions [214]. A general scheme in protein-carbohydrate associations is that

aromatic residues are aligned face-to-face with the hydrophobic face of the sugar ring, thus providing stacking interactions. Polar residues, on the other hand, are thought to make hydrogen bonds with the hydrophilic hydroxyl groups. Thermodynamic analyses of CBM binding have shown that, generally, binding of soluble polysaccharides and oligosaccharides is promoted mainly by a decrease in enthalpy, resulting from several hydrogen bonding contacts between the protein and the ligand [34, 35, 42, 133, 250]. In contrast, binding of a type A CBM to crystalline cellulose has been shown to be driven by an increase in entropy, partly due to dehydration of the ligand-binding surface [64].



**Figure 6.** Examples of structures of type A (a) and type B (b) CBMs. **a.** The family I CBM from the *T. reesei* endoglucanase TrCel7A [145]. **b.** The family IV CBM from the *C. fimi* endoglucanase CfCel9B [42]. Residues implicated in binding are shown in red.

## Cellulose-binding CBMs

Some of the most studied cellulose-binding CBMs are those from the fungus *T. reesei*, and from the bacteria *Cellulomonas fimi* and *Pseudomonas cellulosa* [36, 114, 155, 269]. The cellulose-binding CBMs from *T. reesei* are relatively small (30-40 residues) and classified into family I. A majority of the family I CBMs are connected to cellulases of fungal origin [155, 257]. However, a few fungal hemicellulases, including the *T. reesei*  $\beta$ -mannanase also carry family I CBMs [171, 244]. In *T. reesei* cellulases it has been shown that family I CBMs are important for efficient hydrolysis of cellulose [257, 267, 276]. Two of the most studied *T. reesei* CBMs are those from the endoglucanase Cel7B and the cellobiohydrolase Cel7A [151, 152, 154]. The solved 3D structures of these CBMs reveal that they share a similar overall fold [145, 174]. In addition, structural and mutational work has shown that the three exposed aromatic residues on the flat binding surfaces of the CBMs are important for binding [152, 173, 175, 218, 219]. However, the two CBMs show different binding affinities for cellulose and it has been proposed that this difference depends to a large degree on a single amino acid substitution on the flat binding surface [151]. Furthermore, substitution of the CBM in Cel7A with the CBM from Cel7B demonstrated that an increased binding to cellulose of the hybrid enzyme could be linked to an increase in cellulose hydrolysis [236].

The CBMs from *C. fimi* are mainly classified into family II and IV [36, 269]. The modules in these families are found only in bacteria and are larger than those in family I (100-150 residues). One of the most studied CBMs is the family II CBM from the *C. fimi* enzyme CfXyn10A which

**Table 3.** A list of CBM families, in accordance with the generally accepted classification system [36, 95, 269]. The CBMs in this table include those in which binding to cellulose and/or hemicellulose has been indicated. References give examples of structural and functional studies

Families <sup>a</sup>	Entries <sup>b</sup>	Ligands <sup>c</sup>	Binding surfaces	Amino acids	Catalytic modules attached <sup>c</sup>	Reference
I	87	Cellulose Chitin	Flat	30-40	Mannanase Xylanase Cellulase Esterase	[145, 152]
II	94	Cellulose Xylan	Flat (IIa) Twisted (IIb)	100	Cellulase Xylanase Chitinase	[231, 288]
III	61	Cellulose Xylan	Flat	150	Mannanase Xylanase Cellulase	[271]
IV	18	Cellulose Cellulose (Der. <sup>d</sup> ) $\beta$ -1,3/1,4 Glucan Oligosaccharides	Groove	150	Xylanase Cellulase	[42, 132]
V	71	Cellulose Chitin	Flat	60	Cellulase Chitinase	[43]
VI	32	Cellulosa Xylan $\beta$ -1,3/1,4 Glucan Oligosaccharides	Cleft	120	Xylanase Cellulase Esterase Chitinase	[66]
IX	16	Cellulose Xylan $\beta$ -Glucan Oligosaccharides Monosaccharides	Slot	170	Xylanase	[34, 202]
X	11	Cellulose	Flat	45	Xylanase Mannanase Cellulase	[215]
XI	3	Cellulose	N.D.	180-200	Cellulase	[168]
XV	2	Xylan Oligosaccharides	Groove	160	Xylanase	[189,250]
XVII	8	Cellulose Cellulose (Der. <sup>d</sup> ) $\beta$ -Glucan Oligosaccharides	Groove (shallow)	200	Cellulase	[33, 201]
XXII	41	Xylan $\beta$ -1,3/1,4 Glucan Oligosaccharides	Groove	150	Xylanase	[52]
XXIII	1	Mannan	N.D.	230	Mannanase	[237]
XXVII	4	Mannan	N.D.	180	Mannanase	[247]
XXVIII	9	Cellulose $\beta$ -1,3/1,4 Glucan Oligosaccharides	N.D.	190	Cellulase	[35]
XXIX	1	Cellulose (Der. <sup>d</sup> ) Mannan	N.D.	130-140	Non-catalytic	[87]

<sup>a</sup> CBM families are typed in Roman numerals, to distinguish them from the glycoside hydrolase families

<sup>b</sup> Based on those available at <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html> [63]

<sup>c</sup> This list is not complete, only some examples are included

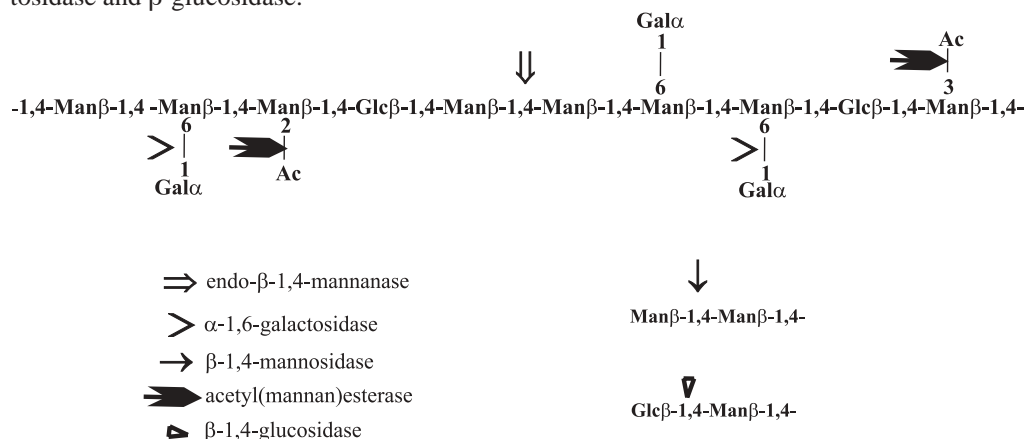
<sup>d</sup> Cellulose derivatives

display mixed glucanase/xylanase activity [97, 98, 288]. This CBM displays a ligand-binding area somewhat resembling the flat surface in family I CBMs [288], and it has been shown to bind tightly to cellulose [97]. Furthermore the hydrolysis of cellulose by CfXyn10A increases in the presence of the attached CBM [98]. In contrast, another family II CBM from *C. fimi* module has been proposed to promote non-hydrolytic disruption of cellulose surfaces, independent of the catalytic module [74]. Family II has been divided into two subfamilies, on the basis of the preferred binding ligand: family IIa modules bind to cellulose and family IIb modules bind to xylan [97, 231]. Remarkably, site-directed mutagenesis of a single amino acid residue on a family IIb CBM changed its binding specificity from xylan to cellulose [232]. The two tandem family IV CBMs in the *C. fimi* endoglucanase Cel9B bind to amorphous cellulose and oligosaccharides [133, 265].

Cellulose-binding CBMs from family II and family X are ubiquitous among polysaccharide-degrading enzymes from *P. cellulosa*, and are found in both cellulases and several types of hemicellulases [114]. In *P. cellulosa*, cellulose-binding CBMs from both cellulases and hemicellulases have been shown to influence hydrolytic activity [28, 29, 109]. In some cases, two CBMs are connected to the same enzyme. For example, the *P. cellulosa* xylanase Xyl10A contains one family II CBM and one family X CBM which bind to cellulose apparently independent of each other [99].

## Galactoglucomannan-degrading enzymes

Due to the complex chemical structure of hemicelluloses, multiple enzymes are often needed for the complete degradation of these polymers. In the case of *O*-acetyl-galactoglucomannan, four different glycoside hydrolases ( $\beta$ -mannanase,  $\beta$ -mannosidase,  $\alpha$ -galactosidase and  $\beta$ -glucosidase) and acetyl esterase are required for the complete conversion of the polymer into unsubstituted monosaccharides (Figure 7).  $\beta$ -Mannanase is the major endo-acting enzyme involved in galactoglucomannan degradation [26, 180, 243]. This enzyme degrades the polymer into smaller oligosaccharides, which are further hydrolysed to monosaccharides by  $\beta$ -mannosidase,  $\alpha$ -galactosidase and  $\beta$ -glucosidase.



**Figure 7.** Schematic overview of the enzymes involved in degradation of *O*-acetyl-galactoglucomannan.

# $\beta$ -Mannanase

## Biochemical properties

Endo- $\beta$ -1,4-D-mannanase ( $\beta$ -mannanase; EC 3.2.1.78) catalyses the random hydrolysis of manno-glycosidic bonds in mannan-based polysaccharides. Most  $\beta$ -mannanases degrade manno-oligosaccharides down to a DP of 4 [26, 180, 243]. In addition, some  $\beta$ -mannanases are also active on mannotriose, although at a much lower rate, thus indicating the presence of at least 4 subsites in several  $\beta$ -mannanases [7, 112]. However, hydrolysis of oligosaccharides by some  $\beta$ -mannanases from anaerobic fungi and bacteria has indicated that binding is required over at least 6 subsites [82, 110, 190]. The main end-products of mannan hydrolysis by  $\beta$ -mannanase are often mannobiose and mannotriose [3, 57, 225, 245, 273], although minor amounts of mannose and mannotetraose also are produced in some cases [273]. In the degradation of heteromannans, the pattern of released oligosaccharides is often more complex, probably due to hindrance of the enzymatic hydrolysis caused by the substituents [182, 210, 240, 259]. It has also been shown that at least some  $\beta$ -mannanases are capable of degrading crystalline mannan (Paper III).

In addition to hydrolysis, several  $\beta$ -mannanases can also perform transglycosylation [62, 107, 111]. For example, the *T. reesei*  $\beta$ -mannanase has been shown to form transglycosylation products with either mannose or mannobiose as glycosidic bond acceptors [111].

The pH and temperature optima of some  $\beta$ -mannanases are shown in Table 4. Generally,  $\beta$ -mannanases have moderate temperature optima (40-70 °C), except some  $\beta$ -mannanases from thermophiles which have higher temperature optima [93, 205, 209, 248]. The pH optima of most  $\beta$ -mannanases are found in the neutral or acidic region. Commonly, the molecular weights of  $\beta$ -mannanases are in the range of 30-80 kDa (Table 4).

However, some modular  $\beta$ -mannanases have molecular weights near or above 100 kDa [46, 239, 248]. Typically, most  $\beta$ -mannanases have isoelectric points between 4 to 8. Frequently, multiple  $\beta$ -mannanases with different isoelectric points and/or molecular weights are found in the same organism [4, 125, 172, 190, 245, 270]. In some cases these enzymes are produced from different genes [172, 190, 199], and in other cases, the enzymes are isoforms produced from the same gene [4, 244]. These isoforms may be caused by differences in post-translational modifications.

**Table 4.** Some properties of  $\beta$ -mannanases from families 5 and 26

Organism	Swissprot <sup>a</sup>	Family	M <sub>w</sub> <sup>s</sup>	pI	Optima		Reference
					pH	Temp	
<i>Agaricus bisporus</i> C54-carb8	Q9P893	5	N.D.	N.D.	N.D.	N.D.	[256]
<i>Agaricus bisporus</i> D649	Q92401	5	44.9 <sup>b</sup>	N.D.	N.D.	N.D.	[289]
<i>Aspergillus aculeatus</i> <sup>c</sup>	Q00012	5	45	4.5	5	60	[56]
<i>Aspergillus niger</i>	-	(5) <sup>*</sup>	40	3.7	3.5	N.D.	[3]
<i>Bacillus circulans</i> K-1	O66185	5	62	Several	6.9	65	[293, 294]
<i>Caldibacillus cellulovorans</i> <sup>d</sup>	Q9RFX5	5	30.7 <sup>e</sup>	N.D.	6 <sup>e</sup>	85 <sup>e</sup>	[248]
<i>C<sup>+</sup>. saccharolyticus</i> <sup>d</sup>	P22533	5	34 <sup>e</sup>	N.D.	6	80	[94, 160]
<i>Clostridium cellulovorans</i> <sup>d</sup>	-	5	38	N.D.	7	45	[255]
<i>G<sup>+</sup>. stearothermophilus</i>	-	5	73	N.D.	5.5-7.5	N.D.	[78, 252]
<i>Mytilus edulis</i>	-	5	39	7.8	5.2	50-55	[287](PaperV)
<i>Lycopersicon esculentum</i>	O48540	5	39 <sup>b</sup>	5.3 <sup>b</sup>	N.D.	N.D.	[25]
<i>Streptomyces lividans</i> 66	P51529	5	36	3.5	6.8	58	[15]
<i>T<sup>+</sup>. polysaccharolyticum</i>	Q9ZA17	5	116	N.D.	5.8	65/75 <sup>f</sup>	[46]
<i>Thermotoga maritima</i> <sup>d</sup>	Q9X0V4	5	76.9 <sup>b</sup>	N.D.	7	90	[205]
<i>Trichoderma reesei</i>	Q99036	5	51-53	Several	3-4	70	[244, 245]
<i>Vibrio</i> sp. Strain MA-138	O69347	5	49	3.8	6.5	40	[253, 254]
<i>Bacillus</i> sp. 5H	O83011	26	37	N.D.	N.D.	N.D.	[140]
<i>Bacillus</i> sp. strain AM-001	P166699	26	58	5.9	9	60	[4, 5, 7]
<i>Bacillus subtilis</i> NM-39	P55278	26	38	4.8	5	55	[186, 187]
<i>C<sup>+</sup>. saccharolyticus</i> Rt8B.4 <sup>d</sup>	P77847	26	N.D.	N.D.	6-6.5	60-65	[92]
<i>Cellulomonas fimi</i>	Q9XCV5	26	100	N.D.	5.5	42	[239]
<i>Clostridium thermocellum</i> <sup>d</sup>	-	26	70 <sup>g</sup>	N.D.	6.5 <sup>e</sup>	65 <sup>e</sup>	[110]
<i>Clostridium thermocellum</i> F1	-	26	55 <sup>h</sup>	N.D.	7 <sup>h</sup>	75 <sup>h</sup>	[147]
<i>Dictyoglomus thermophilum</i> <sup>d</sup>	O30654	26	40	N.D.	5	80	[93]
<i>Piromyces</i> sp. <sup>d</sup>	P55296	26	68	N.D.	N.D.	N.D.	[82]
<i>Piromyces</i> sp.	P55297	26	N.D.	N.D.	N.D.	N.D.	[190]
<i>Piromyces</i> sp.	P55298	26	N.D.	N.D.	N.D.	N.D.	[190]
<i>Pseudomonas cellulosa</i> <sup>d</sup>	P49424	26	46	N.D.	7	N.D.	[40]
<i>Rhodothermus marinus</i>	P49425	26	113	N.D.	5.4	85	[209]

<sup>s</sup> Molecular weights in kDa. Data from SDS-PAGE

<sup>\*</sup> Similarity from N-terminal sequence

<sup>a</sup> Shown when available

<sup>b</sup> Theoretical value from the amino acid sequence

<sup>c</sup> Expressed in *S. cerevisiae*

<sup>d</sup> Expressed in *E. coli*

<sup>e</sup> Catalytic module only

<sup>f</sup> Two optima observed

<sup>g</sup> From Western blot

<sup>h</sup> Lacking the dockerin module

<sup>+</sup> *Caldocellulosiruptor*

& *Thermoanaerobacterium*

<sup>+</sup> *Geobacillus*

## Occurrence and regulation

$\beta$ -Mannanases have been isolated from a wide range of organisms, including bacteria [7, 40, 239], fungi [3, 56, 245], plants [25, 172] and animals (Paper V) [53]. Amongst bacteria,  $\beta$ -mannanases have been found amongst aerobes, anaerobes and different extremophiles such as thermophiles, halophiles and psychrophiles [205, 274, 296]. Most  $\beta$ -mannanases are extracellular, however some appear to remain attached to the cell [91]. In plants,  $\beta$ -mannanase activity has been correlated with seed germination [77, 199, 200], and in some cases also with fruit ripening [24, 38]. Some  $\beta$ -mannanases from molluscs have been isolated from their digestive tract (Paper V) [85].

Expression of many microbial  $\beta$ -mannanases is induced by growth on mannan or galactomannan [3, 13, 221].  $\beta$ -Mannanases from some microbes, including *T. reesei*, have also been produced by growth on cellulose [221, 242]. However, expression of the *T. reesei*  $\beta$ -mannanase is repressed on glucose and several other monosaccharides [170, 228]. In plants, production of  $\beta$ -mannanase has been shown to be regulated by plant hormones, such as gibbrellins and abscisic acid [10, 104]

## Family classification and structural determination

All  $\beta$ -mannanases for which the genes have been cloned have been classified into families 5 or 26 of glycoside hydrolases [115, 119]. Both of these families are included in the GH-A clan of glycoside hydrolases [32, 131] and the retaining mechanism has been confirmed for family 5 and family 26  $\beta$ -mannanases [3, 32, 112]. Both bacterial and eukaryotic  $\beta$ -mannanase have been annotated to family 5. This family also includes endo- and exoglucanases, xylanases and endoglycoceramidases. An alignment of the amino acid sequences of some family 5  $\beta$ -mannanases mainly from eukaryotic sources is shown in Figure 8.

With the exception of a few anaerobic fungi, the  $\beta$ -mannanases in family 26 are of bacterial origin. Besides  $\beta$ -mannanases, some endoglucanases and a few  $\beta$ -1,3-xylanases are also found in this family. In some cases,  $\beta$ -mannanases from the same genus have been classified in different families;  $\beta$ -mannanases from different strains of *Caldocellulosiruptor saccharolyticus* have been classified in both families 5 and 26 [92, 94], and those from different *Bacillus* species are also found in both families [5, 187].

```

Trichoderma M-MMLSKSLLSAAT--AASALAAVLQPV-----PRASSFVTISGTQFNIDGK
Aspergillus --MKLSHMLLSLASLGVATALPRTPNHN-----AATTFAPFSTGLHFTIDGK
Agaricus -----AM---N-----QAF---S---DI-AN
Geobacillus MNKKWSYTFIALLVSVICAVVPIFFSQNNVHAKTKRREPATPTKDNFVVRKGDGKLMIGNK
Orpinomyces --MHFNK--VSALLLTATSVAGQFR-----EGFVQTDGTNEFVVDGK
Lycopersicon --MSYARRSCICGLFLLFLALVCEAN-----SGFIGVKDSHFELNGS
Mytilus -----MLLTALAVLFASTGC-----QARLSVSGTNLNLYNGH
consensus

Trichoderma VGYFAGTNCYWCSEFL---TNHA----DVDSTFHSIHSSGLKVVVWVG-FNDVNTQPSF
Aspergillus TGYFAGTNSYWIGFL---TNND----DVDLVMSQLAASDLKILVWVG-FNDVNTKPTD
Agaricus AG---GT-----T-----VTRTWG-FNEV-TSPN
Geobacillus EFRFVGTNNYYLHYK---SNQ-----MIDDVIESAKKMGKIVLRLWGFDFDGTSENQA
Orpinomyces KRYFSGSNYYL-MV---SNHE----RVDLALETYARHNLNVVRAWA-FCDECEDAT-
Lycopersicon PFLFNFGNSYWLHMVAADPTERY----KVTEVLKDVASVAGLSVCTWTA-FSDGGDRA--
Mytilus HIPLFSGANQAVVNYARDFGHNQYSKGGKSTFESTLSDIQSHGGNSVWVWVW-L-HIEGESTPE-
consensus * * *

Trichoderma GQIWFQK---LSATG--STINTGAD--GLQTLDYVVQSAEQHNKLLKIPFVNNWSDYGG
Aspergillus GTVWYQ----LHANGT-STINTGAD--GLQRLDYVVTSAEKYGVKLLINFNVEWTDYGG
Agaricus GN-YYQS-----WSGAR--PTINTGAS--GLLNFDNVIAAAKANGIRLIVALTNWADYGG
Geobacillus HNTYMQYEMGKYMGEPIPKLEGAQN-GFERLDYTIYKAKQEGIRLVLVLTNNWNNFGG
Orpinomyces -R-LVD-----FSGPE--VTLN-GEN---MEKVDYLLAAAQRNIRVVLTLTNWTDYGG
Lycopersicon ---LQ-----ISPG---IYDERV---FQGLDFVIAEAKK-----YG-
Mytilus ----FD-----NNG---YVTGIDNTLISDMRAYLHAAQRHNLIFFTL--WN--GA
consensus : * *

Trichoderma INAYVNAF-G-GNATT-WYTNTAAQTQYRKYVQAVVSR-----YANSTAI FAWELGN
Aspergillus MQAYVTAAY-G-AAAQDFYNTAIQAAYNKYKAVVSR-----YSSSAAIFAWELAN
Agaricus MDVYVNMQVGNQPHDLFYNTAIKDAFYSYGRAVFSR-----YANEPVMAWELAN
Geobacillus MMQYVNW-I-G-ETNHDLFYTDERIKTAYKNYVHYLNRKNQYTGIIKNEPTIMAWELAN
Orpinomyces MVVWVKQF-G-GKYHDEFYTNKDIIRGYKQYKAMINRVNTYTGQLYKDDPTIFSWQLAN
Lycopersicon --AQI-----SNDEEFYTHPMLKKYLKNHIEKVVTRLNSITKVAYKDDPTIMAWELAN
Mytilus VKQSTH-Y----RLNGLMVDTRKLSYIDHALKPMANA-----LKNEKALGWSDIMN
consensus : * * *

Trichoderma EPRC---NG-----CSTDVIVQ---WA-----TSVSQYV-----KSLDSNHLV
Aspergillus EPRC---QG-----CDTSVLYN---WI-----SDTSKYI-----KSLDSKHLV
Agaricus EPRC---KGSTGTTSGTCTTTVTIN---WA-----KEMSAFL-----KTIDSNHLV
Geobacillus EPRN---DSD-----PTGDTLVR---WA-----DEMSTYI-----KSIDPHHLV
Orpinomyces EARCN---NGPHGLPVKNCNTDTITK---WM-----DEIATFI-----HQEDPNHLV
Lycopersicon EPRD---QAD-----YSKGTVNG---WV-----QEMASFV-----KSLDNKHLL
Mytilus EPEGEIKPGESS-SEPCFDTRHLSGSGAGWAGHLYSAQEIGRFVNWQAAAIKEVDPGAMV
consensus * : * * *

Trichoderma TLGDEGLGLST----GDGAYPYTYGNGTDFAKN-VQIKSLDFGTFFHLPDPSW--GTNYT
Aspergillus TIGDEGFGLDVD----SDGSYPYTYGEGLNFTKN-LGISTIDFGTLHLVDPDSW--GTSYD
Agaricus AIGDEGF-YNQF---GAPTYPYQSGSEGVDFEAN-LAISSVDFATEHSVPEPW--GQGAD
Geobacillus AVGDEGFFRRSSGGFNGEGSYMYTYGYNVDWDLR-IALKNIDYGTFFHLPPEHW--GISPE
Orpinomyces SSGIEIGILTPPAG-VDKNTYVYTYTEGTDYEAI-SALDSIDYNTVHMVYVVGW--GLKDY
Lycopersicon EVMGEGFYGDSIP---ERKSVNPGYQVGTDFISN-HLINEIDFATIHAYTDQVWVSGQSD
Mytilus TVGSWNMKADT----DAMGFHNLYSDHCLVKAGGKQSGTSLFYQVHTLW--DW----QNH
consensus * : * * *

Trichoderma ----WGNNGWIQTH-AAACLAAGKPCVFEIYGAQ-----QNP---CTNEAPWQQTSL
Aspergillus ----WGNNGWITAH-AAACKAVGKPCLEIYGVY-----SNH---CAVESPWQQTAG
Agaricus AKA-WGTQWITDH-AASMKRVNKPVILEIYFGVY-----TNQ---PDTYAEWFN-EI
Geobacillus NVEKWEQYILDH-LAAGKAKKFPVLEIYGISATGV-----QNR---EMIVTDWNRMTF
Orpinomyces AKD--GVTWIKAH-ADVDKKFNKPTVVEEWGLSTADNVPLEQR---DPIYQWMMNEVL
Lycopersicon AQLVWMEKWITSHWEDARNILKPLVLAIEFGKSSRQO-----GSRDIFMSSVYRNVYLNLA
Mytilus ----FGNESPFKH-SFSNFRLLKPMVIGIEFNQEHGAG-----MSS---ESMF-EWAY---
consensus * * * : *

Trichoderma TTRGMGDMFQW-G-----DTFANGAQSNSDPYTVWY-NSSNWQCLVKNHVDAINGG-
Aspergillus NATGISGLYMQY-G-----TTFSWG-QSPNDGNTFY-NTSDFTCLVDHVAAINAQS-
Agaricus ESSGLTGDLIMQA-G-----SHLSTG-DTPNDGYAYVP-DGPVYP-LVKSHASAMKNRA
Geobacillus EHG-GTGAMFLLTGIDDDNPEADENGYYPDYDGRIVN-DHSSVNLKTYAKLFGNDR
Orpinomyces ANDIGMNMFWVCG-----EDYDG---TDGY---LLEE---DEI---
Lycopersicon EGGTMAGSLVWQL-----MAHGMENYDDGYCIVLGTPTSTQIISDQAHVMTALA
Mytilus -TRKYSGAWTMSR-----TDVSWN--NQLRGIQHLK-----SRTDHGQVQFGL-
consensus *

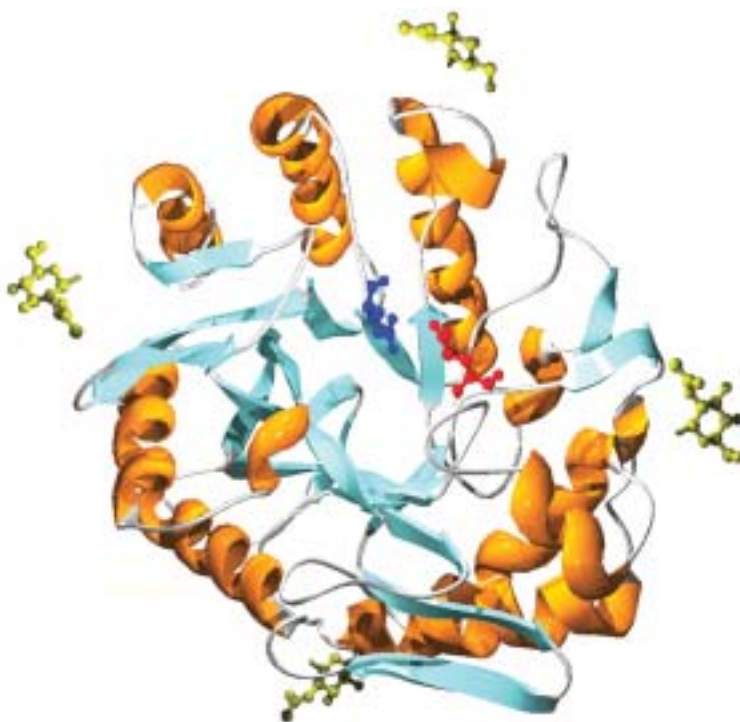
```

**Figure 8.** Sequence alignment of some  $\beta$ -mannanases from family 5. The residues conserved within family 5 are boxed. Conserved (\*) and similar residues (:) are indicated in the consensus line. For references, see Paper VII (Figure 2).



The structures of two family 5  $\beta$ -mannanases – those from *T. reesei* and *T. fusca* – have been determined by X-ray crystallography [121, 223]. The structures of the *T. reesei* and *T. fusca*  $\beta$ -mannanases share the overall  $(\beta/\alpha)_8$  fold, which is conserved in clan GH-A (Figure 9). Interestingly, the *T. reesei*  $\beta$ -mannanase also contained two additional  $\beta$ -sheets which are not conserved in other  $(\beta/\alpha)_8$  enzymes. Furthermore, four glycosylation sites are occupied with N-acetylglucosamine residues (Figure 9).

The active site in the  $\beta$ -mannanase structures is located in a shallow cleft exposed to the solvent. Within the active site, at least seven amino acids conserved in other family 5 enzymes (Figure 8) are present. In the *T. reesei*  $\beta$ -mannanase these correspond to Arg54, Asn168, Glu169, His241, Tyr243, Glu276 and Trp306 [223, 244]. Structures of the *T. fusca* and *T. reesei* enzymes with oligosaccharides bound their active site clefts have also been solved [121, 223]. These structures reveal the presence of several substrate-enzyme interactions in the -2 and -3 (*T. fusca*) and +1 and +2 (*T. reesei*) subsites. The structure of the family 26  $\beta$ -mannanase ManA from *Pseudomonas cellulosa* has also been solved recently [124]. As expected, this  $\beta$ -mannanase also exhibits the  $(\beta/\alpha)_8$  fold and the conserved positions of the catalytic residues in clan GH-A. Very recently, the crystallisation of the family 5  $\beta$ -mannanase (studied in Paper V) from blue mussel was also reported [286].



**Figure 9.** The structure of the catalytic module of family 5  $\beta$ -mannanase (TrMan5A) from *T. reesei* [223].  $\alpha$ -Helices are shown in gold,  $\beta$ -sheets in light blue. The predicted acid/base and nucleophile residues are shown in red and blue, respectively. The four N-acetylglucosamine residues are shown in green.

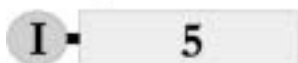
## Modular $\beta$ -mannanases

Several  $\beta$ -mannanases display a modular architecture (Figure 10). Amongst  $\beta$ -mannanases from aerobic fungi, the enzymes from *T. reesei* and *A. bisporus* are composed of a family 5 catalytic module linked to a family I CBM [244, 256, 289]. Some bacterial  $\beta$ -mannanases from families 5 and 26 have more complex structures: the *C. fimi*  $\beta$ -mannanase Man26A contains both a mannan-binding family XXIII CBM, a putative SLH-module and a module of unknown function [237, 239, 240]. The family 5  $\beta$ -mannanase from *Thermoanaerobacterium polysaccharolyticum* also contain an SLH-module and, in addition, two internal family XVI CBMs [46]. Several  $\beta$ -mannanases from anaerobic bacteria and fungi contain dockerin modules which attach the  $\beta$ -mannanases to multienzyme complexes [82, 110, 190, 255]. A family 26  $\beta$ -mannanase from *Caldocellulosiruptor saccharolyticus* contains two family XXVII mannan-binding modules [247].

*T. reesei*



*A. bisporus*



*C. fimi*



*T. polysaccharolyticum*



*C. saccharolyticus*



*C. cellulovorans*



**Figure 10.** A schematic picture of the modular organisation in some  $\beta$ -mannanases. Catalytic modules are shown as boxes, CBMs as ellipses. Other modules are shown as pentagons. The  $\beta$ -mannanases are those from *Trichoderma reesei* [244], *Agaricus bisporus* [256], *Cellulomonas fimi* [239], *Thermoanaerobacterium polysaccharolyticum* [46], *Caldocellulosiruptor saccharolyticus* [247] and *Caldibacillus cellulovorans* [248].

## Applications

$\beta$ -Mannanases have several existing and potential industrial applications. They have been shown to be effective in increasing the brightness of pulps in bleaching experiments [45, 192, 249, 280], most notably in combination with xylanases [45, 58]. In the food and feed industries,  $\beta$ -mannanases are used in the production of fruit juices and soluble coffee [102, 108, 224], and also in the preparation of poultry diets [128].  $\beta$ -Mannanases have also been shown to have a strong potential as viscosity reducers of hydraulic fracturing fluids used in oil and gas production [183]. Furthermore,  $\beta$ -mannanases have potential applications in recycling of copra and coffee wastes [216].

## Exo-acting enzymes

### $\beta$ -Mannosidase

$\beta$ -Mannosidase (EC 3.2.1.25;  $\beta$ -1,4-D-mannoside mannohydrolase) catalyses the hydrolysis of mannose units from the non-reducing end of mannosides. However, some  $\beta$ -mannosidases are active both on glucosides and mannosides [21, 80]. The most commonly employed substrate for analysis of  $\beta$ -mannosidase activity is a chromogenic monosaccharide. In addition, several  $\beta$ -mannosidases are also capable of degrading longer manno-oligosaccharides, with DP over 4 [6, 12, 113]. However, only a few  $\beta$ -mannosidases have been shown to release mannose from the non-reducing end of mannan-based polymers [14, 122, 146] (Papers I, III and VI).

$\beta$ -Mannosidases have been isolated from widely different types of organisms, including eubacteria, archaeobacteria, plants, fungi and animals [12, 21, 53, 176, 239].  $\beta$ -mannosidase appears to carry out different functions, depending on the producing organism.  $\beta$ -Mannosidases from microbes are often employed in the degradation of mannans and heteromannans from decaying plant material for nutritional purposes.  $\beta$ -Mannosidases in plants are involved in the release of storage polysaccharides in the seed endosperm during germination [178]. In contrast, mammalian  $\beta$ -mannosidases appear to function mainly as lysosomal enzymes involved in degradation of protein-linked glycans.  $\beta$ -Mannosidosis is a congenital disorder, which results from the lack of a functional  $\beta$ -mannosidase activity. This disease was first found in ruminants, but has more recently also been described in humans [134].

Despite their functional difference, many  $\beta$ -mannosidases are related to each other and are classified in family 2 of glycoside hydrolases, which is included in the GH-A clan [115, 119, 131]. The molecular weights of most  $\beta$ -mannosidases, as determined from SDS-PAGE, are in the range 50-130 kDa (Table 5). However, some  $\beta$ -mannosidases appear to consist of several subunits [21, 204]. The isoelectric points of most  $\beta$ -mannosidases are in the acidic range, except for some bacterial enzymes which have isoelectric points near neutrality (Table 5). Most  $\beta$ -mannosidases show maximal activity at acidic or neutral pH, and with the exception of some thermophilic enzymes, most  $\beta$ -mannosidases show their maximal activity in the temperature range 40-70 °C.

No three-dimensional structure of a  $\beta$ -mannosidase has been determined at this stage although the crystallisation of a *T. reesei*  $\beta$ -mannosidase has been reported [11]. However, the structures of two

other family 2 enzymes, those of the tetrameric *E. coli*  $\beta$ -galactosidase and the human  $\beta$ -glucuronidase, have been solved [129, 130]. In the *E. coli*  $\beta$ -galactosidase, the active site pocket is situated at a subunit interface [136].

**Table 5.** Properties of some  $\beta$ -mannosidases

Organism	Swissprot <sup>a</sup>	Family	Mw <sup>b</sup>	pI	Optima		Ref
					pH	Temp	
<i>Aspergillus aculeatus</i>	O741682	2	130	4	2	70	[12, 251]
<i>Aspergillus awamori</i>	-	-	96-100	4.55	3.8	66	[195]
<i>Aspergillus niger</i>	Q9UUZ3	2	135	5	2.5-5	70	Paper I & II
<i>Bacillus sp. AM-001</i>	-	-	94	5.5	6	50	[6]
<i>Cellulomonas fimi</i>	-	2	103	N.D.	7	55	[239]
<i>C<sup>s</sup>. tetragonolobus</i>	-	-	59	9.4	5-6	52	[176, 181]
<i>Helix aspera</i> Müller	-	-	77.8	N.D.	3.3	37-42	[53]
<i>Helix pomatia</i>	-	-	94	4.7	4	55	[177]
<i>Homo sapiens</i>	O00462	2	110 <sup>c</sup>	4.7 <sup>c</sup>	4.5 <sup>c</sup>	N.D.	[9, 127]
<i>Polyporus sulfureus</i>	-	-	64	3.9	2.4-3.4	N.D.	[275]
<i>Pyrococcus furiosus</i>	Q51733	1	59	6.9	7.4	105	[21]
<i>Rhizopus niveus</i>	-	-	N.D.	N.D.	5	40	[113]
<i>Sclerotium rolfsii</i>	-	-	57.5	4.5	2.5	55	[106]
<i>Thermotoga neapolitana</i>	-	2	95	5.6	7.7	87	[204, 205]
<i>Tremella fuciformis</i>	-	-	140	N.D.	5	N.D.	[235]
<i>Trichoderma reesei</i>	-	-	105	4.8	3.5	N.D.	[146]
<i>Pomacea canaliculata</i>	-	-	90	4.3	5	45	[122]
<i>Trichosporon cutaneum</i>	-	-	114	N.D.	6.5	40	[203]

<sup>a</sup> Swiss-prot accession numbers are shown when available

<sup>b</sup> Molecular weights in kDa. Data from SDS-PAGE (*i.e.* monomer size)

<sup>c</sup> Determined from  $\beta$ -mannosidase isolated from human placenta

<sup>s</sup> *Cyamopsis*

## Other exo-acting enzymes

**$\alpha$ -Galactosidase** (EC 3.2.1.22) cleaves  $\alpha$ -(1 $\rightarrow$ 6)-linked non-reducing galactose residues. These enzymes have been classified into families 4, 27, 36 and 57 of glycoside hydrolases [115]. Eukaryotic  $\alpha$ -galactosidases are found in family 27 and most of the bacterial  $\alpha$ -galactosidases are found in families 4 and 36 [1]. In hemicellulose degradation,  $\alpha$ -galactosidases release galactosyl side-groups from oligomeric and polymeric mannan substrates. Some of the  $\alpha$ -galactosidases in family 27 can release galactose from polymeric substrates [2]. However, most of the  $\alpha$ -galactosidases in family 36 lack this ability [2, 159].

**$\beta$ -Glucosidase** (EC 3.2.1.21) catalyses the hydrolysis of non-reducing terminal glucose residues. Most  $\beta$ -glucosidases have been classified into families 1 and 3 of glycoside hydrolases [290].  $\beta$ -Glucosidase is an ubiquitous enzyme found in most types of organisms. In hemicellulose degradation,  $\beta$ -glucosidase releases non-reducing end glucose from oligosaccharides released by  $\beta$ -

mannanase. In a few cases it has been shown to release glucose residues from polymeric glucomannan (Paper VI).  $\beta$ -Glucosidase is also important in the degradation of cellulose; it degrades cellobiose released by cellobiohydrolase and endoglucanase

In addition to glycoside hydrolases, **acetyl esterases** also participate in the degradation of acetylated heteromannans. Acetyl esterase catalyses the hydrolysis of acetyl groups from various substrates. In the context of hemicellulose degradation, most studies on deacetylation have been conducted with acetylxyln esterase [26]. However, a few examples of acetyl esterases active on acetylated mannans have been reported [210, 260, 261].

# Present investigation

## Outline

In this text, the results from Papers I-VII of the thesis are presented and discussed (see List of papers, page 5). The text is divided into four parts:

In the first part (**Mannan-degrading enzymes**), the major enzymes studied in this work are presented. The enzymatic characterisation (Paper I) and cloning (Paper II) of a  $\beta$ -mannosidase (AnMan2A) is presented. Next, the characterisation of a  $\beta$ -mannanase (MeMan5A) from blue mussel is described (Paper V). After this, the modular organisation of the  $\beta$ -mannanase (TrMan5A) from *T. reesei* is presented (Papers IV and VII). Finally, the expression of TrMan5A in *T. reesei* (Paper IV) and *Pichia pastoris* (Paper VII) is described.

In the second part of the text (**The CBM of TrMan5A**), the characterisation of the CBM from TrMan5A is presented (Paper IV). In this work, the binding properties of the CBM was studied and its influence on the catalytic performance of this enzyme was investigated. Furthermore the possible role of cellulose-binding modules in  $\beta$ -mannanases is discussed.

The third part (**The catalytic module of TrMan5A**) describes the mutagenesis of specific amino acids in the active site cleft of the TrMan5A catalytic module (Paper VII).

In the fourth part (**Mannan-hydrolysis**), the specificities in mannan-hydrolysis of several hemicellulases are discussed (Papers I, III, VI, V and VI). The hydrolysis of mannan and heteromannans by AnMan2A is presented (Papers I and VI). In addition, the hydrolysis of crystalline mannan by  $\beta$ -mannanase and  $\beta$ -mannosidase is described (Paper III). Finally, the degradation of different heteromannans by hemicellulases and cellulases is compared (Paper VI).

# Mannan-degrading enzymes

## *Aspergillus niger* $\beta$ -mannosidase, AnMan2A

*Aspergillus niger* produces several enzymes involved in hemicellulose degradation [1, 86]. In this work a  $\beta$ -mannosidase isolated from *A. niger* was purified and characterised (Paper I). In accordance with the recommended nomenclature for glycoside hydrolases [120], this enzyme is referred to as AnMan2A (it has earlier been called Mnd2A). The investigation of the molecular organisation and the enzymatic specificity of AnMan2A which has been conducted in this work (Papers I, II, III and VI) reveals some interesting features which are discussed in this section and later (see pages 45, 46 and 48).

In this work, AnMan2A was purified to homogeneity in three steps (Paper I). The molecular weight of the purified enzyme was analysed by gel filtration and SDS-PAGE. Interestingly, the results suggest that the  $\beta$ -mannosidase is a homodimer. This is an unusual observation since most other  $\beta$ -mannosidases analysed to date are monomeric. Only in a few previous cases have oligomeric  $\beta$ -mannosidases been described [21, 204]. One example is the tetrameric  $\beta$ -mannosidase from *Pyrococcus furiosus* [21].

Furthermore, an investigation of the enzymatic properties of AnMan2A showed that this enzyme is able to degrade manno-oligosaccharides (Paper I). The standard substrate used in many  $\beta$ -mannosidase studies is a small substrate composed of a chromophore linked to a mannosyl residue. In this work we showed that oligosaccharides up to a DP of 6 are degraded by AnMan2A (Paper I). An assessment of the activity of AnMan2A on longer saccharides was not made. However, as will be described later (see page 45) AnMan2A was also active on polymeric mannan substrates. Thus, it can be speculated that AnMan2A probably also degrades longer oligosaccharides. AnMan2A was also active on galactosyl-substituted manno-oligosaccharides (Papers I and II). However, galactosyl groups appear to pose some restrictions on AnMan2A, since hydrolysis is blocked at the point of the first substituent. Degradation of oligosaccharides above DP 3 and galactosyl substituted substrates has previously only been reported for a few  $\beta$ -mannosidases [6, 12, 113, 240].

Further in this work, the gene encoding AnMan2A was cloned (Paper II). Most  $\beta$ -mannosidase genes analysed previously have been isolated from mammals (*Bos taurus*, *Mus musculus*, *Homo sapiens*, *Capra hircus*) and bacteria (*C. fimi*, *Thermotoga maritima*, *Thermotoga neapolitana*) [9, 22, 54, 148, 205, 239]. Apart from the  $\beta$ -mannosidase from the archaeon *Pyrococcus furiosus* which has been assigned to family 1 [21], all  $\beta$ -mannosidases with known sequences have been classified into family 2. In the present case, AnMan2A was assigned to family 2 of glycoside hydrolases on the basis of sequence analysis (Paper II). Besides *man2A*, the only previously described gene encoding a fungal  $\beta$ -mannosidase was isolated from *Aspergillus aculeatus* [251].

The catalytic acid and nucleophile of AnMan2A were predicted to be Glu479 and Glu584, respectively (Paper II). The only experimentally identified catalytic residue among  $\beta$ -mannosidases is the catalytic nucleophile (Glu519) in the  $\beta$ -mannosidase from *C. fimi*, which was identified by mass-spectrometry using a mannosyl fluoride inhibitor [238]. Analysis of the AnMan2A sequence

also revealed 13 putative N-glycosylation sites (Paper II). The presence of N-linked glycans was also indicated by enzymatic deglycosylation of AnMan2A, which yielded a decrease in molecular weight of the enzyme (Paper I).

### **The *M. edulis* $\beta$ -mannanase, MeMan5A**

Both in terms of species and individuals, the molluscs (Phylum *Mollusca*) constitute one of the largest groups of animals and display a wide diversity in terms of feeding habits [65]. Filter-feeding mussels (*Polysyringia*) are a group of molluscs which thrive on microscopic algae and dissolved organic matter. As part of their digestive system, these organisms produce digestive enzymes which are secreted into the stomach from a style sac [212]. Several polysaccharide-degrading enzymes, including cellulases and amylases, have been found in the digestive tract of filter-feeding mussels [212], but there has been no previous report on  $\beta$ -mannanases in these organisms. In this work (Paper V),  $\beta$ -mannanase from the common blue mussel (*Mytilus edulis*) was purified and characterised. Two  $\beta$ -mannanase variants with approximately similar molecular weights and isoelectric points were identified. Both variants also had similar pH and temperature optima (Paper V). Furthermore, the N-terminal sequences of both variants were identical and showed significant similarity to two unclassified  $\beta$ -mannanases from the molluscs *Littorina brevicula* and *Pomacea insularis* [291, 292].

Very recently the corresponding  $\beta$ -mannanase gene was cloned [287]. Sequence analysis revealed similarities to family 5 of glycoside hydrolases and the encoded gene product will hereafter be referred to as MeMan5A. Furthermore, since the gene was isolated from a tissue separate from the digestive tract, possible contaminations from organisms inhabiting these organs were avoided and unequivocal proofs of the endogenous nature of the  $\beta$ -mannanase was gained. Variants of  $\beta$ -mannanases apparently encoded by one gene have been observed in several other organisms, including *T. reesei* [244]. For other organisms different  $\beta$ -mannanases appear to be encoded by several genes [172, 190].

The hydrolysis of manno-oligosaccharides by MeMan5A was analysed in this work (Paper V). MeMan5A showed no activity on manno-oligosaccharides of DP up to 3. Moreover, mannotetraose was degraded at a lower rate than mannopentaose. Thus, it was concluded that binding in at least five subsites is probably required for optimal activity. It was recently reported by others that crystals of this  $\beta$ -mannanase has been obtained [286]. A 3D structure of this enzyme will possibly provide some information about the specific enzyme-polysaccharide interactions in the active site cleft.

Mannan-degrading enzymes have previously been found in several other types of molluscs. A majority of these have been isolated from snails [53, 85, 122, 177, 246]. Due to the variability of molluscs in terms of nutrition, it is tempting to believe that these enzymes are used for different purposes. In the case of *M. edulis* it could be speculated that the  $\beta$ -mannanase participate in the degradation of mannan from algal cell walls [88, 156, 208].

### **The modular *T. reesei* $\beta$ -mannanase, TrMan5A**

The filamentous fungus *T. reesei* is a potent producer of hemicellulose-degrading enzymes involved in the degradation of different mannans and xylans [26]. One of the major hemicellulases



produced by this fungus is a  $\beta$ -mannanase (TrMan5A), which is expressed in media containing cellulose and galactomannan [221, 242]. Previously it was shown that several  $\beta$ -mannanase isoforms with different molecular weights and isoelectric points are produced by *T. reesei* [245]. Later, a  $\beta$ -mannanase encoding gene (*man1*) was isolated from *T. reesei* and it was concluded that the different isoforms are products of the same gene [242, 244]. Thus, it was suggested that the isoforms are likely to differ mainly in their patterns of glycosylation or other types of post-translational modifications. Analysis of the  $\beta$ -mannanase sequence suggested that it is a modular enzyme comprised of a N-terminal catalytic module and a C-terminal CBM connected by a Ser/Thr/Pro rich linker sequence. On the basis of sequence alignment and hydrophobic cluster analysis the catalytic module was classified into family 5 of glycosyl hydrolases according to the classification by Henrissat *et al* [115]. The C-terminal module showed extensive sequence similarity with CBMs from *T. reesei* cellulases and was classified [244] into family I according to the classification of Tomme *et al.* [269].

TrMan5A appears to be specific for mannosidic linkages [244, 245]. Furthermore, in the present work, no hydrolysis was observed when TrMan5A was incubated with cellulose or cello-oligosaccharides (unpublished results).

In this study (Papers III, IV, VI and VII), the functions of the two modules of the *T. reesei*  $\beta$ -mannanase TrMan5A were investigated. In order to study the properties of the modules, a mutant lacking the C-terminal CBM (TrMan5A $\Delta$ CBM), was constructed, and expressed in *T. reesei* as described below (Paper IV). The full-length enzyme was also expressed under the same conditions and both enzymes were purified to electrophoretic homogeneity. Furthermore, several mutants of specific amino acids in the active site of TrMan5A $\Delta$ CBM were expressed in *Pichia pastoris* and characterised, along with the wild type TrMan5A $\Delta$ CBM (Paper VII).

**Homologous expression in *T. reesei*.** As stated earlier, *T. reesei* is a very efficient producer of secreted enzymes and was thus an attractive candidate as a host for gene expression of TrMan5A. Furthermore, since *T. reesei* is the native host of TrMan5A, correct processing of the enzyme is more likely. Since *T. reesei* is utilised as a host for heterologous expression [206], several methods for transformation of foreign genes into *T. reesei* have been developed [164], which can be employed also for the expression of homologous proteins. The main problem posed for expression of mutated homologous enzymes, such as TrMan5A $\Delta$ CBM, is to avoid the expression of the endogenous chromosomal gene. However, it has been shown that  $\beta$ -mannanase expression in *T. reesei* is repressed when it is grown in a medium with glucose as the sole carbon source [170]. Furthermore, it is known that genes under the control of the *Aspergillus nidulans* glyceraldehyde phosphate dehydrogenase (*gpdA*) promoter are constitutively expressed in the presence of glucose [211].

Thus, in this work, the *gpdA* promoter was utilised for expression of mutants of *T. reesei*  $\beta$ -mannanase (Paper IV), as has been done previously with other glucose-repressed enzymes [59, 207]. The expression of TrMan5A and TrMan5A $\Delta$ CBM in *T. reesei* under the control of the *gpdA* promoter was successful and enzyme yields of approximately 5 mg/ml were obtained (Paper IV). Furthermore, the culture filtrate was composed of a limited number of proteins and protein purification was thus facilitated. Pure enzyme preparations were isolated in one or sometimes two chromatographic steps.

**Expression in *Pichia pastoris*:** Traditionally, *Saccharomyces cerevisiae* has been the model organism for yeast expression and also used for heterologous expression. In this specific case,

TrMan5A was previously expressed in *S. cerevisiae* but the enzyme yield was very low [244]. The molecular weight of TrMan5A expressed in *S. cerevisiae* was higher (75 kDa) than that of the *T. reesei* expressed enzyme (52-54 kDa) and it was suggested that the enzyme was likely to be overglycosylated [242]. Expression of a  $\beta$ -mannanase from *Aspergillus aculeatus* also resulted in a higher molecular weight compared to the enzyme expressed in the wild-type organism [229]. Expression in the methylotrophic yeast *P. pastoris* shares many of the advantages of *S. cerevisiae*, such as high expression yields of extracellular enzymes and simple techniques for molecular genetic manipulations. Furthermore, *P. pastoris* has been reported to attach glycans of lower molecular weight than those found in *S. cerevisiae* [103].

In the present case, an expression system which utilises the alcohol oxidase promoters in *P. pastoris* was used. This promoter is strongly induced when the organisms are grown on methanol as sole carbon source. In this work, TrMan5A $\Delta$ CBM and a number of mutants thereof were expressed and secreted by *P. pastoris* using the native signal sequence from the *T. reesei* enzyme (Paper VII). The expression levels were approximately 10 mg/ml and pure enzyme preparations were achieved after two chromatographic steps

## The CBM of TrMan5A

### Sequence and structure

Family I CBMs are common among polysaccharide-degrading enzymes from *T. reesei* and other fungi. A majority of the *T. reesei* cellulases carry family I CBMs either at their N- or C-termini [155, 257]. Amongst *T. reesei* hemicellulases, family I CBMs have been found only in TrMan5A [244] and an acetyl xylan esterase [171]. However, family I CBMs have also been found in some xylanases from *Humicola insolens* [68] and in  $\beta$ -mannanases from *Agaricus bisporus* [256, 289]. The C-terminal family I CBM of TrMan5A is proposed to be composed of 37 amino acids [242]. Figure 11 shows a sequence alignment of the CBM from TrMan5A with several other family I CBMs derived from *T. reesei* endoglucanases and cellobiohydrolases. The three aromatic residues which are exposed on the flat binding surface in the structure of TrCel7A (Figure 12a) are conserved in this alignment – in TrMan5A these residues correspond to Tyr378, Trp403 and Tyr404. The importance of these conserved aromatic residues in several family I CBMs linked to *T. reesei* cellulases has been extensively studied by site-directed mutagenesis [151-153, 218, 219].

TrMan5A	373	SCSPLYGQCGGSGYTGPTCC-AQGT-CIYSNYWY	SOCLNT	410
TrCel7A	461	PTQSHYGQCGGIGYSGPTVC-ASGTTCQVLN	PFYYSQCL	497
TrCel6A	2	ACSSVWGQCGGQNWSGPTCC-ASGSTCVYSND	YYSQCL	38
TrCel7B	401	CTQTHWGQCGGIGYSGKTC-TSGTTCQYSND	YYSQCL	437
TrCel5A	1	QQTVWGQCGGIGWSGPTNC-APGSACSTLN	PYYAQCI	36
TrCel45A	205	GQQTLYGQCGGAGWTGPTTCQAPGT-CKVQN	QWYYSQCLP	242
		x  x  x  x  x          x          x                          x  x  x  x		

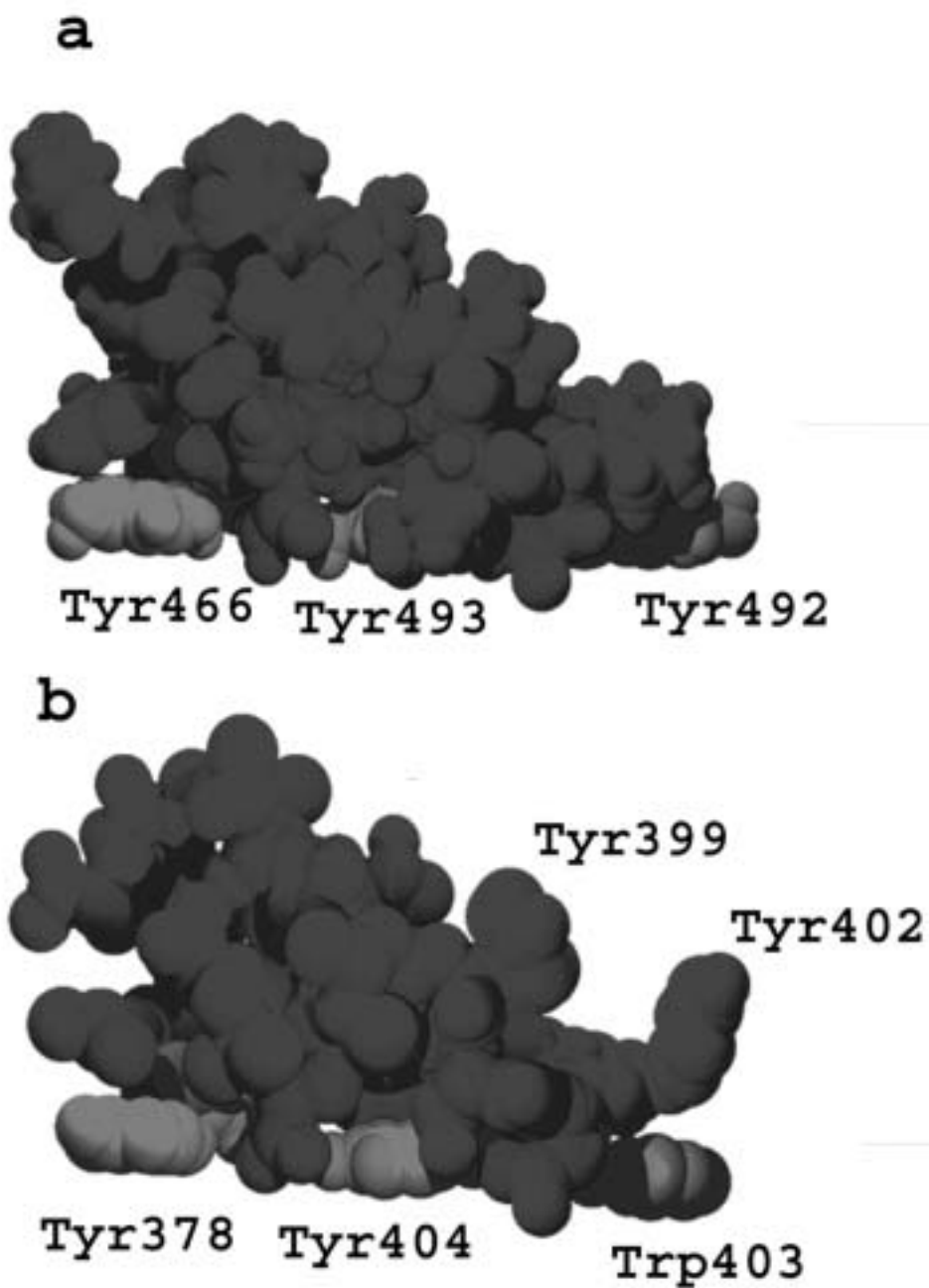
**Figure 11.** Sequence alignment of family I CBMs from TrMan5A and a number of *T. reesei* cellulases. The three conserved aromatic residues are boxed. Identical residues are also indicated (x). The numbers of the amino acids at the N- and C-termini are shown. Adapted from [244].

Based on the structure of the CBM from TrCel7A [145], the structures of the CBMs of the *T. reesei* cellulases TrCel6A, TrCel7B, TrCel45A and TrCel5A were studied by molecular dynamics and were predicted to display a relatively similar structure [123]. Subsequently, the structure of the CBM from TrCel7B was solved and the similarity with the CBM from TrCel7A was validated [174]. In the current study, a model of the CBM from TrMan5A was generated using the program Swiss-PdbViewer [105]. The modelling was based on the structure of the CBM from TrCel7A [145]. As seen in Figure 12b, the flat surface from the CBM of TrCel7A is preserved in this model. Interestingly, two bulky tyrosines protrude from the surface on the rough side of the CBM model. One of these tyrosines (Tyr399) is present in a roughly similar position in the TrCel7B structure [174]. The other tyrosine (Tyr402) appears to be unique for TrMan5A as no counterpart could be found in an extensive sequence alignment [222]. However, it should be pointed out that this is a preliminary model. The positions of these two residues and their possible involvement in polysaccharide binding need to be further studied.

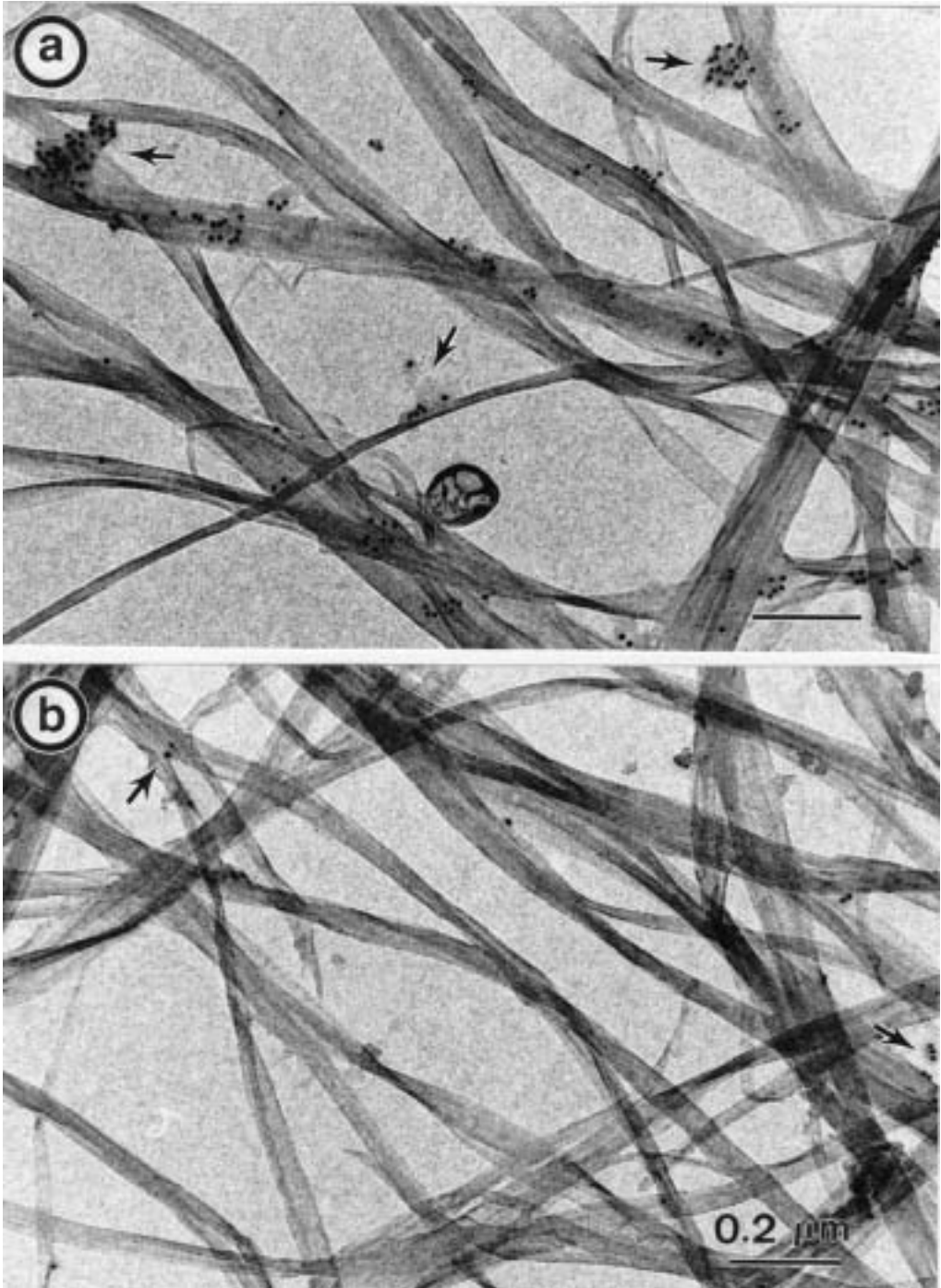
### **Binding properties of the CBM**

Binding to cellulose and kraft fibers with TrMan5A has been observed previously [3, 90, 258], and it was postulated that this binding is mediated by the C-terminal CBM [242]. In this work, the quantitative and qualitative binding properties of the CBM were studied (Paper IV). TrMan5A and TrMan5A $\Delta$ CBM were incubated with cellulose and the amount of free enzyme was analysed. Only TrMan5A adsorbed significantly to cellulose and it could thus be inferred that the binding of TrMan5A to cellulose is indeed mediated by its C-terminal CBM.

The binding of TrMan5A to cellulose was also visualised by immuno-gold labelling (Figure 13). Again, TrMan5A and TrMan5A $\Delta$ CBM were incubated with cellulose. After several washes and incubations with primary antibodies and protein A conjugated to gold-particles, the cellulose samples were analysed by transmission electron microscopy. As seen in Figure 13, cellulose microfibrils incubated with TrMan5A were more strongly labelled in comparison with those incubated with TrMan5A $\Delta$ CBM.



**Figure 12.** **a.** Structure of the CBM from TrCel7A. **b.** Model of the CBM from TrMan5A, based on the structure of the CBM from TrCel7A. The numbers of the aromatic residues on the flat sides of the CBM from TrCel7A and the model of the TrMan5A CBM are indicated. The numbers of the tyrosines on the rough side of the model of the CBM from TrMan5A are also indicated.



**Figure 13.** Visualisation of the adsorption of TrMan5A to ribbons of bacterial cellulose by gold labelling and transmission electron microscopy. Electron micrographs of cellulose labelled with TrMan5A (a) and TrMan5A $\Delta$ CBM (b) are shown.

A quantitative comparison of cellulose binding with a *T. reesei* cellulase carrying a family I CBM, TrCel7B, was made (Paper IV, Figure 4). Analysis of the binding permitted an estimation of the relative binding constant ( $K_p$ ) for the two enzymes. The results showed that the values of this constant were fairly similar with TrCel7B (1.05) and TrMan5A (0.84). This similarity indicates that the C-terminal CBM is a true cellulose-binding CBM. Thus, it can be suggested that TrMan5A shares some of the properties which have been observed in other family I CBMs.

An investigation of the binding of TrMan5A toward mannan-based polysaccharides was also conducted (Paper IV). Earlier, a *Clostridium thermocellum*  $\beta$ -mannanase was shown to bind insoluble mannan [110]. Furthermore, the  $\beta$ -mannanase Man26A from *Cellulomonas fimi* and a  $\beta$ -mannanase from *Caldicellulosiruptor saccharolyticus* were shown to bind to soluble galactomannan via mannan-binding modules from families XXIII and XXVII, respectively [237, 247]. In contrast, no binding of TrMan5A to insoluble mannan, soluble galactomannan and crystalline mannan was detected in the current work (Paper IV). However, TrMan5A did bind to a mannan sample which contained some cellulose (Paper IV, Figure 6b). This mannan/cellulose complex had been isolated from ivory nut and contained approximately 15 % cellulose. Further binding analysis with an *in vitro* mixture of mannan and cellulose, suggested that TrMan5A binds to the cellulose component in this mannan/cellulose complex (Paper IV).

### Effect on hydrolysis

In cellulose degradation, cellulose-binding CBMs are very important for efficient hydrolysis [98, 109, 267, 276]. In particular, several family I CBMs have been shown to increase the activity towards insoluble cellulose substrates [155, 257]. The effect of hemicellulase-linked CBMs has been investigated only to a limited extent and most studies have been related to xylan degradation. The presence of cellulose-binding CBMs have been shown to influence the hydrolysis of complex cellulose/xylan substrates by a xylanase and an arabinofuranosidase from *P. cellulosa* [28, 29, 99]. Also, xylan-binding CBMs from family VI and XXII have been shown to potentiate the hydrolysis of insoluble but not soluble xylan by xylanases from *Clostridium thermocellum* [52, 84]. Amongst  $\beta$ -mannanases, the mannan-binding CBM of Man26A from *Clostridium thermocellum* has been shown to increase the activity towards insoluble ivory nut mannan [110]. Furthermore, hydrolysis of soluble galactomannan by a family 5  $\beta$ -mannanase from *Caldicellulosiruptor saccharolyticus* was reduced in the absence of the two internal family III CBMs [92].

In the present case, the possible influence of the family I CBM of TrMan5A on the hydrolysis of mannan substrates was investigated by comparing the catalytic activity of TrMan5A and TrMan5A $\Delta$ CBM on a range of mannan-containing substrates (Paper IV). Analysis of the hydrolysis of ivory nut mannan and galactomannan showed that the CBM had no effect on degradation of either insoluble or soluble mannan. On the contrary, in the hydrolysis of a mannan/cellulose complex, an increased hydrolysis rate was observed for TrMan5A in comparison with TrMan5A $\Delta$ CBM (Paper IV, Figure 5b). Thus, it was concluded that the CBM has a positive influence on the degradation of this substrate under the conditions used.

Interestingly, the results from these hydrolysis experiments fit well with the data from the binding experiments: binding of the CBM to the mannan/cellulose complex could be correlated to the

increase in activity towards this substrate (Paper IV, Figures 5b and 6b). In the cases where no binding was detected, there was no influence on hydrolysis (Paper IV, Figures 5a and 6a). The lack of influence on hydrolysis of soluble substrates agrees with what has been observed with family I CBMs linked to cellulases where no major influence of the CBMs on hydrolysis of soluble cellulose derivatives has been observed [155].

### **Why a cellulose-binding CBM on a $\beta$ -mannanase?**

In cellulose-degrading enzymes, the presence of cellulose-binding CBMs makes clear sense since the CBM binds to the preferred substrate of the enzyme, thus increasing the effective substrate concentration. The rationale behind the presence of cellulose-binding CBMs in hemicellulose-degrading enzymes is more ambiguous. Previous results have indicated that some cellulose-binding CBMs may promote non-hydrolytic disruption of cellulose surfaces [74]. It could be speculated that such activities of a CBM in a plant cell wall could reveal previously inaccessible parts of the hemicellulose to mannan-degrading enzymes.

On the contrary, results with some other CBMs have shown that, when isolated, these CBMs cannot enhance hydrolysis independently of their catalytic modules [31, 191]. Hence, it has been suggested that the increased hydrolysis obtained with CBMs is due to an increase in effective substrate concentration [31]. Furthermore, it has been postulated that cellulose functions as a general receptor for CBMs of both cellulases and hemicellulases [191]. Since cellulose is a major component in the plant cell wall it might be an easy available target for CBMs. Moreover, since cellulose has a uniform chemical composition it is possible that it displays a large number of similar binding sites for CBMs.

Several studies have shown that mannan and cellulose are closely associated in the plant cell wall [227, 297]. Therefore, it could be speculated that the binding to cellulose of the CBM in TrMan5A positions the catalytic module in the close vicinity of accessible mannan chains. There are some indications that at least some CBMs bind to different sites on cellulose [47]. However, more studies need to be carried out in order to assess any possible cellulose binding preferences of the CBM from TrMan5A.

It is instructive to compare the modular structure of TrMan5A with those in other  $\beta$ -mannanases. CBMs have been predicted for several  $\beta$ -mannanases from family 5 and family 26 (Figure 10). However, only in a few cases has the binding been experimentally confirmed. In addition to TrMan5A, family I CBMs have been indicated in the fungal  $\beta$ -mannanases from *Agaricus bisporus* [256, 289]. However, the  $\beta$ -mannanases from *Aspergillus aculeatus* and *A. niger* appear to lack cellulose-binding CBMs [3, 56].

Some other family 5  $\beta$ -mannanases have CBMs which have been classified into family III and XVI (Figure 10). Moreover, some family 26  $\beta$ -mannanases, have family XXIII and XXVII CBMs which have been shown to bind to mannan [237, 247]. However, many  $\beta$ -mannanases from both families 5 and 26 appear to be sole catalytic modules. Thus, at present, it is difficult to establish any obvious patterns in modularity among  $\beta$ -mannanases. It clearly appears as if several different strategies in mannan-hydrolysis exists.

# The catalytic module of TrMan5A

## The active site cleft

The specificity of catalytic modules is governed mostly by its interaction with the substrate in the active site cleft. Thus, an understanding of the function of specific amino acid residues positioned at the active site or in its vicinity, is essential to an in-depth understanding of the function of an enzyme. In order to pinpoint the function of a given amino acid, site-directed mutagenesis is a powerful method which allows for comparative analyses with the wild-type enzyme. In the present study, a number of mutants (listed in Paper VII, Table 1) of specific amino acids in the active site of the catalytic module of TrMan5A were designed (Paper VII). The basis for the choices of mutants was the solved structure of the catalytic module of TrMan5A, in complex with mannobiose [223].

Figure 1 (Paper VII) shows a close up of the active site of TrMan5A, highlighting the catalytic residues and one of the residues involved in mannobiose binding. As predicted from sequence analysis [244], the catalytic residues (Glu169 and Glu276) are positioned in the active site in the positions conserved within family 5 of glycoside hydrolases [71, 76, 121]. Also shown in Figure 1 (Paper of VII), is the mannobiose molecule positioned in subsites +1 and +2 [223]. Interestingly, the mannose residue in subsite +2 is predicted to hydrogen bond to an arginine residue (Arg171) [223]. The donor in this hydrogen bond is the C-2 hydroxyl of the mannose residue. The conformation of the C-2 hydroxyl (which is axial in mannose) is the only conformational difference from its C-2 epimer – glucose (in which the C-2 hydroxyl is equatorial). Thus, the hydrogen bond between Arg171 and the mannose residue in the +2 subsite, can potentially be an interaction which is important in determining mannan specificity.

Previous kinetic studies on oligosaccharide hydrolysis with TrMan5A has indicated that the +2 subsite is likely to be occupied in substrate binding, at least in the case of saccharides of DP higher than 3 [112]. Furthermore, analysis of the transglycosylation pattern of TrMan5A indicates that the enzyme can produce transglycosylation products of DP  $n+2$  after incubation with a substrate of DP  $n=5$  [111]. Thus, it can be envisaged that the interaction between mannose in the +2 subsite and Arg171 influences the rate of transglycosylation and potentially also the hydrolytic activity of the enzyme.

In summary, Arg171 is likely to be involved in substrate binding and was thus a strong candidate for mutagenesis. In order to maintain the charge balance, a lysine mutant was constructed (Arg171Lys). Furthermore, mutants of the catalytic residues were constructed in order to confirm their role in catalysis. The aim was also to construct an inactive mutant for use in crystallisation of an enzyme/substrate complex.

## Characterisation of mutants

The analysis of the catalytic rate for the active site mutants constructed in this work (Paper VII) gave interesting results. Comparison with the wild-type enzyme indicated that the Arg171Lys



mutant was almost equally active on polymeric galactomannan:  $k_{\text{cat}}$  values of 262 and 241  $\text{s}^{-1}$  were obtained for Arg171Lys and the wild-type, respectively (Paper VII, Table 2). However, the apparent  $K_{\text{M}}$  value for the mutant (1.3 g/l) was somewhat higher than the corresponding value for the wild-type (0.59 g/l). Furthermore, a reduction in hydrolysis rate of this mutant was observed on an oligomeric substrate (mannopentaose). It could be speculated that the catalytic activity of the mutant is impaired only on shorter substrates, and that a certain DP higher than 5 is required for this mutant to show activity in a range close to the wild-type enzyme. Thus, it would be interesting to study the rate of degradation of a range of oligosaccharides with different DP.

Interestingly, an indication of a shift in the activity optimum of Arg171Lys toward a more alkaline pH was detected (Paper VII). Alkaline conditions are preferred in several industrial processes in which cellulases and hemicellulases can be used [44]. Accordingly, there is an interest to engineering these enzymes to increase their pH optima. Several previous studies have focused on mutating charged or polar residues in the close vicinity of the catalytic residues [23, 81]. In the present case, the mutated residue is located at the +2 subsite. However, it is also at hydrogen bond distance to Glu205, which is situated closer to the catalytic acid/base (Paper VII, Figure 1). However, further kinetic studies need to be carried out in order to elucidate the mechanisms behind the observed activity pH-optimum shift.

The activities of the mutants of the predicted catalytic residues (Glu169 and Glu276) were largely reduced (Paper VII). No activity at all was detected for these mutants, with the exception of a mutant of the acid/base (Glu169Gln) which did display activity, albeit very low (a 25-fold decrease in  $k_{\text{cat}}$  (Paper VII, Table 2)). The low or abolished activities of these mutants support the postulated involvement of Glu169 and Glu276 in the catalytic mechanism. In accordance with the current observations, previous site-directed mutagenesis of either the nucleophile or the catalytic acid/base residue of glycoside hydrolases has often resulted in a dramatic decrease in activity and frequently no activity was detected [161]. In several family 5 enzymes, the role of catalytic residues has been established by site-directed mutagenesis [20, 48, 194, 213] or by mass spectrometry, using covalently linked inhibitors [165, 277].

## Mutant Glu169Ala

A 3D-structure of an enzyme can provide important information concerning the overall structure of the enzyme and the position of its active site. However, in order to gain knowledge about specific substrate interactions, it is often necessary to achieve a structure of the enzyme in a complex with a substrate analogue. As stated earlier, TrMan5A has previously been crystallised with a substrate in subsites +1 and +2 [223]. In order to get more information about the substrate interactions in the active site cleft, it would be of interest to crystallise TrMan5A in the presence of a substrate which covers more subsites. In particular, a structure with a substrate residue bound in the -1 subsite might provide information about the catalytic mechanisms of mannoside hydrolysis. However, due to the hydrolytic activity of the wild-type enzyme, there is a big risk that the substrate is degraded in the crystallisation process. Therefore, it was of interest to construct a catalytically inactive mutant. In this work, one of the mutants (Glu169Ala) of the catalytic acid/base residue which did not display any catalytic activity (Paper VII) was chosen as a candidate for crystallisation.

# Mannan-hydrolysis

## Degradation of unsubstituted mannan

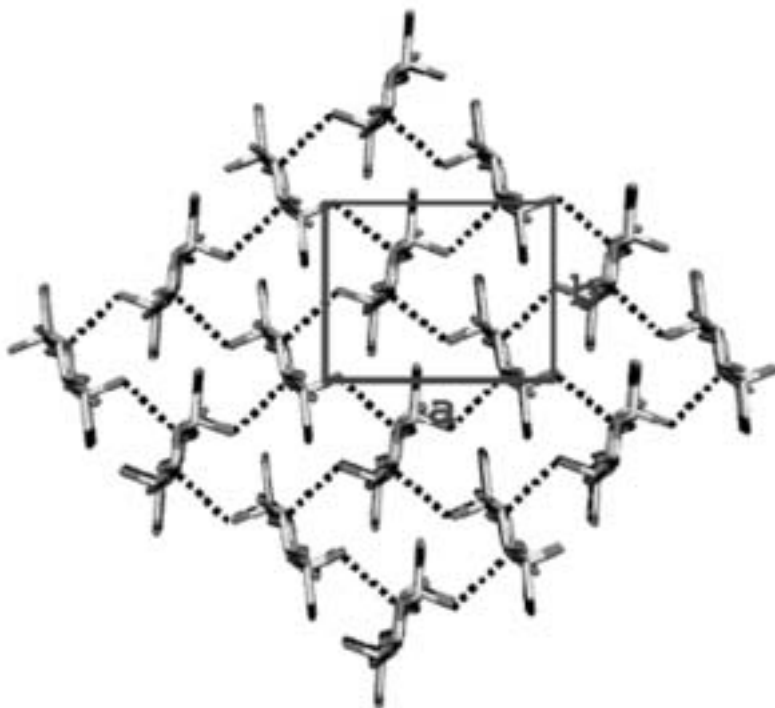
In this work, the hydrolysis of unsubstituted mannan by different mannoside-hydrolases was investigated (Papers I, IV and V). MeMan5A, TrMan5A and AnMan2A were incubated with mannan and the amount of hydrolysis was analysed. Mannan was degraded by TrMan5A (Paper IV), MeMan5A (Paper V) and, interestingly also by AnMan2A (Paper I). The mannan used in these studies was the ivory nut mannan extracted from seeds of *Phytelephas macrocarpa*. This mannan has been reported to have a DP of 20 [184] and was shown in the current work to be free of oligosaccharides of DP lower than 7 (Paper I). AnMan2A was capable of releasing approximately 25 % of the mannose residues in ivory nut mannan from the non-reducing end (Paper I). The ability of  $\beta$ -mannosidases to degrade polymeric mannan has been studied only to a limited extent and very few previous reports have revealed the hydrolysis of mannan by  $\beta$ -mannosidases [14].

The main products formed after extensive hydrolysis of mannan with TrMan5A were mannobiose and mannotriose (Paper IV). This product pattern agrees with previous reports on TrMan5A [245], and other fungal  $\beta$ -mannanases [3, 229]. MeMan5A also degraded mannan to mainly mannobiose and mannotriose, but also produced significant amounts of mannotetraose (Paper V). Production of mannotetraose has previously been reported after limited hydrolysis of ivory nut mannan with a  $\beta$ -mannanase from *A. niger* [272, 273].

## Crystallisation of mannan

As stated earlier (page 16), two polymorphs of crystalline mannan are found in the native state – mannan I and mannan II [51]. These two crystal forms can also be obtained upon recrystallisation of mannan from solution. Recrystallisation of mannan in either polymorph is dependant on the crystallisation conditions: a high molecular weight of the polysaccharide, a high polarity of the crystallisation medium and a low temperature favour the formation of mannan II crystals, and the reverse conditions favour the formation of mannan I [50, 51]. In this study, mannans from ivory nut and *Acetabularia crenulata* were crystallised as mannan I and mannan II, respectively (Paper III). The morphologies of mannan I and mannan II crystals are strikingly different. Crystallisation in the mannan I form yields lozenge-shaped, laminated single crystals (Paper III, Figure 1c). Mannan II, on the other hand, shows a ribbon-like morphology (Paper III, Figure 3).

The molecular structure of mannan I has been studied with electron diffraction and X-ray diffraction [19, 49, 88, 197]. Mannan I crystals are composed of anti-parallel sheets of mannan chains packed with their molecular axes perpendicular to the base plane of the crystal (Figure 14). The molecular structure of mannan II is less well known. However, based on X-ray diffraction analysis a model has been proposed [188, 295], in which the mannan chains are packed anti-parallel within sheets which themselves are anti-parallel to neighbouring sheets.

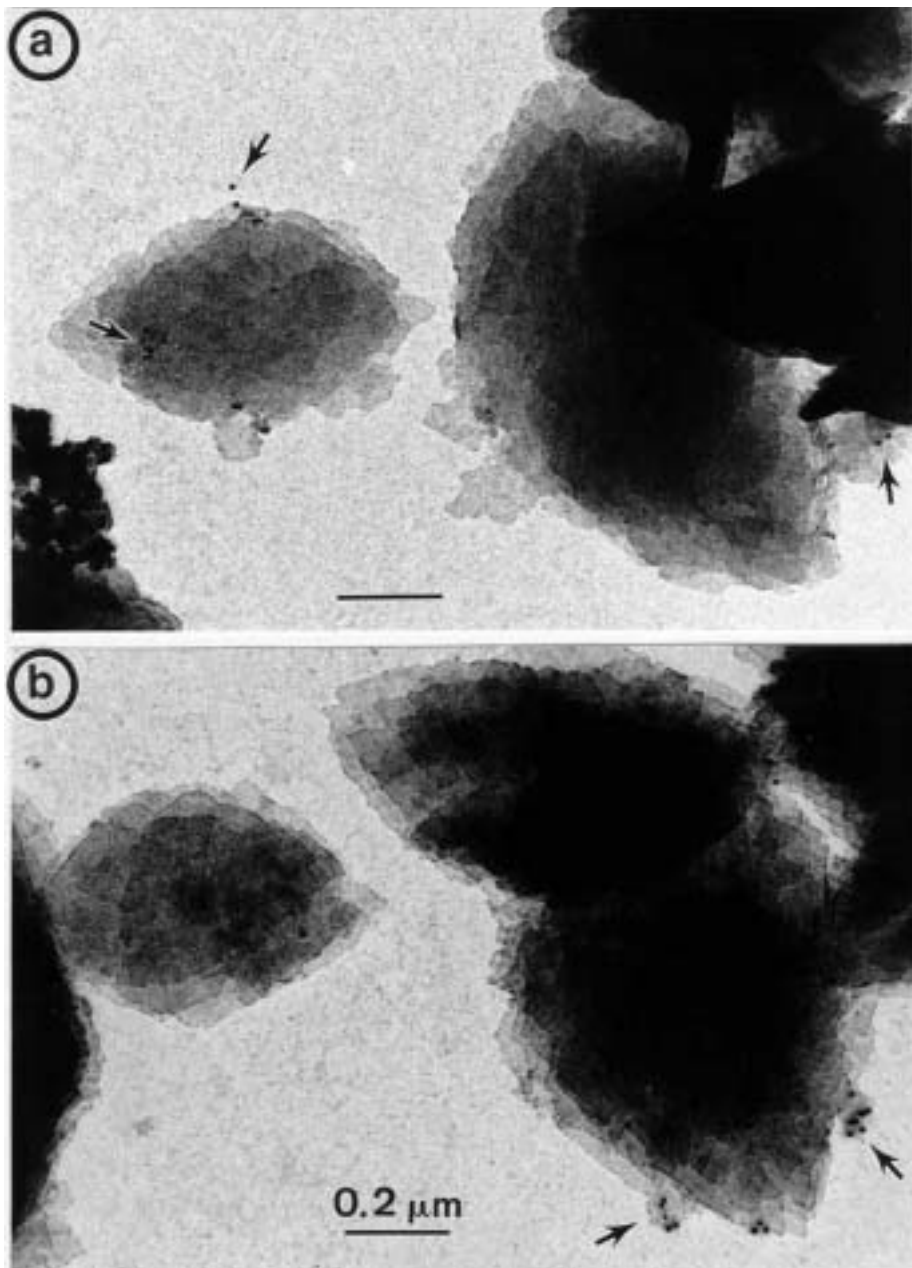


**Figure 14.** Structure of mannan I viewed as a projection of the unit cell onto the *ab* plane. The unit cell dimensions are  $a=8.92 \text{ \AA}$ ,  $b=7.21 \text{ \AA}$  and  $c=10.27 \text{ \AA}$ . Adapted from [222].

## Degradation of mannan crystals

In the present study (Paper III), crystals of mannan I and II were degraded by TrMan5A, TrMan5AΔCBM and AnMan2A. In addition, mannan I crystals were also degraded by the *A. niger*  $\beta$ -mannanase (AnMan5A). The enzymatic degradation of the crystals was studied with transmission electron microscopy, electron diffraction and sugar analysis. The results show that both mannan I and mannan II crystals can be degraded with the enzymes used in this study. However, only a limited degradation was seen with AnMan2A, especially in the case of mannan II. Based on the results from these enzymatic degradations and the established structure of mannan I crystals, a model for degradation of mannan I crystals by the mannan-degrading enzymes was proposed (Paper III, Figure 6). According to this model, the initial sites of degradation by the mannan-degrading enzymes are the corners of the crystal lamellae. After the initial degradation of the corners, the endo-acting  $\beta$ -mannanase will continue to hydrolyse the interior of the lamellae.  $\beta$ -Mannosidase, on the other hand, will be unable to degrade the crystals after the initial attack on the corner chains. Due to the antiparallel chain packing of the mannan I crystal every other layer of mannan chains will have its non-reducing end buried in the interior of the crystal and thus is unavailable for attack by  $\beta$ -mannosidase (Paper III, Figure 6).

The proposed enzymatic attack at the crystal corners was further supported by immuno-gold labelling with  $\beta$ -mannanase (Figure 15). TrMan5A and TrMan5A $\Delta$ CBM were incubated with mannan I crystals and subsequently analysed by transmission electron microscopy. As seen in Figure 15, the labelling appears to be clustered at the crystal corners to some extent.



**Figure 15.** Visualisation of the adsorption of TrMan5A to mannan I crystals by gold labelling and transmission electron microscopy. Electron micrographs of crystals labelled with TrMan5A (a) and TrMan5A $\Delta$ CBM (b) are shown.

The degradation of mannan II crystals by TrMan5A $\Delta$ CBM altered the morphology of the crystal samples (Paper III, Figure 4). After prolonged incubation, the ribbon-like crystallites disappeared and a granular matrix remained. However, the electron diffraction pattern of the degraded sample is similar to the pattern prior to degradation which is typical of crystalline mannan II. Thus, it seems possible that the obtained granular matrix consists of undegraded mannan II crystals.

## Degradation of heteromannans

In this study, the degradation of different types of heteromannans by mannoside-hydrolases was studied (Papers I, V, VI and VII). These investigations revealed several interesting features concerning the catalytic properties of mannoside-hydrolases, in particular regarding  $\beta$ -mannosidase. AnMan2A was able to liberate mannose from galactomannan, glucomannan and galactoglucomannan (Paper I and VI). Furthermore, AnMan2A also released acetyl-substituted mannose from the non-reducing end of *O*-acetyl-galactoglucomannan (*O*-acetyl-GGM) (Paper VI). The hydrolysis of heteromannans by  $\beta$ -mannosidase has previously only been studied to a very limited extent [106, 122, 146], and this is the first indication that a  $\beta$ -mannosidase have the ability to liberate acetyl-substituted mannose. However, as was described in Paper I, AnMan2A is restricted by galactosyl substituents.

Degradation of heteromannans also enabled some comparisons between several  $\beta$ -mannanases. In these studies TrMan5A, MeMan5A and the  $\beta$ -mannanase from *C. fimi* (CfMan26A) were used (Papers V, VI and VII). Hydrolysis of LBG galactomannan by TrMan5A and MeMan5A under assay conditions permitted an estimation of the Michaelis constant ( $K_M$ ) for the two enzymes (Paper V and Paper VII). The apparent  $K_M$  value for LBG obtained with MeMan5A was 3.95 g/l (Paper V). This was considerably higher than the corresponding value obtained with TrMan5A 0.6-0.7 g/l (Paper VII, Table 2). Interestingly, the  $K_M$  values for LBG in previous reports on  $\beta$ -mannanases from molluscs are generally considerably higher than those in reports on fungal  $\beta$ -mannanases. The  $K_M$  values for three  $\beta$ -mannanases from snails lie in the range (1.4-3 g/l) [53, 85, 180]. In contrast, reports on fungal  $\beta$ -mannanases from *Trichoderma* and *Aspergillus* have given values between 0.0015-0.7 g/l [17, 56, 57, 229].

The degradation of glucomannan by CfMan26A and TrMan5A was also compared in the present study (Paper VII, Figure 2). Analysis of the DP of the products formed after prolonged hydrolysis revealed relatively similar patterns with the two enzymes; in both cases, mannobiose and mannotriose were the major products formed. Slightly more mannotriose was formed with CfMan26A in comparison with TrMan5A. Thus, no major difference in the product formation of glucomannan degradation was observed under the conditions used. This may indicate that the two  $\beta$ -mannanases from families 5 and 26 have similar binding requirements with respect to mannose and glucose residues. However, it must be stated that the products were analysed only with respect to their DP. In order to further assess any possible differences/similarities in substrate binding requirements between family 5 and family 26  $\beta$ -mannanases, a larger number of enzymes should be included and a more detailed product analysis should be made.

In the context of mannan degradation, investigations on the hydrolysis of isolated glucomannans and galactoglucomannans have previously been conducted, which have provided information about the specific enzyme-polysaccharide interactions of  $\beta$ -mannanases. In particular, the subsite

specificity of the *T. reesei* and *A. niger*  $\beta$ -mannanases has been investigated to some extent [182, 259]. In both these enzymes glucose is allowed in the +1 subsite, but not in the -1 site.

In the present study, a comparison of hydrolysis of glucomannan and *O*-acetyl-GGM by TrMan5A was made (Paper VII). Analysis of the hydrolysis products showed that the DP profiles were different for *O*-acetyl-GGM (mainly DP 2-5) and glucomannan (mainly DP 2-3). This indicates that some restrictions exist for the  $\beta$ -mannanase with regard to either galactosyl- or acetyl-substituents. The restrictions of galactosyl-substituents for TrMan5A [259] and the *A. niger*  $\beta$ -mannanase [182], has previously been observed and it has been concluded that galactose substituents can be accommodated in the -1 subsite, but not in the +1 subsite [182, 259]. Moreover, Puls *et al.* [210] demonstrated that acetyl-groups appears to limit hydrolysis of galactoglucomannan by the *A. niger*  $\beta$ -mannanase. However, a more detailed study on the restrictions of acetyl-substituents on  $\beta$ -mannanase activity has not yet been made. The degradation of *O*-acetyl-GGM by  $\beta$ -mannanases is important, not only from an enzymologic point of view. Previous work in our group has shown that  $\beta$ -mannanases can be used as tools to analyse the structure of heteromannans [158].

Interestingly, results from analysis of *O*-acetyl-GGM hydrolysis also indicated that an endoglucanase (TrCel7B) from *T. reesei* has a limited depolymerisation activity on this polymer (Paper VII). It has previously been shown that TrCel7B and several other *T. reesei* cellulases are active on glucomannan [138]. Furthermore, it has been reported that TrCel7B is active on xylan [27]. In addition, the endoglucanase TrCel5A from *T. reesei* display activity on galactomannan [163]. These cross-activities of cellulases are interesting in view of the close association between cellulose and hemicellulose in the plant cell wall. A more detailed product analysis will possibly reveal more information about specific enzyme-polysaccharide interactions in the degradation of *O*-acetyl-GGM by TrCel7B. Of special interest here is to detect the specificity of interactions in the -1 subsite.



# Conclusions & future perspectives

In this thesis, new results concerning the enzyme-substrate interaction of mannan-degrading enzymes have been presented and discussed. In particular, novel information has been put forward regarding the function of a modular  $\beta$ -mannanase (TrMan5A). It was shown that the CBM of TrMan5A binds to cellulose and potentiates the hydrolysis of complex substrates. Thus, the substrate preference of this enzyme is influenced by interactions in both modules. An interesting fact is that, in the present case, the binding of the CBM is not directed to the mannan-substrate *per se*. However, these results may be a reflection of the tight and complex organisation of cellulose and hemicellulose in the plant cell wall.

This observation opens up new questions regarding the role of CBMs in  $\beta$ -mannanases and other hemicellulases. It would be interesting to investigate if the cellulose-binding CBMs in these enzymes bind to cellulose at specific positions where the cellulose and hemicellulose are closely associated. It would also be interesting to compare the *T. reesei*  $\beta$ -mannanase with other modular  $\beta$ -mannanases and other hemicellulases. CfMan26A, which carries a family XXIII mannan-binding CBM, is an example of an interesting candidate for such a study.

The results presented here concerning the roles of specific amino acid in a  $\beta$ -mannanase is somewhat unique in the area of  $\beta$ -mannanases. Few studies of this kind have been carried out with these enzymes. The results presented here have given some hints about the influence of Arg171, positioned in the +2 subsite, on hydrolysis. Therefore, it would be interesting to study this residue and further assess its role in substrate recognition. Furthermore, a new complex-structure with an inactive mutant will potentially provide new information about the specific enzyme/carbohydrate interactions in the active site cleft.

This work has also included studies on mannan hydrolysis by different mannan-degrading enzymes. The investigation of degradation patterns from hydrolysis of different heteromannans has provided new insights into the specificity of different enzymes on these substrates. Interestingly, a  $\beta$ -mannosidase was capable of degrading both mannans and heteromannans. A limited amount of degradation of mannan crystals was also detected with this  $\beta$ -mannosidase. These observations indicate that the *A. niger*  $\beta$ -mannosidase can participate in the direct enzymatic attack on polymeric substrates. However, the nature of the enzyme-polysaccharide interaction remains to be elucidated.

Due to the complex nature of the plant cell wall and mannan-based substrates, several issues need to be addressed in order to achieve a better understanding of mannan-degradation. Firstly, the enzyme-polysaccharide interaction of mannoside-hydrolases in the degradation of the more complex heteromannans, like *O*-acetyl-galactoglucomanan, should be studied in more detail. In particular, the influence of different substituents on the rate of hydrolysis needs to be investigated further. Secondly, a larger comparative study of mannan-degrading enzymes from different enzyme families would possibly reveal any differences or similarities in substrate specificity. In general, improved methods for the separation and detection of polysaccharides and oligosaccharides would be very useful in these types of investigations.



Furthermore, the results from this work indicate that an endoglucanase from *T. reesei* can depolymerise galactoglucomannan. This observation is interesting to discuss in the framework of modular enzymes. Several modular enzymes possess both cellulase and hemicellulase catalytic modules. Furthermore, modular enzymes may contain multiple CBMs with affinity for both cellulose and hemicellulose. Thus, it appears that, at least in some cases, the enzymatic degradations of cellulose and hemicellulose are interlinked with each other. These connections between catalytic modules and CBMs with affinity for cellulose and hemicellulose are in accordance with the observed enzyme-polysaccharide interaction of the modular  $\beta$ -mannanase from *T. reesei* and clearly illustrate the complexity of the plant cell wall.

In the study of complex substrates, integrated approaches including several other types of cell wall-degrading enzymes, might prove to be useful. Since the results in this work showed that an endoglucanase is capable of depolymerising isolated galactoglucomannans, it would be interesting to assess if the enzyme also perform this activity in the plant cell wall. Possibly, such an activity may facilitate the degradation of mannans by hemicellulases.

In summary, the work presented here has provided new information about enzyme-polysaccharide interaction in the modular  $\beta$ -mannanase from *T. reesei* which will be valuable for further studies on this enzyme and other modular enzymes. Furthermore, hydrolysis of mannans by mannoside- and glycoside-hydrolases has opened up new questions regarding the substrate specificity in the subsites of these enzymes. Hopefully, the work presented in this thesis will be an incitement for continued studies on the specific polysaccharide interactions in modular enzymes.

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