Probing Promiscuity:

Structural studies of *Phanerochaete chrysosporium* Laminarinase 16A

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2009

Acta Universitatis agriculturae Sueciae
2009:98

Cover: Subsite numbering scheme for glucose residues in Lam16A. Black: mandatory binding sites. Blue: β-1,3/(1,6)-glucans; periods denote β-1,6-branches. Yellow: $(G4G3G4)_n$ mixed linkage β-glucans. Grey circles: alternate binding site for G6G3G3G. Inverted numbers mean that the C1 carbon is pointing away from the viewer.

ISSN, 1652-6880
ISBN, 978-91-576-7445-6

Print: SLU Service/Repro, Uppsala 2009

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Abstract

The glycoside hydrolase Laminarinase 16A (Lam16A) from the white-rot fungus *Phanerochaete chrysosporium* is able to bind and cleave two different substrates in a specific manner. It has been called "non-specific", but we find that binding promiscuity and catalytic promiscuity better define this phenomenon. We have published high-resolution structures of wildtype Lam16A alone (Paper I) and in complex with oligosaccharide products from hydrolysis of laminarin and lichenan[1] (up to 1.1 Å resolution) (Paper II) as well as structures of the catalytic nucleophile mutant Lam16A E115S in complex with laminariheptaose and α-laminariheptaosyl fluoride (Paper III, submitted). A β-sandwich forms the substrate binding cleft, evenly open at both ends. The Lam16A ligand/complex structures, especially the catalytic mutants (Paper IV, manuscript), illustrate why two β-1,3-linked glucose residues are required at the −2 and −1 subsites and why no β-1,6-branch can be accomodated at these sites. Also, catalytic mutants enabled the trapping of substrate in the +1 subsite, showing how Lam16A can hydrolyze both β-1,4- and β-1,3 glycosidic bonds.

Keywords: 1,3(4)-beta-D-glucanase, promiscuous, enzyme, protein-ligand structure, lichenan, lichenin, laminarin, glycoside hydrolase family 16

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[1] we used the term lichenin instead of lichenan in Paper II.

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Jonas Vasur, Rie Kawai, Anna M. Larsson, Kiyohiko Igarashi, Mats Sandgren, Masahiro Samejima and Jerry Ståhlberg (2006). X-ray crystallographic native sulfur SAD structure determination of laminarinase Lam16A from *Phanerochaete chrysosporium*. *Acta Cryst D* 62(Pt. 11), 1422–1429.

II Jonas Vasur, Rie Kawai, Evalena Andersson, Kiyohiko Igarashi, Mats Sandgren, Masahiro Samejima and Jerry Ståhlberg (2009). X-ray crystal structures of *Phanerochaete chrysosporium* Laminarinase 16A in complex with products from lichenin and laminarin hydrolysis. *FEBS Journal* 276(14), 3858–3869.

III Jonas Vasur, Rie Kawai, K. Hanna M. Jonsson, Göran Widmalm, Åke Engström, Martin Frank, Evalena Andersson, Henrik Hansson, Zarah Forsberg, Kiyohiko Igarashi, Masahiro Samejima, Mats Sandgren, Jerry Ståhlberg (2009). Synthesis of cyclic β-glucan using Laminarinase 16A glycosynthase mutant from the basidiomycete *Phanerochaete chrysosporium*. (submitted to *JACS* in October)

IV Jonas Vasur , Rie Kawai , Evalena Andersson , Mats Sandgren, Kiyohiko Igarashi , Masahiro Samejima , Jerry Ståhlberg (2009). Mimics of bound β-1,3/1,6-glucan and lichenan in mutant *Phanerochaete chrysosporium* Laminarinase 16A. *manuscript*

1 Introduction

A lot of the action in the wild happens in dark places. Take fungi, for example. Fungi are often hidden from sight, feeding off the dead or in symbiotic relationships with the living. Representatives of the Kingdom of Fungi can be the largest of organisms yet they do not present themselves as dramatically as animals do, nor as blatantly as plants (which by definition require sunlight and thus have only a limited ability to elude discovery). Of course some basidiomycete fungi produce mushrooms, which do appear rather dramatically, but these fruiting bodies are mere details compared to the vastness of the intricate mycelial network underneath them, a network that for some species can extend for kilometers, and challenge our very notion of the term "organism". Fungi may elude us at first glance, but their contributions are vital to the ecosystems in which they live. Fungi can define the structure of the media in which they grow while quickly and quietly cycling its nutrients, to the benefit (and occasional detriment) of plants and animals.

The basidiomycete *Phanerochaete chrysosporium*[2] is known as white-rot fungus because of its ability to degrade (brown) lignin in wood while leaving the (white) cellulose initially intact (Burdsall and Eslyn [1974]). It can be found underneath leaves or logs in the forest. In 1984, a sporoform specimen of *P. chrysosporium* was isolated in Sweden (Johnsrud and Eriksson [1985]). This specimen, named K-3, was especially good at degrading lignin. At the time, the ability of white-rot fungi to digest the lignin in wood and subsequently expose the cellulose was seen by some visionaries as a possible alternative to the expensive and dirty removal of lignin by mechanical or chemical means (Johnsrud and Eriksson [1985]). Furthermore, the constituents of lignin are similar to many environmental pollutants, making *P. chrysosporium* a useful tool in bio-remediation. The potential of such applications prompted the funding of basic research on the extracellular enzymes of *P. chrysosporium* and the decoding of the *P. chrysosporium* genome (Wymelenberg et al. [2006]). Sometimes the profitable practical applications envisioned in funding proposals do not come true, and other promises of profit are needed to fill the funding void (Laudel [2006]). Nevertheless, the basic research enabled by these dreams can add important understanding to fundamental concepts in biology.

The research described in this thesis is part of a larger quest to understand how *P. chrysosporium* degrades wood. In 1991, electron microscopy results (Ruel and Joseleau [1991]) showed how fungal hyphae were encap-

[2] previously classified as *Chrysosporium lignorum*, and subsequently as *Sporotrichum pulverulentum*

sulated by a mucilagenous β-1,3 1-6-glucan[3]. This extracellular β-1,3- 1,6-glucan network[4] serves several functions. First, it is a connecting matrix, or interface, between the fungal hyphae and plant cell walls or other solid substrate. Second, the β-1,3 1,6-glucan, produced by the fungus, can serve as a temporary carbon source for the fungus. Finally, a high concentration of extracellular fungal enzymes can be found here. Many of these enzymes facilitate the fungal attack of the plant cell. As the hyphae grow closer toward and into the plant cells they are invading, the extracellular β-1,3- 1,6-glucan network changes with it. β-glucanase-modifying enzymes found in the hyphal sheath could facilitate modifications of the latter.

One such extracellular enzyme is *P. chrysosporium* Laminarinase 16A (Lam16A). Laminarinase 16A is able to hydrolyze different substrates (Kawai et al. [2006]), and is therefore a promiscuous enzyme. This thesis describes the structures of Lam16A together with different substrates. It is the first structure of a promiscuous subfamily of glycoside hydrolase of family 16. Lam16A is one of few promiscuous enzymes to be structurally elucidated together with bound substrate. Because of their inherent versatility and evolvability, promiscuous enzymes can be interesting targets for modification for use in the synthesis of novel molecules. As an indication of such potential usefulness, we also describe the first successful synthesis and validation of cyclic laminariheptaose using mutant Lam16A and modified substrate.

[3]While earlier research had isolated β-glucans from various fungal species (Bartnicki-Garcia [1968]) this had been thought to be an integral part of the fungal cell wall, not an extracellular matrix, or sheath.

[4]hyphal sheath or β-glucan sheath

2 Substrates and Ligands

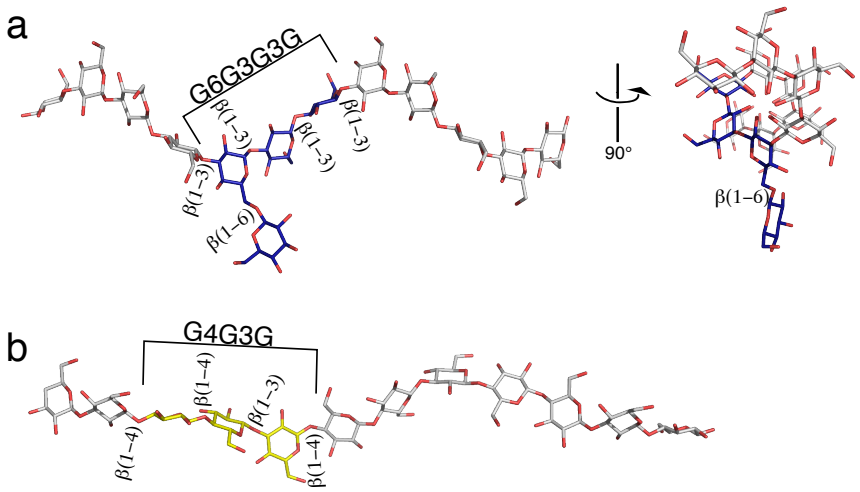

Figure 1: Twelve-unit-main-chain theoretical models of (a) laminarin and (b) lichenan (Bohne et al. [1999]). The ligands G6G3G3G (blue) and G4G3G (yellow) were used in Papers II and IV. Notice the pronounced helical nature of laminarin.

Before describing Lam16A and the details of its binding cleft, we must understand the two model substrates, the polysaccharides laminarin and lichenan, which it cleaves. To understand their structure, some fundamentals of carbohydrate geometry will be reviewed. "Linear" is often used to describe non-cylcic polysaccharides, but polysaccharides are never linear in the sense that they are "straight"; instead they are helical.

2.1 Glycosidic Bonds Shape the Polysaccharide

β-glucans are polysaccharides of β-D-glucopyranose (glucose). The most likely conformations of a β-glucan are determined by the bonds between its glucose residues[5]. If some force were to "straighten out" this helix (or dramatically change its pitch), then it would need to overcome the geometric constraints within the glucose residues themselves. The ϕ (phi) and ψ (psi) dihedral angles of the glycosidic bond (between the glucose units) are thus constrained by the conformations and relative positions of the glucose residues it connects.

[5]This applies to all carbohydrates, not just β-glucans

The ϕ and ψ angles of glucans (and of other polysaccharides) can be represented in ϕ/ψ plots (Frank et al. [2007]). Such plots allow us to visualize and analyze how well the observed ϕ and ψ dihedral angles match theoretical minima. Unless there is some force acting upon a glycosidic bond, then its ϕ and ψ angles should not be dramatically different from theoretical minima for the polysaccharide in question[6].

2.2 β-1,3 glucan: Laminarin and Curdlan

Plain β-1,3 glucan is commonly known as curdlan and can be extracted from a variety of sources. In its loosest conformation, β-1,3 glucan forms a helix of six repeating glucose residues with a pitch of 23 Å and a diameter of 10 Å. In the described conformation, it can appear as a single helix (Okuyama et al. [1996]) or as a triple helix (Deslandes et al. [1980]). Although it has three different states, depending on the degree of hydration, the helical geometry persists for all energy minima (McIntosh et al. [2005]).

Laminarin is like curdlan except that it is shorter, readily soluble in water, and contains occasional β-1,6 branches. It is extracted from brown algae (kelp) *Laminaria digitata* (oarweed). Laminarin is popular as a substrate in β-glucan studies because it is well-characterized, water-soluble and commercially available. The median length of a laminarin molecule is less than 30 glucose units, with no more than 4 β-1,6 branches (Read et al. [1996]). Laminarin is useful in the study of Lam16A because the position of a β-1,6 branch can inhibit binding in the catalytic site.

Lam16A produced a G6G3G3G product upon reaction with laminarin (Kawai et al. [2006]) . Crystallization experiments were performed with this product bound in Lam16A to discover how the enzymatic cleft was accommodating the β-1,6 branch.

2.3 Lichenan $(G4G3G4)_n$

Lichenan is extracted from *Cetraria islandica* (Iceland moss) and resembles β-glucan from oat and barley. It is comprised of β-1,4- to 1,3-linkages in a 2:1 ratio. The structure has been solved using X-ray fiber diffraction crystallography (Tvaroska et al. [1983]), confirming the $(G4G3G4)_n$ repeat as well as the theoretical structure based on this pattern. The (G4G3G4) mixed

[6]These ϕ ψ plots for carbohydrates are similar to Ramachandran ϕ/ψ plots for the peptide bonds of proteins - albeit the dihedral angles are defined differently for peptide bonds. While amino acids have the same peptide bond between them, carbohydrate ϕ and ψ minima, by contrast, differ depending on which hydroxyls form the glycosidic bond

linkages produce a very tight helical twist[7]. Lichenan is well-characterized and commercially available, but it is not readily soluble in water.

G4G3G is a product of Lam16A hydrolysis of lichenan. By crystallizing G4G3G together with Lam16A (Paper II) and its mutants (Paper IV), we showed how Lam16A was able to hydrolyze β-1,4 glycosidic bonds and why these points of hydrolysis had to be preceded by a β-1,3 bond.

[7] An intuitive conclusion might assume that mixed linkages confer an "averaged" shape to the polysaccharide helix; this is false, as the wider-than-curdlan helix of the hypothetical example $(G3G4G3)_n$ would demonstrate.

3 *P. chrysosporium* Laminarinase16A

The focus of this thesis is how the catalytic cleft of Lam16A is able to accommodate two different substrates. It is this ability to bind two different substrates that makes the enzyme promiscuous. Binding of the substrate is a prerequisite for hydrolysis of the glycosidic bond, making substrate binding more important for substrate specificity than the enzymatic mechanism itself. Hampering the mechanism can be a useful tool in studying binding.

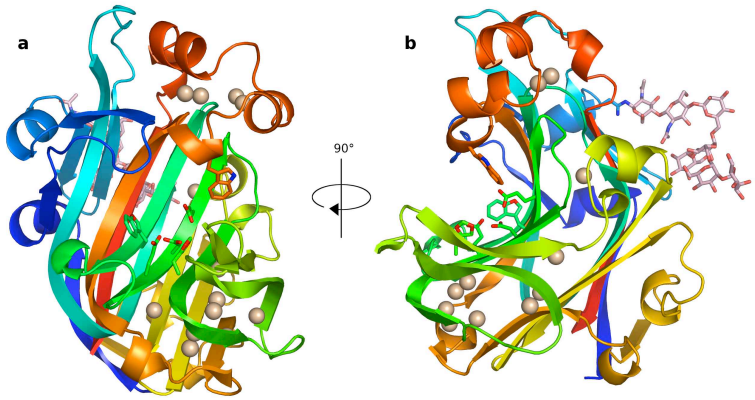

Figure 2: Secondary structure representation of laminarinase Lam16A from *P. chrysosporium*, rainbow-colored from N- (blue) to C-terminus (red). Beta sheets were assigned using Beta-Spider (Parisien and Major [2005]).

3.1 Enzyme Structure

Lam16A is comprised of antiparallel β-strands that form a curved β-sandwich, 7 strands wide in the concave/inner sheet and 6 strands wide in the convex/outer sheet of the sandwich. There are also ten single- or double-turn α-helices interspersed on the periphery of the structure. The protein forms an irregular ellipsoid with approximate dimensions of 60 Å × 40 Å × 30 Å. The concave β-sheet and connecting regions at the periphery combine to form a cleft approximately 30 Å in length, 12 Å deep and 8 Å wide, which cuts across the middle of the enzyme. Aromatic residues lining this participate in binding of the polysaccharide substrate. The catalytic residues, Glu115 (nucleophile) and Glu120 (Brønsted acid/base) are located on the same β-strand near the middle of the cleft.

The β-sandwich fold of the catalytic cleft of Lam16A is similar to the β-sandwich fold found in the majority of non-catalytic CBMs (carbohydrate-binding modules) (Boraston et al. [2004]). In the context of a non-enzymatic binding scaffold such as the CBM, the β-sandwich has been been described as "extremely plastic" (Boraston et al. [2002]). Hence, it is probably not a coincidence that Lam16A, which has the same type of fold, should be promiscuous in binding of substrate. It would thus not be surprising in the future to discover that Lam16A can also bind carbohydrates or molecules other than the well-characterized lichenan, curdlan and laminarin used in the studies herein.

3.2 Mechanism

Glycosidases (such as Lam16A) catalyze the hydrolysis of glycosidic bonds. There are two types of glycosidases: retaining glycosidases (such as Lam16A) and inverting glycosidases. The nucleophile and Brønsted acid/base (proton giver/taker (Brønsted [1928])) are vital for their function. For retaining glycosidases, the (deprotonated) nucleophile attacks the anomeric carbon of the glycosidic bond while the charge polarization that results is stabilized by the proton-donating Brønsted *acid*/base. Thus a covalent intermediate is formed (Davies et al. [1998]). A water molecule attacks the vulnerably exposed anomeric carbon of the covalent intermediate, loses a proton to the Brønsted acid/*base*, and becomes the anomeric hydroxyl of the reducing end of a new (shorter) carbohydrate[8]. The process is depicted in Fig 3.

In some cases, the anomeric carbon of the covalent intermediate can be attacked by the hydroxyl of another glucan instead of water. This phenomenon, known as transglycosylation, is the only reaction that occurs in some structurally related enzymes[9] (Johansson et al. [2004], Mark et al. [2009],) and to some extent also in Lam16A (Kawai et al. [2006]).

3.3 Interrupting the Mechanism: Catalytic Mutants

To study substrate binding, key amino acid can be replaced using point mutations, and the enzymatic mechanism can be hampered. This way, ligands can remain unaltered by enzymatic processes and hence remain in the catalytic cleft of the mutant enzyme instead of being expelled by default.

[8] inverting glycosidases (where water acts as a nucleophile) willl not be covered here.

[9] Glycoside hydrolases are classified into families according to certain similarities in the amino acid sequence (Henrissat [1991]). These sequence similarities have proven themselves to be a much better predictor of protein structure than substrate specificity has.

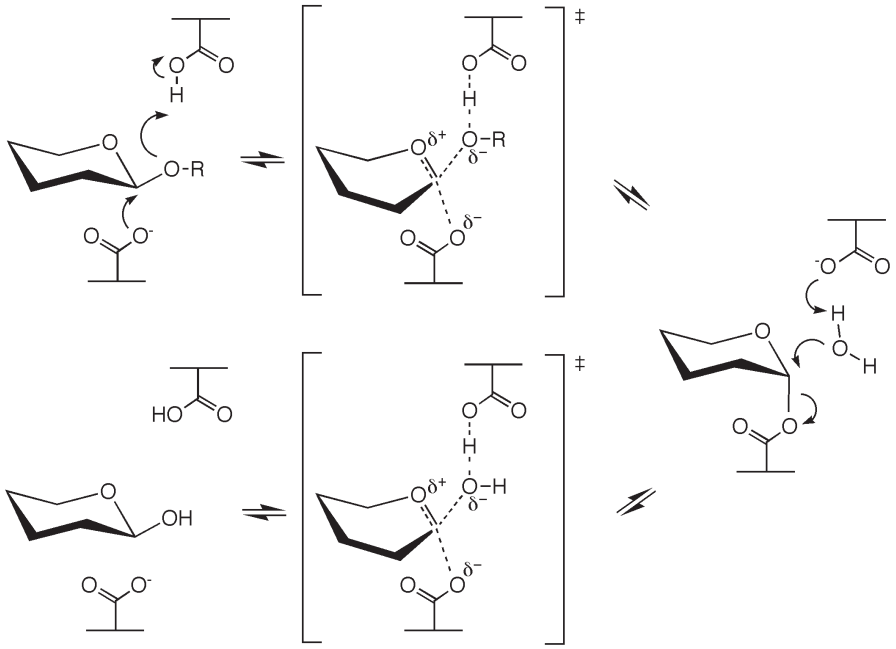

Figure 3: Schematic depiction of glycosidase retaining mechanism. On the right: covalent intermediate; in between: transition states (image by Anna S. Larsson).

The Lam16A E115S mutants and E120A mutants replaced the glutamate residues of the nucleophile and the Brønsted acid/base, respectively.

3.4 Describing Glucan Binding by Numbers

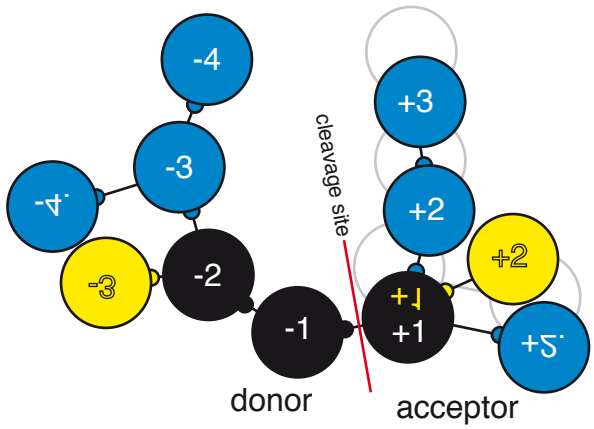

Figure 4: Subsite numbering scheme for glucose residues in Lam16A. Black: mandatory binding sites. Blue: β-1,3/(1,6)-glucans; periods denote β-1,6-branches. Yellow: $(G4G3G4)_n$ mixed linkage β-glucans. Grey circles: alternate binding site for G6G3G3G. Inverted numbers mean that the β-anomer is pointing away from the viewer.

Lam16A is an endolytic glycoside hydrolase, meaning that it can cleave its glucan substrate in the middle, instead of solely at the ends. A nomenclature has been devised to describe subsites where glycones (glucose residues in this case) bind (Davies et al. [1997]). The convention is to draw the glyconic end on the left, binding in the donor site, and the aglyconic end on the right, binding in the acceptor site. Subsites are numbered as integers emanating from the glycosidic bond: negative integers in the donor site and as positive integers in the acceptor site. This numbering system is a neat way to describe unbranched oligosaccharide binding in unique positions along a catalytic cleft.

When branches are involved, then the naming of subsites becomes trickier (Fig 4) and there is no consensus to date on how to best name branches. In our papers, we have used n_{branch} to denote β-1,6 branches, where n denotes the integer counting from the cleavage site.

Another peculiarity for a promiscuous glycosidase is that the glucose residues will bind to different subsites in the enzyme as their glycosidic bond patterns diverge. In Lam16A, only subsites -1, -2 and $+1$ are the same for mixed-linkage lichenan and β-1,3 glucan. These subsites can be considered mandatory subsites. Beyond these subsites the name of the ligand or substrate needs to be specified. Despite the variety of binding, the positioning of the glucose residues is nevertheless always distinct and discrete.

3.5 Obligatory β-1,3-bond Between −1 and −2 Subsites

All Lam16A enzyme-ligand complexes so far studied show alignment at the -2 and -1 subsites. Two hydrophobic platforms, Trp103 and Trp110, seem to be responsible for this alignment. Arg73 holds a hydroxyl in place in the -2 subsite. Other than that, there are also important water-mediated hydrogen bonds (Paper II).

3.6 Conserved Waters at the −1 Subsite

Conserved waters are those within the set of known structures [10] which do not move in the presence of ligand. Conserved waters are more tightly bound than transient waters (Barillari et al. [2007]) and can be considered as extensions of the peptide chain (Ball [2008]). The conserved waters in the catalytic cleft of Lam16A act as intermediaries for hydrogen bonding between glucan hydroxyls and amino acids (Fig 6d). Three such water molecules line a cavity at the -1 subsite and hydrogen-bond to O4 and O6 hydroxyls of the -1 glucose residue. From an evolvability perspective, conserved waters such as these could make it easier for an enzyme to adapt to new substrate, as the water molecules can be shed more easily than the side-chain of an amino acid.

3.7 Congruent Glucose Residues at the +1 Subsite

The glycosidic O-atom must be β-anomeric for the glycosidic bond to be hydrolyzed. Both a β-1,4 bond and a β-1,3 bond between the -1 and $+1$ subsites are able to span the catalytic site and allow the aglyconic (acceptor) end to extend out of the cleft. This is evident when superposing lichenan and laminarin complexes. The constraints of the $+1$ subsite force the glucose residue to be roughly congruent (Fig 5). This way, Lam16A can hy-

[10] This thesis contains 10 Lam16A structures.

drolyze either a β-1,4 bond or a β-1,3 bond, provided the bond is preceded by a β-1,3-bond between the −2 and −1 subsites.

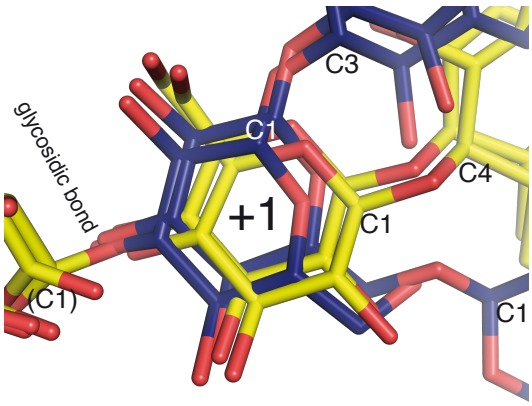

Figure 5: Glucose residues from mixed-linkage lichenan (yellow) and laminarin (blue) face opposite at the +1 subsite, but are parallel and congruent. Numbered carbons are to help orient the reader. Labeled glycosidic bond is at cleavage site.

4 Summary of the Present Investigation

4.1 Paper I: The Ligand-less Lam16A

Laminarinase Lam16A from *Phanerochaete chrysosporium*, recombinantly expressed in *Pichia pastoris*, was crystallized and the structure was solved at 1.34 Å resolution using native sulfur SAD X-ray crystallography. It is the first structure of a promiscuous 1,3(4)-β-D-glucanase from glycoside hydrolase family 16 (GH16). Lam16A is an extracellular protein that is expressed when laminarin is used as the sole carbon source for *Phanerochaete chrysosporium*. The protein folds into a curved β-sandwich, homologous to that of other known GH16 enzyme structures.

This first paper is only concerned with the structure of the apo-enzyme. It contains some topics which are not covered in later papers. For example, there is the N-glycosylation structure from the glycosylated enzyme. Also, much effort was put into structural comparisons with structurally related enzymes.

4.2 Paper II: Reaction Products as Ligands

The 1,3(4)-β-D-glucanases of glycoside hydrolase family 16 provide useful examples of promiscuous protein-carbohydrate interactions. Here we report the X-ray structures of the 1,3(4)-β-D-glucanase *Phanerochaete chrysosporium* Laminarinase 16A in complex with β-glucan products from laminarin (1.6 Å) and lichenin (1.1 Å) hydrolysis. The G6G3G3G glucan, in complex with the enzyme, showed a β-1,6 branch in the acceptor site. The G4G3G ligand-protein complex showed that there was no room for a β-1,6 branch in the -1 or -2 subsites; furthermore, the distorted residue in the -1 subsite and the glucose in the -2 subsite required a β-1,3 bond between them. These are the first X-ray crystal structures of any 1,3(4)-β-D-glucanase in complex with glucan products. They provide details of both substrate and product binding in support of earlier enzymatic evidence.

This is the first of the enzyme-ligand complex papers. It contains the only example of a co-crystalized ligand (G4G3G in wild-type). The rest are all enzymes soaked with ligand. This may be a reason why Asn162 moves and hydrogen-bonds with a glucan hydroxyl in subsite -1 in the Lam16A/G4G3G complex (Fig 6).

4.3 Paper III: Synthesis of Cyclic L7

Glycosynthases are precise molecular instruments for making specifically linked oligosaccharides. X-ray crystallography screening of ligands bound

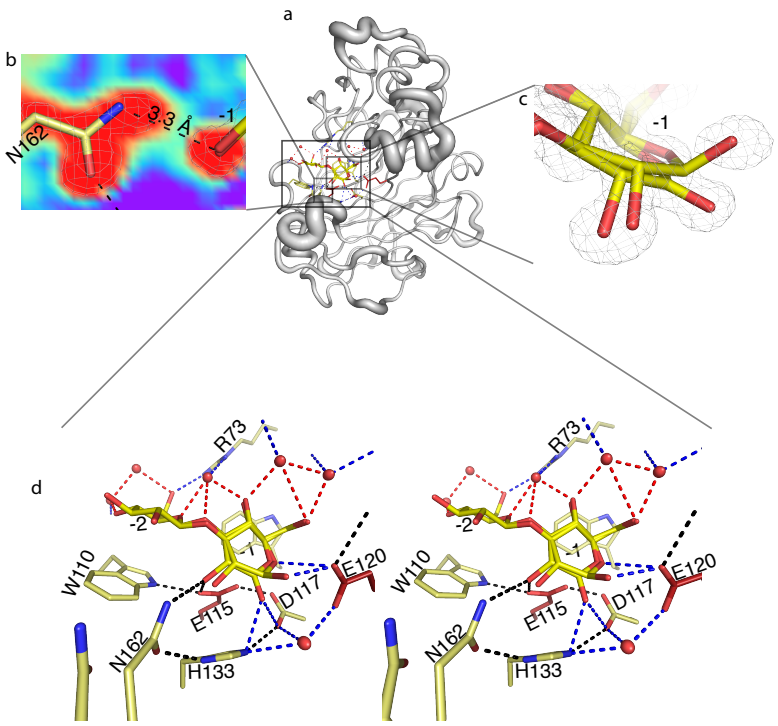

Figure 6: The Lam16A P3 structure with a well-defined disaccharide occupying subsites −2 and −1. (a) Electron density (0.35 e Å^{-3}) at subsite −1. (b) Asn162 Nδ2 displays elongated electron density (Maia et al. [2005]) towards O2 of the −1glc residue, suggesting protonation. (c) Stereo image of protein/ligand interactions; red dotted lines: H-bonds to conserved waters; black: H-bond network from Asn162 "swung-in" conformer to nucleophile Glu115; blue: H-bonds between amino acids and ligand or between amino acids and conserved waters.

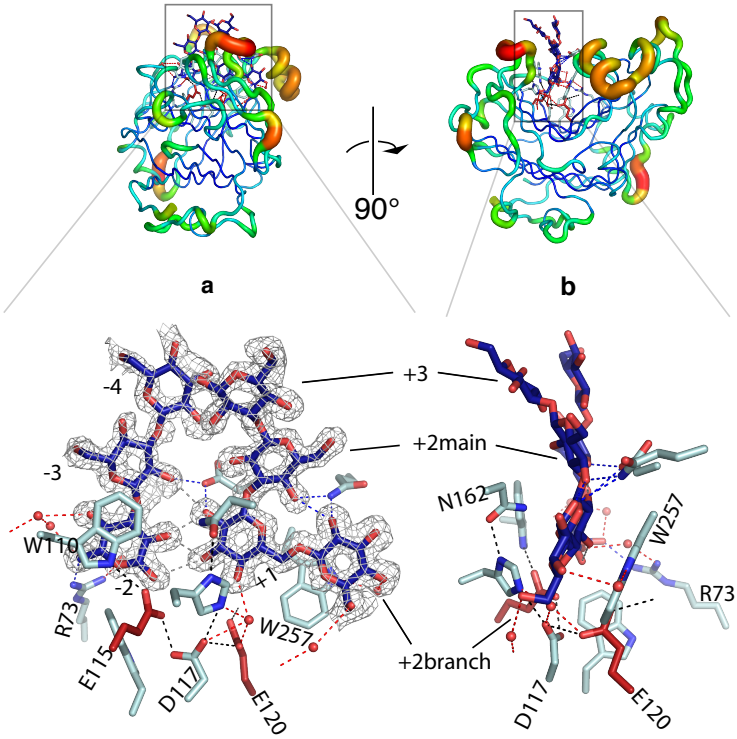

Figure 7: The Lam16A P4 structure with two ligand molecules, occupying both the donor and acceptor sites of the substrate-binding cleft, viewed (a) across and (b) along the binding cleft. Electron density (0.35 e $Å^{-3}$) reveals three glucose residues (−4, −3 and −2) in the donor site, and four residues in the acceptor site (+1, +2main, +2branch, and +3). The catalytic residues Glu115 (nucleophile) and Glu120 (acid/base) are shown in red, other selected residues around the catalytic centre with light-blue carbon atoms, and conserved waters as red spheres. Red dotted lines: H-bonds to conserved waters; black: H-bonds from and including the catalytic triad; blue: H-bonds between amino acids and ligand or between amino acids and conserved waters; gray: ligand-to-ligand H-bonds.

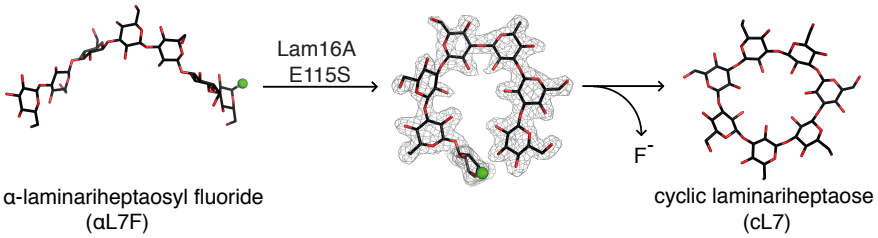

Figure 8: A glycosynthase reaction is performed using the nucleophile mutant E115S Lam16A with an α-fluorinated laminariheptaose. A cyclical β-glucan is formed.

to the 1,3(4)-β-D-glucanase nucleophile mutant E115S of Lam16A showed that laminariheptaose (L7) bound in an arch with the reducing and non-reducing ends occupying either side of the catalytic cleft of the enzyme. The X-ray structure of Lam16A E115S in complex with α-laminariheptaosyl fluoride (αL7F) revealed how αL7F could make a nucleophilic attack upon itself. Indeed, when Lam16A E115S was allowed to react with αL7F the major product was a cyclic β-1,3-heptaglucan (Fig 8) as shown by mass spectrometry. NMR confirmed uniquely β-1,3-linkages and no reducing end. Molecular dynamics simulations indicate that the cyclic laminariheptaose molecule is not completely planar and that torsion angles at the glycosidic linkages fluctuate between two energy minima. This is the first report of a glycosynthase that joins the reducing and non-reducing ends of a single oligosaccharide, and the first reported synthesis of cyclic β-glucan.

This is probably the article that best follows a hypothesis from beginning to end. In the validation of the product, every step is essential to ascertain the identity of the product. In the the context of theme of this thesis, this paper exemplifies the helical nature of β-1,3-glucan.

Does the cyclic product have any economic value? Maybe. For Lam16A research, it should at least be used as a test substrate, to compare the efficacy of future mutants. Transient transglycosylation products have complicated earlier enzymatic studies with Lam16A (Kawai et al. [2006]). With a cyclic substrate and a functional enzyme, the chances of transglycosylating back into the original cyclic product would seem insignificant, improving the chances of interpretable measurements.

4.4 Paper IV: Modelling Substrate Binding

Promiscuous substrate binding and characteristic oligosaccharide products are hallmarks of endohydrolytic 1,3(4)-β-D-glucanases. To visualize how different β-glucan substrates bind in the catalytic cleft of *Phanerochaete chrysosporium* Laminarinase 16A, catalytically deficient enzymes together with bound ligands were elucidated using X-ray crystallography to at least 1.50 Å resoultion. By aligning five such new crystallographic structures and an earlier structure (from Paper II), we were able to model the binding of mixed linkage β-1,3-1,4-glucan (lichenan) from subsites -3 to $+2$ and of branched β-1,3/1,6-glucan from subsites -5 to $+3$. The models are congruent at the -1 and -2 subsites, hexose-ring-congruent at the $+1$ subsite, while diverging at peripheral subsites according to the substrate linkage pattern.

Paper IV is a sequel to Paper II, adding more structural data and confirming our prediction about the position of $+1$ glucose in the acceptor site. Also, the alternate binding position of the G6G3G3G tetraglucan in the acceptor site (Fig 7) was again observed for the Lam16A/ E120A complex (see grey circles, Fig 4). This may be due to hydrogen bonding between the hydroxyls of the $+1$ glucose residue and Asp256 together with hydrophobic interactions and hydrogen-bonding between the $+2$ branch and Trp257. These residues, Trp257 and Asp256, could be helping the G6G3G3G ligand inhibit the function of Lam16A. Asn162, on the opposite side of the catalytic cleft with respect to Asp256, may have a similar role with the G4G3G ligand (Fig 6).

5 Conclusions

Lam16A has sometimes been dubbed a "non-specific" β-1,3(4) glycosidase (Strohmeier et al. [2004]). This epithet can be misleading, because Lam16A is indeed specific - but for more than one substrate. This phenomenon is known as promiscuity[11] (Khersonsky et al. [2006], Aharoni et al. [2005], Farinas et al. [2001]). Binding promiscuity is when an enzyme binds different substrates differently. Lam16A exhibits binding promiscuity because it binds both mixed-linkage β-1,4-1,3 glucans and branched and unbranched β-1,3 glucans. While it may be standard practice to name an enzyme according to the substrate that yields the highest k_{cat}/K_M values, this classification system may exclude the possibilities of promiscuous binding and of ongoing evolutionary processes.

Catalytic promiscuity is when an enzyme makes use of different catalytic mechanisms, resulting in different products. The mechanism for wild type Lam16A is the hydrolysis of the glycosidic bond. It could be argued, however, that because Lam16A hydrolyzes both β-1,3 and β-1,4 bonds, then it is *catalytically* promiscuous as well.

The recruitment hypothesis (or patchwork hypothesis) Jensen [1976] postulates that primitive enzymes possess a broad substrate specificity and that gene duplication has allowed for increased specialization and efficiency. However, an optimization toward maximum k_{cat}/K_M for a narrowly defined substrate may not always be practical. The selective pressure may be toward a more generalist/promiscuous enzyme that is able to maintain its original functionality while adapting to new conditions. *In vitro* experiments have shown that enzymes can evolve without the need to revert to gene duplicates (Khersonsky et al. [2006], Farinas et al. [2001]).

Furthermore, promiscuous enzymes would make better candidates for directed evolution experiments (O'Loughlin et al. [2006]). Promiscuity can also be preferable in oligosaccharide synthesis (Hancock et al. [2005]). Already we have an indication of the latter in Paper III. If binding promiscuity is a sign of an evolvable enzyme, then it may be possible to find closely related, functioning mutants of the same enzyme in different specimens of the same species. After all, the *Phanerochaete chrysosporium* genome (Wymelenberg et al. [2006], Martinez et al. [2004]) is from a single organism. Intraspecial variance of fungi could be studied by sequencing more specimens from different environments in the wild[12].

[11] The terms multi-specificity and cross-reactivity have also been used

[12] Already, mycologists have found high diversity within *Aspergillus* species (Geiser et al. [1998]), for example.

6 Sammanfattning på svenska

Glykosidhydrolaset Lam16A från vitrötesvampen *Phanerochaete chrysosporium* kan binda och klyva (genom hydrolys) två olika sorters betaglukankedjor. Vi har gjort röntgenkristallografiska experiment med vildtypsenzym i komplex med substratnedbrytningsprodukterna G6G3G3G[13] (från laminarin) och G4G3G (från lichenan) samt med längre beta-1,3-glukaner. Totalt tio olika strukturer i denna avhandling, för att bättre förstå hur detta enzym binder de substrat som det hydrolyserar. Först strukturbestämdes Lam16A utan ligand (papper I). Därefter två strukturer med vildtyp och vardera ligand (papper II). När vi kristalliserade muterat Lam16A med heptaglukan såg vi hur den band i en bågform, och båda ändorna möttes (papper III). Detta ledde till syntes av en cyklisk betaglukan (papper III). Till sist gjorde vi modeller av inbundet substrat med hjälp av ligander bundna i muterat Lam16A (papper IV).

[13] G är glukos och siffran visar hur den binder i kedjan.

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Acknowledgements

First of all, thank you Jerry Ståhlberg and Mats Sandgren for giving me the opportunity to work with X-ray crystallography. Mats, thank you for giving me a temporary position in your lab, a first step toward my PhD studies. Thanks for getting me a computer that worked, teaching me the details of data processing, and making sure I had all the tools I needed. Jerry, from your example I have learned the virtues of a friendly and mild demeanor. Your curiosity, perseverance and enthusiasm are contagious. Upon being asked at the first meeting with delegates from the University of Tokyo what suggestions you had on how to collaborate further, you replied that you would be sending me to Tokyo for a year. It's as if you had read my mind!

Working in Kiyohiko's and Samejima's lab at the University of Tokyo was a very good educational experience, and I will always remember it fondly. Rie Kawai, Kiyohiko Igarashi and Masahiro Samejima: without you, none of this would have been possible. Thank you Kiyo for helping me focus on what was important and helping me see the context of my work. Thank you also Shinya Fushinobu, for helpful insight on high-resolution electron densities. Thank you Sweden-Japan Foundation, for funding my stay in Tokyo.

Thank you Evalena Andersson, for your technical expertise during and after your PostDoc with Jerry. Thank you Zarah Forsberg, for picking up where the rest of us left off. I think you have produced some of the most exciting Lam 16A glucosynthase results so far - and they should be published! Anna Larsson, for working AutoSharp and ShelX. Henrik Hansson and Zarah, for helping with separation of glucans. Åke Engström, for running mass spec on our samples. Gerard Kleywegt, for being an excellent mentor, for lending me your ear, your advice and your side-office. Terese Bergfors, for your fantastic lectures, keeping the lab in order, for your advice on crystallization and on other matters. Thomas Norberg, for lending me your vacuum pump and for helping me identify the gluconolactone. Andreas Bohne-Lang and Tomas Lütteke for introducing me to carbohydrate structure. Martin Frank, for modeling cyclic β-glucan and for discovering its two energy minima. In Stockholm, Göran Widmalm and Hanna Jonsson, for producing the α-fluorinated glucans and for contributing to our project with NMR results.

Reminiscing back further, I want to thank my first real science teachers: in chemistry and math, Bruce Rinker, my first biology teacher Rosie Daggett, and my Earth science teacher in high school, Jim Sproull. Had it not been for you, I might never have learned to see beauty, patterns and adventure where others see only a marsh. Thank you for demanding and

expecting the best of us.

If anyone did not get mentioned in a professional capacity, please notify me as soon as possible so I can compensate with a beer or a public thank you of some sort. For gratitude of a more personal nature, please accept my handwritten praise below: