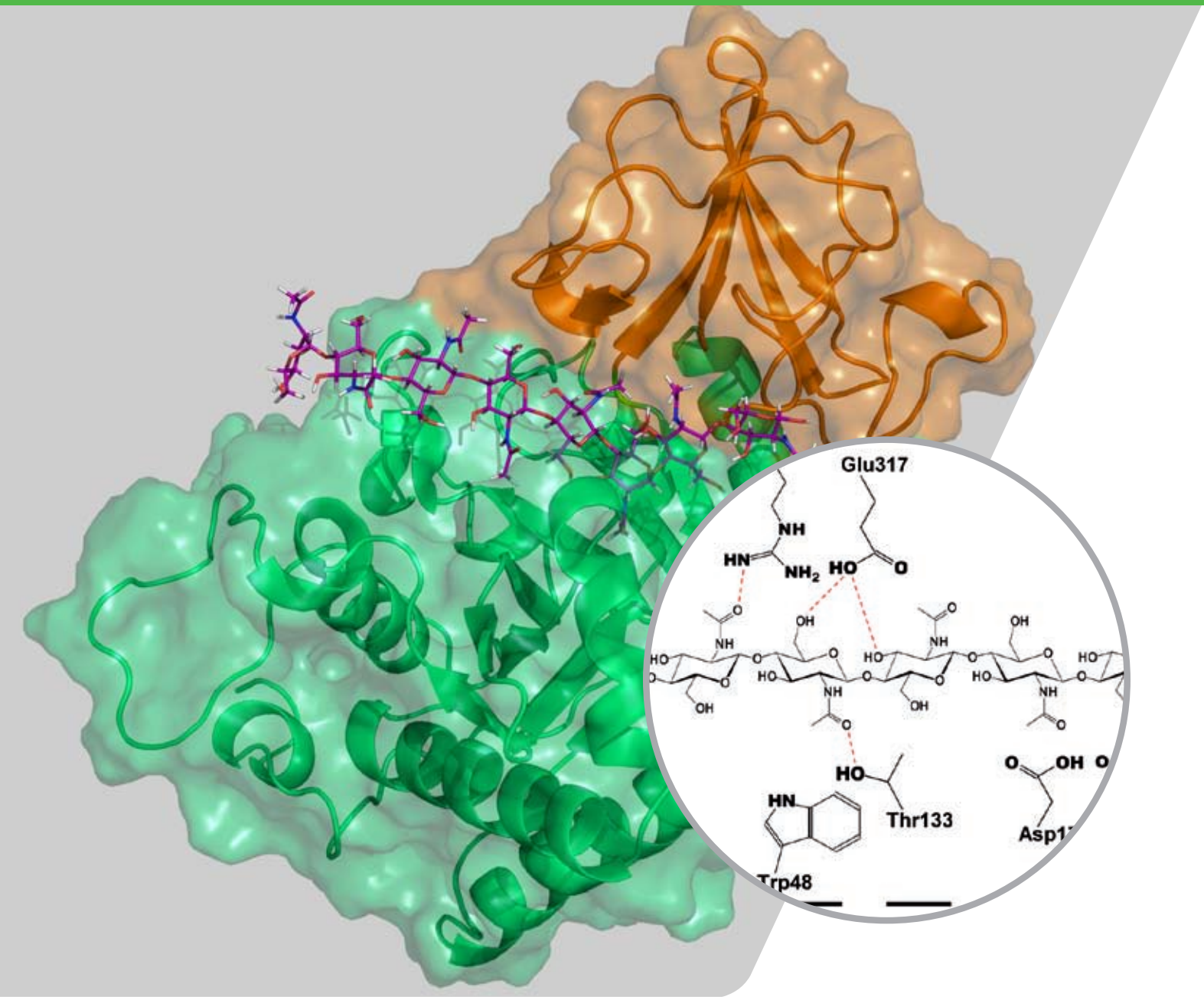
VTT PUBLICATIONS 735



Michael Lienemann

Characterisation and engineering of protein–carbohydrate interactions

VTT PUBLICATIONS 735

Characterisation and engineering of protein–carbohydrate interactions

Michael Lienemann

ISBN 978-951-38-7391-2 (soft back ed.)
ISSN 1235-0621 (soft back ed.)

ISBN 978-951-38-7392-9 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)

D93

JULKAISIJA – UTGIVARE – PUBLISHER

VTT, Vuorimiehentie 5, PL 1000, 02044 VTT
puh. vaihde 020 722 111, faksi 020 722 4374

VTT, Bergsmansvägen 5, PB 1000, 02044 VTT
tel. växel 020 722 111, fax 020 722 4374

VTT Technical Research Centre of Finland, Vuorimiehentie 5, P.O. Box 1000, FI-02044 VTT, Finland
phone internat. +358 20 722 111, fax + 358 20 722 4374

Technical editing Leena Ukskoski

Text preparing Tarja Haapalainen

Edita Prima Oy, Helsinki 2010

Characterisation and engineering of protein–carbohydrate interactions

Von der Fakultät Energie-, Verfahrens- und Biotechnik der Universität Stuttgart zur Erlangung der Würde eines Doktors der Naturwissenschaften (Dr. rer. nat) genehmigte Abhandlung

vorgelegt von

Michael Lienemann

aus Bielefeld

Hauptberichter: **Prof. Dr. Georg A. Sprenger**
Mitberichter: **Prof. Dr. Vlada B. Urlacher**

Tag der mündlichen Prüfung: 15. März 2010

Institut für Mikrobiologie der Universität Stuttgart

2010

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Espoo in Finnland, den 26.4.2010

_____

(Michael Lienemann)

Michael Lienemann. Characterisation and engineering of protein–carbohydrate interactions [Charakterisierung und Engineering von Protein–Kohlenhydrat-Wechselwirkungen]. Espoo 2010. VTT Publications 735. 90 p. + app. 30 p.

Keywords chitinase, mutagenesis, protein–carbohydrate interaction, Trichoderma harzianum, surface plasmon resonance, self-assembled monolayer, AFM force spectroscopy, wheat germ agglutinin

Abstract

Protein−carbohydrate interactions play a crucial role in many biological processes such as cell−cell recognition and receptor−ligand interactions and catalysis. This thesis explores the possibilities of engineering the protein–carbohydrate interactions between carbohydrate-binding proteins and their ligands. Protein–carbohydrate interactions were characterised using different techniques, such as atomic force microscopy (AFM) and surface plasmon resonance (SPR).

Two different chitinoligosaccharide-binding proteins were used in the presented experiments, i.e. the plant lectin wheat germ agglutinin (WGA) and a neolectin created by inactivation of the fungal chitin-degrading enzyme Chit42. The structures of WGA and Chit42, which were available as X-ray diffraction data and a homology model, respectively, revealed differences between their binding site architectures. The interaction of monomeric and polymeric *N*-acetyl-D-glucosamine (GlcNAc) with WGA, that normally binds chitinoligosaccharides on cell surfaces, was investigated with nanomechanical force measurements using AFM. These measurements aimed at determining the effect of ligand length and binding site clustering (multivalent binding) on binding strength between the protein and the carbohydrate ligand. Here, the GlcNAc ligand was presented at the surface of a planar self-assembled monolayer (SAM) of neoglycoconjugates and in a polymeric form as chitin beads. In adsorption measurements using the quartz crystal microbalance (QCM) it was shown that alkanethiols adsorb rapidly to a gold surface, covering it completely within a few minutes as an SAM. In AFM force measurements, GlcNAc-specific binding events were detected with a WGA-modified probe on a GlcNAc-neoglycoconjugate SAM at bond rupture forces of 47 ± 15 pN. When this experiment was repeated on a polymeric substrate, manyfold higher forces were

detected. This indicated a high increase of affinity with additional binding subsites which were able to interact with the chitinous ligand. SPR measurements confirmed that WGA has higher affinity towards the immobilized GlcNAc-neoglycoconjugate SAM than towards the soluble free monosaccharide providing evidence of a significant affinity increase as a result of binding site cooperativity. Furthermore, two different SAM formats have been tested for their suitability for studying protein–carbohydrate interactions. Here, non-specific adhesion was identified as a critical factor that was mainly related to the hydrophobic parts of the neoglycoconjugates and could be attenuated by the introduction of a tetraethylene glycol spacer into the neoglycoconjugate. The binding measurements on neoglycoconjugate SAMs demonstrated that this type of carbohydrate presentation is a useful reference for interactions on naturally occurring two-dimensional glycan arrays and demonstrated that the minimization of non-specific adhesion of proteins is often required in order to obtain meaningful binding data.

In addition to WGA–carbohydrate interactions this thesis also deals with the carbohydrate binding cleft of the glycoside hydrolase *Trichoderma harzianum* chitinase Chit42, which in the wild-type form, solubilises polymeric crystalline chitin (composed of *β*-4 linked GlcNAc units). Nine different catalytically inactive Chit42 variants were created, carrying amino acid alterations along the binding site cleft (at sugar-binding subsites -4 to +2). These Chit42 variants were characterised with regard to their affinity towards chitinous and non-chitinous oligosaccharides by SPR. As a result, hydrogen bonding between subsites -2/-3 and particularly stacking interactions by tryptophanes at subsites -3 and +2 were seen to be very important for the carbohydrate binding. The exchange of the corresponding amino acids did not cause a change of binding specificity, although the selective binding of GlcNβ-4$(GlcNAc)_4$ could be improved by providing a counter charge through an amino acid substitution at subsite -3, replacing threonine with aspartic acid. In addition the introduction of glutamine and particularly an asparagine residue at this position appeared to broaden the substrate preference towards Galβ-4$(GlcNAc)_4$, and the site was thereby implicated as a binding groove hot spot for creating binding proteins, or hydrolytic enzymes with novel substrate specificity towards e.g. structurally related and medically significant oligosaccharides. Analysis of the Chit42 variants with modified active sites showed how the binding selectivity and affinity of neolectins can be engineered and may thereby function as a model for further neolectins and glycoside hydrolases with new ligand and substrate specificities, respectively.

Michael Lienemann. Characterisation and engineering of protein–carbohydrate interactions [Charakterisierung und Engineering von Protein–Kohlenhydrat-Wechselwirkungen]. Espoo 2010. VTT Publications 735. 90 S. + App. 30 S.

Stichwörter chitinase, mutagenesis, protein–carbohydrate interaction, Trichoderma harzianum, surface plasmon resonance, self-assembled monolayer, AFM force spectroscopy, wheat germ agglutinin

Zusammenfassung

Protein−Kohlenhydrat-Wechselwirkungen sind von entscheidender Bedeutung in zahlreichen biologischen Prozessen, wie der gegenseitigen Erkennung von Zellen und Rezeptor–Ligand-Wechselwirkungen sowie bei der Katalyse. In dieser Doktorarbeit werden die Möglichkeiten erforscht Protein–Kohlenhydrat-Wechselwirkungen zwischen kohlenhydratbindenden Proteinen und ihren Liganden mittels Protein Engineering gezielt zu manipulieren. Die untersuchten Wechselwirkungen wurden mit Hilfe von verschiedenen Techniken zur Messung von Bindungsprozessen wie Rasterkraftmikroskopie (AFM) und Oberflächenplasmonresonanz (SPR) charakterisiert.

In den dargestellten Experimenten wurden zwei Chitinoligosaccharid-bindende Proteine verwendet um die Wechselwirkungen zwischen Proteinen und Kohlenhydraten zu untersuchen. Hierbei handelte es sich um Weizenkeimagglutinin (WGA) und ein Neolektin, welches durch Inaktivierung des chitinabbauenden fungalen Enzyms Chit42 erzeugt wurde. Bei einem Vergleich der Strukturen von WGA und Chit42, welche auf Röntgenstrahlstreuungsmessungen beziehungsweise auf einem Homologiemodell basieren, wurde deutlich, dass sich beide Proteine im Aufbau ihrer Bindungstaschen unterscheiden. Mit Hilfe von nanomechanischer AFM-Kraftmessungen wurden die Wechselwirkungen zwischen monomerem sowie polymerem *N*-Acetyl-D-Glukosamin (GlcNAc) und WGA untersucht, welches normalerweise an auf Zelloberflächen befindliche Chitinoligosaccharide bindet. Das Ziel dieser Messungen war die Bestimmung des Einflusses von Ligandenlänge und Bindungstaschenzusammenlagerung auf die Stärke mit der das Protein den Kohlenhydratliganden bindet. Hierbei wurde der GlcNAc-Ligand als Monomer an der Oberfläche einer planaren selbstangeordneten Monoschicht (SAM) von Neoglykokonjugaten und in polymerer Form

als Chitinkügelchen eingesetzt. Mittels Adsorptionsmessungen mit der Schwingquarzmikrowaage (QCM) wurde gezeigt, dass Alkanthiole sehr schnell an eine Goldoberfläche binden und diese innerhalb von wenigen Minuten vollständig als SAM bedecken. In den dargestellten AFM-Kraftspektroskopieexperimenten wurden mit Hilfe einer WGA-modifizierten Sonde GlcNAc-spezifische Bindungsereignisse bei einer Bindungsabrisskraft von 47 ± 15 pN an einer GlcNAc-Neoglykokonjugat-SAM aufgezeichnet. Während einer Wiederholung dieses Experiments auf einem Polymersubstrat wurden Kräfte gemessen, welche diese auf der GlcNAc-Neoglykokonjugat-SAM gemessene Bindungsabrisskraft um ein Vielfaches überstiegen. Dies wies auf einen starken Anstieg der Bindungsaffinität unter Beteiligung zusätzlicher Bereiche der Lektinbindungstaschen hin, die mit dem Chitinliganden in Wechselwirkung treten konnten. SPR-Messungen zeigten, dass WGA auf Neoglykokonjugat-SAMs immobilisierte GlcNAc-Monosaccharide stärker band als das gelöste Kohlenhydrat was einen bedeutenden Affinitätszuwachs aufgrund von Bindungstaschenkooperativität anzeigte. Zusätzlich hierzu wurden zwei weitere SAM-Formate auf ihre Eignung zur Untersuchung von Protein–Kohlenhydrat-Wechselwirkungen hin untersucht. Hierbei wurde nicht-spezifische Adhäsion als kritischer Faktor identifiziert, die hauptsächlich auf die hydrophoben Teile der Neoglykokonjugate zurückzuführen war und durch die Einführung von Tetraethylenglykolelementen abgemildert werden konnte. In den Bindungsstudien mit Neoglykokonjugat-SAMs wurde gezeigt, dass diese Art von Kohlenhydratpräsentation eine nützliche Referenz für Wechselwirkungen darstellt, die an natürlich auftretenden zweidimensionalen Glykananordnungen stattfinden. Außerdem wurde demonstriert, dass hierbei eine Minimierung von nicht-spezifischer Proteinadhäsion nötig ist um aussagekräftige Bindungsdaten zu erhalten.

Neben WGA–Kohlenhydrat-Wechselwirkungen wird in dieser Doktorarbeit auch die Substratbindungstasche der Glykosylhydrolase *Trichoderma harzianum* Chitinase Chit42 untersucht, welche in ihrer unveränderten Form polymeres kristallines Chitin (aufgebaut aus β-4-verknüpften GlcNAc-Einheiten) solubilisiert. Neun verschiedene katalytisch inaktive Chit42-Varianten wurden hergestellt, welche Modifikationen entlang der Substratbindungstasche zwischen den kohlenhydratbindenden Bindungstaschenuntereinheiten -4 und +2 trugen. Diese Chit42-Varianten wurden durch Bestimmung ihrer Affinität zu chitinartigen und nicht-chitinartigen Oligosacchariden mittels SPR charakterisiert. Hierbei wurde gezeigt, dass Wasserstoffbrückenbindungen und aromatische Wechselwirkungen, die von polaren Gruppen in den Bindungstaschenuntereinheiten -3 und -2

bzw. von Tryptophanresten in den Bindungstaschenuntereinheiten -3 and +2 ausgehen entscheidend zur Bindung des Kohlenhydratliganden beitragen. Der Austausch der betreffenden Aminosäuren hatte keine messbare Auswirkung auf die Chi42-Bindungsspezifität. Eine weitere Veränderung der Bindungstaschenuntereinheit -3 durch einen Austausch von Threonin gegen die saure Aminosäure Asparaginsäure führte jedoch zu einer Erhöhung der Bindungsselektivität für das Pentasaccharid GlcNβ-4$(GlcNAc)_4$, was auf eine Paarung entgegengesetzter Ladungen zurückgeführt wurde. Außerdem konnte eine Erweiterung des Substratspektrums zu Galβ-4$(GlcNAc)_4$ durch den Austausch des Threonins mit Glutamin und Asparagin erreicht werden. Diese Region in der Substratbindungstasche wurde somit als “Hot spot” für die Erzeugung von Kohlenhydratbindungsporteinen oder Glykosidhydrolasen mit neuartigen Ligand- bzw. Substratspezifitäten für z.B. strukturell verwandte und medizinisch bedeutsame Oligosaccharide identifiziert. Die Untersuchung der Chit42-Varianten mit modifizierter Bindungstasche hat gezeigt wie die Bindungsselektivität und Affinität von (Neo-)Lektinen gezielt verändert werden kann und könnte daher als Vorlage in der Entwicklung weiterer Neolektine und Glykosylhydrolasen mit neuen Ligand- bzw. Substratspezifitäten dienen.

Preface and acknowledgements

The experiments presented in this work were conducted at VTT Technical Research Centre of Finland during the period January 2006 to May 2009. I thank Prof. Juha Ahvenainen and Vice President Biotechnology Prof. Anu Kaukovirta-Norja for providing excellent research facilities at VTT. Financial support from the European Union (MRTN-CT-2004-005645), VTT and the German Academic Exchange Service (DAAD) is gratefully acknowledged.

I am very grateful to Professor Georg Sprenger for agreeing to supervise my doctoral thesis and to Dr. Anu Koivula for inviting me to her research team and providing the subject of my thesis. My supervisors Drs. Anu Koivula, Arja Paananen and Harry Boer deserve my deepest gratitude for guiding me throughout the work at VTT and providing me access to excellent research facilities within and outside the VTT organisation. Especially I would like to acknowledge their efforts to revise the manuscripts and reports related to this thesis, despite their tight schedules which often required working during weekends and late into the night. I am very grateful to Drs. Arja Paananen and Harry Boer for introducing me to biochemical and biophysical techniques, their readiness for discussions, their constant encouragement, and friendship. I would like to thank Arja Kiema for sharing her extensive knowledge on techniques of protein and molecular biology with me and for creating a friendly atmosphere in the lab. Dr. Kristiina Kruus deserves my gratitude for her trust in me in management of the team's SPR equipment and for creating a pleasant atmosphere in her team.

I would like to thank all my co-authors. Dr. Jesús Maria de La Fuente, Isabel García, Dr. Marco Marradi, and Professor Soledad Penadés (IIQ-CSIC, Sevilla), Dr. Martina Lahmann and Viviane Fourniere (Bangor University, Bangor) and Sami Hallila, Dr. Eric Samain and Professor Sylvain Cottaz (CERMAV, Grenoble) participated in helpful discussions on self-assembled monolayers and diligently prepared the functionalised alkanethiols which were studied in this thesis.

I am grateful to my office colleague Sanni Voutilainen for her assistance in resolving organisational and language-related issues and being available for discussions on topics of science and everyday life. Dr. Peter Richard, Tuija Teerinen, Dr. Markus Linder, Dr. Geza Szilvay, Tuomas Kulomaa and Katri Kurppa (VTT Espoo) deserve my gratitude for creating a friendly atmosphere at VTT and enthusiastically participating in discussions on the experiments of this thesis.

All past and present members of the VTT groups Development of Industrial Enzymes, Molecular Sensors, Immunotechnology and Molecular Diagnostics are thanked for their amicable collaboration. Oili Lappalainen is thanked for providing me access to numerous important articles.

I would like to thank my parents and grandparents as well as the family of my fiancé and friends for supporting me during my studies in Finland and for providing me with regular language lessons, company and the important fun behind the scenes during hard times. I would like to thank my friend Andrew James and Michael Bailey for revising the language of this thesis. My fiancée Taru deserves my special thanks for her understanding, support, love and efforts to drag me out of the lab once in a while to explore the world outside VTT.

Contents

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

Abbreviations

AFM	Atomic force microscopy
CBM	Carbohydrate binding module
CD	Circular dichroism
Chit42	Chitinase 42
FC	Flow cell
GlcNAc	*N*-Acetyl-D-glucosamine
ITC	Isothermal titration calorimetry
NMR	Nuclear magnetic resonance
NTA	Nitrilotriacetic acid
QCM-D	Quartz crystal microbalance with dissipation monitoring
R_{Eq}	Equilibrium response
RU	Response unit
SAM	Self-assembled monolayer
SPM	Scanning probe microscopy
SPR	Surface plasmon resonance
STM	Scanning tunnelling microscopy
tEG	Tetraethylene glycol
WGA	Wheat germ agglutinin

1. Introduction

Carbohydrates, or saccharides, are highly abundant in nature, occurring in a wide variety of sizes and shapes. The shortest carbohydrates are the soluble monosaccharides which serve as energy source in catabolic reactions and are the starting material of anabolic pathways such as the pentose phosphate cycle (Stryer 1999) and glycosylation reactions (Freeze and Elbein 2008). Oligosaccharides and short polysaccharides, on the other hand, often play a role in recognition events where they are presented as protein or lipid conjugates and interact with specific proteins (Sharon and Lis 2004, Varki and Sharon 2008). In oligosaccharide recognition, nature exploits the possibility of creating an immense number of distinct structures with a minimum of conjugation effort. For example, by combining three different monomer units of nucleic or amino acids only six distinguishable molecules can be made, whereas over 1000 different structures can be generated from three different monosaccharides (Davis and Wareham 1999). Apart from functioning as a recognition signal, conjugated carbohydrates can shield certain functions of a conjugated protein, increase its rigidity and change its protease resistance and stability or quaternary structure due to their conformational freedom (Dwek 1996). Long polysaccharides are usually less complex than recognition-related oligo- and polysaccharides, generally consisting of a single monosaccharide unit or a repeated short oligosaccharide. These long polymers function either as means of energy storage such as starch (Buleon *et al.* 1998) or as structural elements as for example cellulose (Cosgrove 2005), and are very common in nature; cellulose is the most abundant organic polymer on earth (Klemm *et al.* 2005).

Protein–carbohydrate interactions are important in many essential biological processes such as signalling, recognition and catalysis (for review see Sharon and Lis 2004 and Davies *et al.* 2005). Catalytically inactive proteins involved in such events can be classified as lectins, antibodies or carbohydrate-binding

modules (CBMs), and have been associated with important biological processes such as attachment of the influenza virus to sialic acids on the surface of human cells through haemagglutinin (Gottschalk 1952), infection of the urinary tract by *Escherichia coli* strains involving a mannose-binding surface lectin (Aronson *et al.* 1979) and targeting of hydrolases from the rough endoplasmic reticulum to lysosomes through mannose-6-phosphate-specific receptors (Hoflack and Kornfeld 1985). Carbohydrate-active enzymes, on the other hand, fulfil important roles in metabolism and combine the ability to transiently bind carbohydrates and catalyse various reactions such as hydrolysis and transfer of a glycosidic bond (glycoside hydrolases), β-elimination (polysaccharide lyases), synthesis of glycosidic bonds (glycosyltransferases) and de-*O*-acetylation of sugars (carbohydrate esterases) (Davies *et al.* 2005). These functions enable carbohydrate-active enzymes to cleave and build the glycosidic bonds of glycoconjugates, oligosaccharides and polysaccharides and therefore play an essential part in carbon turnover on earth, biological developmental processes and interaction of the cell with its environment. Despite the vast amount of already existing knowledge, there are still many challenging questions due to the complexity of the carbohydrate structures and their interactions with proteins.

1.1 Diversity of naturally occurring glycans

Oligo- and polysaccharides are jointly classified as glycans and occur naturally in a vast structural diversity. Some more complex examples are first presented that are important in recognition events. Later the larger but simpler polysaccharides will be discussed. A rationalisation of the structure and function of glycans involved in recognition has not been possible due to the complexity of this class of molecules. The difficulty in working out such a model is further increased by the fact that the role of a glycan can change during different developmental phases and that it can have different functions at the surface of different cells. In general, the functions of these conjugates can be divided into two categories: (1) those which do not require recognition by other molecules during structural and modulatory effects and (2) those that interact with glycan binding proteins from the same (intrinsic) or a foreign (extrinsic) organism in a specific manner (Varki and Lowe 2008). Intrinsic recognition occurs between two cells, on the same cell or with extracellular molecules. Extrinsic glycan binding proteins are mainly produced by microbial cells (adhesins, agglutinins or toxins) or represent a component of the immune system (e.g. plant lectins and mannose-binding protein on

macrophage cells). These two types of recognition fulfil opposing purposes as an organism-specific signal transduction and an exploitation of this system to evade the immune system and interact with host cells in a beneficial (e.g. rhizobial nodulation factors (Xie *et al.* 1995)) or a pathogenic manner. The use of new glycans will help a potential host for a pathogen to maintain an exclusive glycan code and prevent infection, which can at least partly explain the high diversity of glycans which has evolved during evolution.

Carbohydrates involved in recognition processes on cell surfaces are usually found as conjugates with lipids (glycosylphosphatidylinositol anchors, glycolipids, lipopolysaccharides) or proteins (glycoproteins) to which carbohydrates can be coupled through *N*-glycosylation to Asn residues within an Asn-X-Thr/Ser sequence or through *O*-glycosylation to *β*-hydroxyl groups of certain serines, threonines or hydroxylserines (Dwek 1996). The latter group includes proteoglycans, which carry one or more negatively charged glycosaminoglycan chains attached to a linear protein core and are hydrated under physiological conditions thereby acting as a filler between cells. An example of an unconjugated glycan is hyaluronan, which is a polymer of alternating glucosamine and glucuronic acid linked through *β*-3 and *β*-4 glycosidic bonds. This polysaccharide plays an important role in cell motility and transformation. *N*-Linked proteoglycans contain the common core saccharide Manα-6(Manα-3)Manβ-4GlcNAcβ-4GlcNAc and occur as four different types: High mannose-, complex-, hybrid-, and poly-*N*-acetyllactosamine (Figure 1 A–D). These glycans are composed of a rather limited variety of monosaccharides, but a high structural variety is obtained through variation of the monosaccharides in the outer chains and the degree of branching (from two up to five antennae). Among *O*-glycans, more than one core structure exists (at least six according to (Dwek 1996)), which can be elongated by *β*-linked galactose or glucosamine. It should be noted that proteins with a single *β*-linked GlcNAc also exist, which are exclusively found in the cytoplasm and nucleoplasm. This modification is removed and attached multiple times within the lifetime of a single protein and thereby resembles protein phosphorylation (Hart and Akimoto 2008). Some glycans contain accessible glucosamine residues, which is relevant for this study as they resemble the original substrate of Chit42, polymeric *β*-4 linked *N*-acetyl-D-glucosamine. Examples are the “i” blood group antigen consisting of *β*-linked galactose and glucosamine and Lewis blood antigens carrying fucose and sialic acid residues *α*-linked to *N*-acetyllactosamine, which can be found on *N*- and *O*-glycosylated proteins as well as some glycolipids (Stanley and Cummings 2008). In addition to protein-conjugated glycans, carbohydrates involved in

recognition can also occur as lipid conjugates in the form of glycolipids and glycosylphosphatidyl anchors and lipopolysaccharides. These three groups are distinguished on the basis of the presence of a phosphate group which is present in the two latter groups and absent in glycolipids. Glycosylphosphatidyl anchors target certain proteins to the cell wall and contain the common core pentasaccharide Manα-2Manα-6Manα-4GlcNAcα-6myo-inositol that joins an attached protein and at least two lipophilic groups (Ferguson *et al.* 2008). Lipopolysaccharides are the major constituent of the outer leaflet of the outer membrane of gram-negative bacteria and are composed of a variable *O*-specific polysaccharide, the connecting phosphorylated core polysaccharide and the membrane-associated lipid A (Madigan *et al.* 2001). Lipid A is responsible for pathogenic effects in infected mammalian hosts and contains two β-6 linked GlcNAc residues to which six fatty acids and three phosphate groups are conjugated through ester bonds (Esko *et al.* 2008). Glycolipid is a common term for glycosphingolipids and glycoglycerolipids, of which the former is far more abundant than the latter (Schnaar *et al.* 2008). In glycosphingolipids, one fatty acid is coupled to a glycan through a ceramide group, whereas in glycoglycerolipids two fatty acids are connected through diacylglycerol to a glycan. The function of glycosphingolipids is the mediation of carbohydrate-based interactions in *trans* (glycan recognition by complementary receptors on apposing membranes) and in *cis* (modulation of activity of proteins in the same plasma membrane).

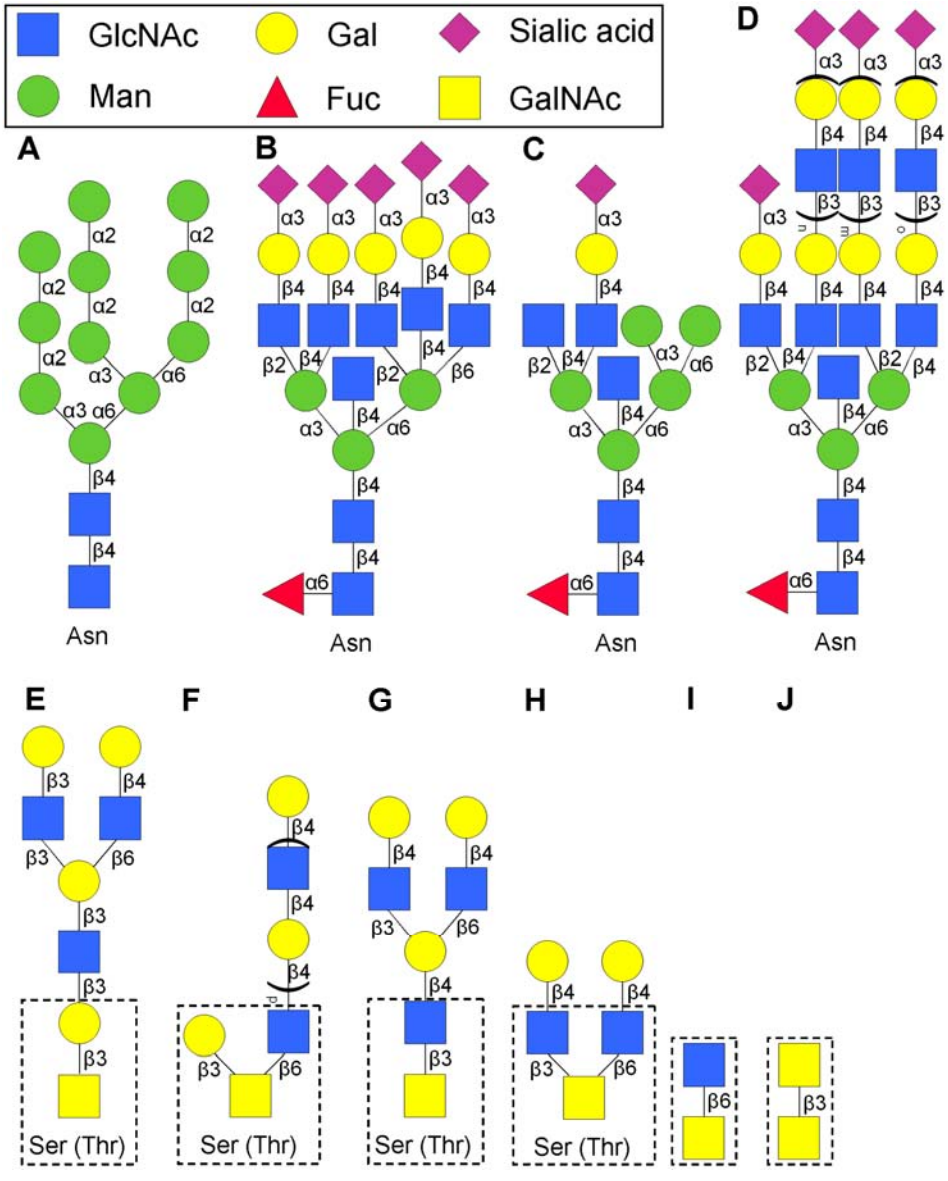


Figure 1.Types of *N*- and *O*-linked glycans. *N*-Glycans share the common core structure Man*α*-6(Man*α*-3)Man*β*-4GlcNAc*β*-4GlcNAc, are often highly branched dendrimers and occur as high-mannose (A), complex (B), hybrid (C), and poly-*N*-acetyllactosamine type (D; o > m > n). *O*-glycans have several short core structures (boxed in E–J) (Reproduced and modified with permission of the publisher after (Dwek 1996)).

Glycans with a high degree of polymerisation can be found throughout nature and serve as energy storage or lend structural properties to associated structures (see Table 1). The polysaccharides cellulose (poly-*β*-D-glucose) and chitin (poly-*β*-*N*-acetyl-D-glucosamine), for example, are the first and second most abundant natural polymers on this planet, respectively (Klemm *et al.* 2005, Rinaudo 2006). Both stabilise the shape of the producing organisms, which are in the case of cellulose plants, algae, fungi and bacteria, and in the case of chitin arthropods, fungi, nematodes and some marine life forms, including species of mollusca, coelenterata and annelida (Klemm *et al.* 2005, Araujo *et al.* 1993, Lehane 1997, Rinaudo 2006). A more complete overview of the diversity of mono-, oligo- and polysaccharides can be found elsewhere (Zamora 2005: http://www.scientificpsychic.com/fitness/carbohydrates.html, Varki *et al.* 2008).

Table 1. Composition and occurrence of selected common high molecular weight polysaccharides.

Polysaccharide	Structural element	Occurrence and function
Starch[1]	*α*4-D-glucose with *α*6-linked branches	Energy storage in plants
Glycogen[1]	*α*4-D-glucose with *α*6-linked branches	Energy storage in animals
Agarose[1,2,3]	*α*3-D-galactose-*β*4-3,6-anhydro-L-galactose	Imparts flexibility to leaves of marine algae (*Rhodophyta*)
Cellulose[1,4]	*β*4-D-glucose	Cell wall stabilisation in plants, algae, fungi and bacteria
Pectin[1]	*α*4-Galacturonic acid	Stabilisation of cell walls of plant leave and seed tissues
Inulin[2,5]	*β*1-D-fructose with terminal D-glucose	Energy storage in plants
Chitin[1]	*β*4-GlcNAc	Tissue stabilisation in various species (see main text)
Peptidoglycan[6]	*β*4-GlcNAc-*β*4-*N*-acetylmuramic acid	Stabilisation of bacterial cell wall
Hyaluronan[7]	*β*4-GlcNAc-*β*3-glucuronic acid	Streptococcus capsules; lubricant in vertebrate and invertebrate tissues
Dextran[8]	*α*6-D-glucose with few *α*2-, *α*3- and *α*4-branching in some species	Bacterial mucous layers and capsules; energy storage in yeasts and bacteria

[1] Madigan *et al.* 2001
[2] Sigma-Aldrich learning center: http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center.html
[3] Stanley 2006
[4] Klemm *et al.* 2005
[5] Zamora, 2005: http://www.scientificpsychic.com/fitness/carbohydrates.html
[6] Scheffers and Pinho 2005
[7] Hascall and Esko 2008
[8] Stryer 1999

A variety of different shapes is adopted by long polysaccharides, ranging from amorphous structures (assemblies without any recognisable order) to highly organised structures. In cellulose, for example, the polysaccharide chain forms sheets of different types (I_α, I_β, II, III, IV and V; Zugenmaier 2001) by alignment in a parallel or antiparallel orientation (Figure 2). Similarly to cellulose, crystalline chitin consists of sheets of carbohydrate chains which are arranged in an antiparallel (*α*-form) or a parallel fashion (*β*-form) (Rudall 1963). In addition, a third type of chitin crystal exists, which contains a mixture of *α*- and *β*-chitin and is called *γ*-chitin.

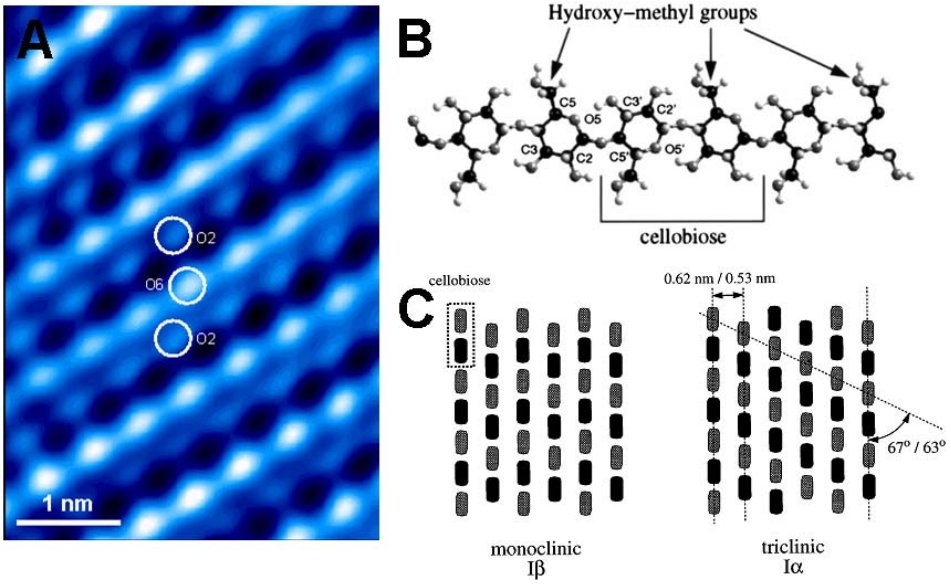


Figure 2. (**A**) Filtered topographical image of a cellulose microcrystal from the alga Valonia. The three circles indicate examples of peaks due to O2, O6, and O2. O6 is displaced slightly to the right of the two O2 peaks and is sited almost midway between them. The secondary O2 peak is lower in height than the O6 peak and is due to the C2 hydroxyl group or possibly to the combined effects of adjacent C2 and C3 hydroxyls; (**B**) A schematic diagram of the structure of cellulose. The prominent hydroxymethyl groups and the cellobiose repeat interval (1.04 nm) are indicated on the diagram; (**C**) A schematic diagram of the differences between the monoclinic and triclinic forms of cellulose I. Each rectangle represents a single glucose unit, with a pair of glucose units constituting the true crystallographic repeat, cellobiose. In the monoclinic form, the cellobiose units stagger with a shift of a quarter of the c axis period, whereas the triclinic form exhibits a diagonal shift of the same amount. Two spacings and angles are given, the first referring to the (100) face and the second to the (010) face of the triclinic crystal. Subfigure A is modified from Baker *et al.* 2000 and subfigures B and C are adapted from Baker *et al.* 1997, both with permission from the publisher.

1.2 Carbohydrate-binding proteins

1.2.1 Carbohydrate-active enzymes

Enzymes classified as carbohydrate-active comprise glycoside hydrolases, glycosyltransferases, polysaccharide lyases and carbohydrate esterases, all of which display a wide variety of protein scaffolds and are essential in the formation, modification and breakdown of glycoconjugates, oligosaccharides and polysac-

charides (Davies *et al.* 2005). Carbohydrate-active enzymes have been classified within each of four enzyme classes into families which define their amino acid sequence similarity, overall fold of the active domain and the active site as well as the catalytic mechanism (CAZy database at http://www.cazy.org/fam/acc_GH.html, Cantarel *et al.* 2009). This classification system comprises altogether 244 families and is constantly being updated. This resource reveals structural relatedness of enzymes, which is generally not obvious when enzymes are classified on the basis of their catalysed reaction as in the Enzyme Commission (EC) classification system (Nielsen and Borchert 2000). The CAZy database is therefore useful for predicting the structure and function of carbohydrate-active enzymes based on their genetic sequence or primary structure. The best characterised subgroup of carbohydrate-active enzymes are the glycoside hydrolases (also known as glycosyl hydrolases or glycosidases and transglycosidases) (Davies *et al.* 2005), and those will be thoroughly described below due to their relevance for this thesis.

In the CAZy database, glycoside hydrolases are currently classified into 115 families which contain e.g. *β*-glycosidases, cellulases, chitinases, lysozymes and various hemicellulases (Davies and Henrissat 1995). Many of these enzymes are of microbial origin and are specific for polymeric substrates or polymer-derived oligosaccharides. They are often composed of several protein domains or modules including the catalytic module and the carbohydrate-binding module (CBM). CBMs increase the concentration of the enzyme on polysaccharide substrates and are therefore important for the hydrolysis of recalcitrant substrates (see section 1.1). Efficient degradation of polymeric substrates requires the action of several different enzymes, which act synergistically in order to degrade the polymer into short soluble sugars (Lynd *et al.* 2002). The complete enzymatic hydrolysis of chitin, for example, involves the concerted action of endochitinases, exochitinases and *N*-acetylglucosaminidases. Members of the former two groups of enzymes are considered chitinases (EC 3.2.1.14) and belong to the glycoside hydrolase families 18 and 19. The endochitinases and exochitinases release chitinoligosaccharides from chitinous substrates through hydrolysis of *β*-4 glycosidic bonds, either at random positions in the internal part of the substrate chain or at the end of the chitin chain, respectively (Seidl 2008). *N*-Acetylglucosaminidases (EC 3.2.1.52) belong to the GH-20 family and produce GlcNAc monosaccharides through hydrolysis of chitinbiose or degradation of chitin from the non-reducing end. Some chitinases cleave chitin in a processive manner which is thought to be an important feature of polysaccharide degradationin which the enzyme performs several cata-

lytic cycles without detaching from the polymeric substrate (Breyer and Matthews 2001). Processivity requires high affinity towards the substrate and a special active site architecture, such as long binding sites consisting of multiple subsites providing interactions for the glycosyl units of the polymer. The binding site is more (tunnel shaped) or less closed (binding groove) and lined by hydrogen bond-forming residues and aromatic side chains for tight and at the same time "fluid" binding (Zakariassen *et al.* 2009).

1.2.1.1 GH-18 family chitinases

Chitinases (EC 3.2.1.14) hydrolyse chitin, a linear polymer of *β*-4 linked GlcNAc units, and produce soluble chitinoligosaccharides. The catalytic domains of chitinases belong to the glycoside hydrolase families 18 and 19 (http://www.cazy.org/fam/acc_GH.html). Chitinases occur in many organisms including viruses, bacteria, fungi, insects, higher plants and animals where they fulfil various roles such as in protection against pathogens, autolysis, nutrition, morphogenesis and pathogenesis (Dahiya *et al.* 2006). In addition to chitinases the GH-18 family also contains non-catalytic, lectin-like proteins such as concanavalin B (Hennig *et al.* 1995) and narbonin (Hennig e*t al.* 1990) as well as non-chitinolytic enzymes, such as the endo-*β*-*N*-acetylglucosaminidase H which is involved in the processing of high-mannose asparagine-linked oligosaccharides in glycoproteins (Rao *et al.* 1995). All GH-18 family proteins share the common $(\beta\alpha)_8$ (TIM) barrel fold, where the substrate binding cleft is formed by loops connecting the carboxyl-terminal end of the *β*-strands with the amino-terminal end of the *α*-helices (Robertus and Monzingo 1999). The members of this protein family contain an extended substrate-binding cleft which can accommodate several GlcNAc units (Papanikolau *et al.* 2001, van Aalten *et al.* 2001, Terwisscha van Scheltinga *et al.* 1994). Some chitinases contain one or several chitin binding domains which are presumed to promote the activity on insoluble substrates (Boraston *et al.* 2004).

In this thesis work a GH-18 family chitinase secreted by the filamentous fungus *Trichoderma harzianum* (Chit42) was studied. This enzyme, along with other chitinases secreted by *T. harzianum*, has previously received attention because of its potential in biocontrol against pathogens and nematodes causing diseases in agricultural crops (de la Cruz *et al.* 1992, Hayes *et al.* 1994). It was suggested that chitinases can protect crops from pathogen infection by hydrolysis of their chitin-containing cell walls. *T. harziamum* Chit42 is a monomeric enzyme with a mo-

lecular weight of 42 kDa (nucleotide base sequence and annotated primary structure are shown in Appendix I) (de la Cruz *et al.* 1992). It cleaves preferentially between the second and third sugar unit from the reducing end of a substrate, and hydrolyzes chitinhexaose ($(GlcNAc)_6$) into three identical disaccharides ($(GlcNAc)_2$) (Boer *et al.* 2004). Chit42 shares the highest amino acid sequence identity with the fungal chitinase CiX1 from *Coccidioides immitis* (Table 2).

Table 2. Comparison of mature *T. harzianum* Chit42 gene product (388 amino acids) with other well-characterised GH-18 family chitinases.

Name	**Length [Amino acids]**	**Identity with Chit42 [%]**
Coccidioides immitis Chi1	427	53
Bacillus circulans ChiA1	699	34
Serratia marcescens ChiA	563	28
Serratia marcescens ChiB	499	27
Hevea brasiliensis Hevamine A	311	14

Based on the similarity between the Chit42 and CiX1, a three-dimensional structural model of the *T. harzianum* Chit42 protein has been created (Boer *et al.* 2004). The structural model of Chit42 shows a typical GH-18 $(\beta\alpha)_8$ barrel fold where a subdomain composed of anti-parallel β-sheets, that can also be found in the CiX1 structure (Figure 3), is located between the β-sheet 7 and the α-helix 7 of the $(\beta\alpha)_8$ barrel. The loops of this subdomain and the mentioned loops of the $(\beta\alpha)_8$ barrel form together a 35 Å long substrate binding groove (Hollis *et al.* 2000; Zees *et al.* 2009) (Figure 4). The Chit42 binding site can accommodate up to seven GlcNAc units of a chitinous substrate at subsites -5 to +2, (Figure 4). The seven subsites are lined by amino acid residues which interact with bound carbohydrates through hydrogen bonds and stacking interactions. The catalytic mechanism of Chit42 is expected to proceed through a substrate-assisted catalysis mechanism in which the conserved residues Asp170 and Glu172 are involved (Boer *et al*. 2007) (Figure 4) and which is described below in more detail (see section 1.3). Modification of these two residues resulted in inactivated enzymes, which have maintained their ability to bind a substrate. It has also been shown that the wild-type Chit42 can recognize animal type β-4 galactosylated and α-3 fucosylated chitinoligosaccharides (Boer *et al*. 2004), which offers a good starting point for engineering the binding specificity towards more mammalian-type oligosaccharides.

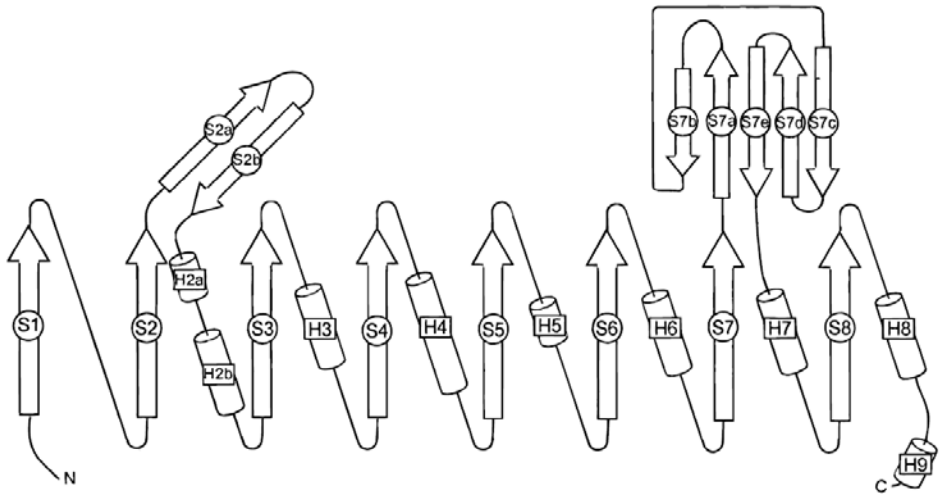


Figure 3. Cartoon presentation of the tertiary structure of CiX1 (adapted from (Hollis *et al.* 2000) with permission from the publisher).

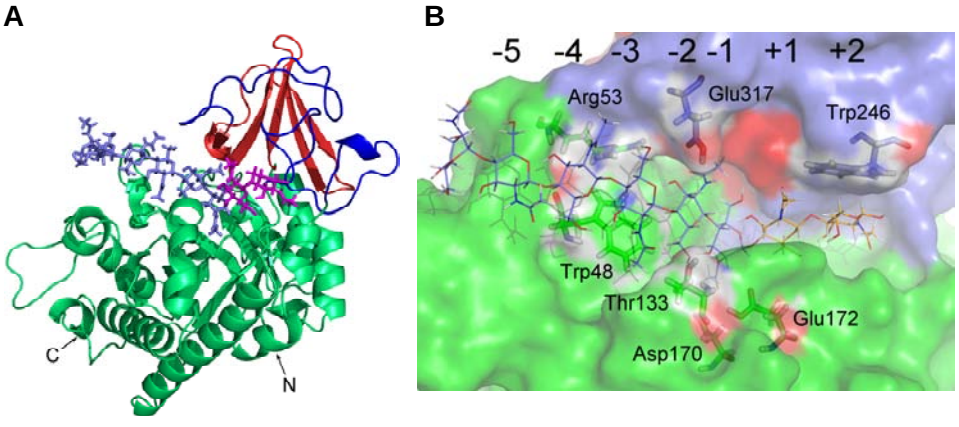


Figure 4. Homology model of the Chit42–substrate complex based on the fungal chitinase CiX1 complex structure (53% amino acid identity). Chit42 has a 35 Å long groove composed of seven putative binding sites (-5 – +2) for the GlcNAc units of a chitin chain. Here, the substrate chitinoctaose is bound in the chitin binding site, with the product part at the reducing end (in subsites +1 and +2) and the remaining part coloured differently. (A) Overall structure of the complex with the *N*- and *C*-termini marked. The $(\beta\alpha)_8$-barrel fold (green), and the (α + β)-domain, containing two flexible loops (dark blue) and an $\alpha\beta$-plane fold (red) are visible; (B) Modified amino acid residues in the substrate binding groove are displayed as annotated sticks with element-based surface colourisation. The "nick" in the substrate chain at the site of cleavage can be seen. Both images were created using PyMol (DeLano 2002).

1.2.2 Carbohydrate-binding antibodies

Antibodies are known for being high affinity binders, but in the case of oligosaccharide ligands the affinities are typically several orders of magnitude lower than those measured with other immunoglobulin types such as peptide-specific ones (Seto and Evans 2000). This low affinity is usually compensated by multivalent binding, leading to avidity in a similar manner as with many lectins (see below section 1.2.3). The antibodies specific for the ABO blood group trisaccharide antigens are prominent examples of this type of carbohydrate-specific proteins (Patenaude *et al.* 1998). Substrate binding was seen at groove-shaped surface structures and was proposed to depend on hydrophobic interactions (Toone 1994) and hydrogen bonds (Bundle and Young 1992).

1.2.3 Lectins

Oligomeric proteins of non-immune origin and which bind specific oligosaccharides mono- or multivalently are classified as “lectins” (Singh *et al.* 1999). Lectins are abundant in nature, where they can be found in viruses, bacteria, fungi, plants and animals (including humans) (Singh *et al.* 1999) and are the basis of many important recognition events (see section 1). Subsite multivalency, in contrast to carbohydrate hydrolysing enzymes, appears to be less important than subunit multivalency as the bound ligands are mostly monosaccharides or short oligosaccharides. The first report on lectin activity appeared in 1860 when Mitchell observed “co-agglutination” of erythrocytes from pigeon blood following the addition of rattle snake venom (Mitchell 1860). In the following decades lectin research gained momentum after the discovery of several plant proteins which could be used to distinguish between different types of red blood cells by agglutination tests. This was the impetus for Boyd and Shapleigh in 1954 to term this protein class “lectins”, from the Latin word “legere” meaning “to pick out” or “choose” (Boyd and Shapleigh 1954). The research performed on lectins so far (see Gabius *et al.* 2004 for a more complete historical overview) has provided new well-characterised lectins with known crystal structure at atomic resolution. Furthermore, lectin biosynthesis has been clarified and this knowledge has allowed their heterologeous production using bacterial hosts. This achievement was the basis for mutant studies aiming at determining the roles of their amino acid residues. With the aid of X-ray crystallography a substantial number of lectin structures could then be determined (813 at the time of writing;

http://www.cermav.cnrs.fr/lectines/). The tertiary structure employed by lectins for providing a scaffold for ligand binding can be very diverse and may involve multiple subunits and contain, amongst other architectures, structural motives such as the jelly roll fold (two sheets of antiparallel *β*-strands), the *β*-propeller (4–8 blade-shaped beta sheets arranged toroidally around a central axis with each sheet typically made up from four antiparallel *β*-strands twisted so that the first and fourth sheets are almost perpendicular to each other) and the *β*-trefoil fold (12 *β*-strands folded into three similar *β*-*β*-*β*-loop-*β* (trefoil) units) (see (Rini 1995) for a more comprehensive overview). The substrate binding of lectins and carbohydrate-binding antibodies occurs at shallow grooves or pockets at their surface (Bundle and Young 1992). Lectins can therefore be distinguished from carbohydrate-binding periplasmic transport proteins and some glycosyl-hydrolysing enzymes that completely envelope their carbohydrate ligands in deep binding pockets (Bourne *et al.* 1993, Toone 1994, Vyas 1991). The lectin binding site usually contains aromatic amino acids that provide van der Waals interactions (e.g. aromatic ring stacking) for the hydrophobic side of the sugar ring and polar residues that solvate the ligand through hydrogen bonds with the hydroxyl functions of the ligand. This binding region can be extended over several subsites or limited to a single subsite, which can accommodate only a single monosaccharide unit (Watson *et al*. 1994).

1.2.3.1 Wheat germ agglutinin

The monocot lectin wheat germ agglutinin (WGA) isolated from *Triticum vulgaris* (wheat) was used in the presented experiments, as it is known to tightly bind relatively short carbohydrate ligands such as the GlcNAc monosaccharide (Nagata and Burger 1974). In this study, it was intended to measure its binding affinity to *N*-acetyl-D-glucosamine at the solvent-accessible sites of planar self-assembled monolayers. WGA is a dimeric protein with a molecular weight of 36 kDa which occurs as the three isoforms WGA1, WGA2 and WGA3 (Monsigny *et al.* 1979, Wright and Raikhel, 1989). The isoforms are very similar and are all composed of two identical subunits that separate below pH 3.5 (Nagata and Burger 1974). Each subunit consists of four globular hevein domains of 42 or 43 amino residues (Wright 1987). This structural motif is common among legume lectins and comprises a cysteine-rich core that is stabilised by three to five disulfide bonds, also known as the “chitin-binding domain” (Sinha *et al.* 2007). In WGA, these domains of one protein subunit are aligned with the do-

mains of the other subunit in a head-to-tail fashion forming a horse-shoe shaped complex (see Figure 5A) (Wright 1980). The dimer contains eight putative carbohydrate-binding sites which are each formed between the interfaces of opposing domains of different subunits (Wright 1992). Based on binding studies, it was proposed that four high-affinity sites are formed by binding domains 2 and 3 and four low-affinity sites by the domains 1 and 4 (Wright 1980, Wright and Kellogg 1996). Binding ligands of WGA are chitinoligosaccharides and neuraminic acid, for which aromatic stacking interactions and hydrogen bridges are provided in the WGA binding sites (Figures 5B and 5C) (Wright and Kellogg 1996). Here, the domain which contains the aromatic residues stacking with the sugar ring is called the principal binding domain and the domain participating in substrate binding through hydrogen bonding is termed the helper domain (Wright 1992). The latter domain cannot be found in all binding sites (Wright and Kellogg 1996). Despite the considerable crystallographic work published on the WGA substrate complexes, the number of functional binding sites is still the subject of discussion. Some estimates are based on reports describing WGA crystals which contain either two or four binding sites/dimer occupied by a chitinoligosaccharide (Wright 1980; Muraki *et al.* 2002). It has been suggested that up to three consecutive GlcNAc units of a chitinoligosaccharide can be accommodated in the high affinity binding sites (Wright 1980).

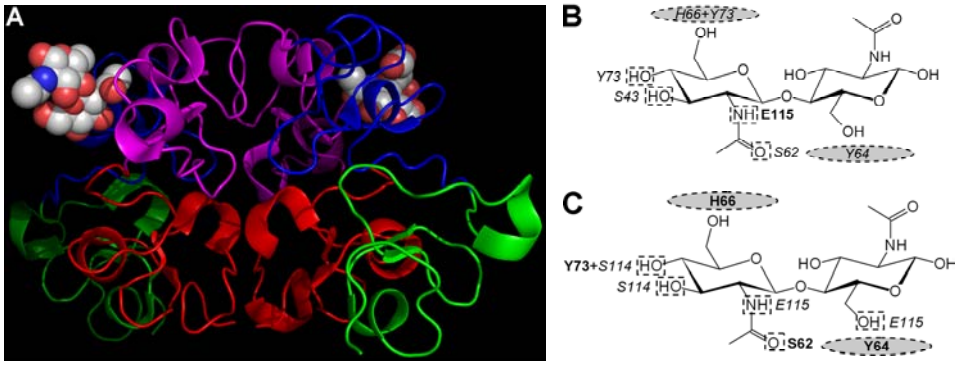


Figure 5. Carbohydrate binding by WGA. Crystal structure of WGA3 complexed with chitinbiose (published in (Muraki *et al.* 2002); PDB-ID 1K7U). The head-to-tail arrangement of the domains of the two subunits can be seen (domain 1 = red, 2 = magenta, 3 = blue, 4 = green). Two $(GlcNAc)_2$ molecules are bound in the high-affinity/primary binding sites at the interface of domains 2 and 3 of different subunits. Image created using PyMol (DeLano 2002). In (B) and (C), residues participating in binding of the two ligands are indicated. Stacking by aromatic side chains is indicated by grey ovals next to the contacted disaccharide ring. Bold and italic types indicate the affiliation to the protomers of dimeric WGA. In both sites aromatic interactions are provided exclusively from domains 2 (principal binding domains) and the opposing domains only provide helper domains.

1.3 Molecular basis of protein–carbohydrate interactions

Protein–carbohydrate interactions are important in signaling, recognition and catalysis, and include various non-catalytic binding proteins such as CBMs (of polysaccharide-degrading enzymes), antibodies, receptors and lectins. In addition, protein–carbohydrate interactions are important in enzymatic reactions catalysed by carbohydrate-active enzymes and which involve cleavage and building of the glycosidic bonds in glycoconjugates, oligosaccharides and polysaccharides. Proteins employ various amino acid functions for carbohydrate binding which frequently include polar and hydrophobic interactions and rarely electrostatic interactions (ion pairing) and metal ion coordination (Sharon 2006, Bourne *et al.* 1993). Polar interactions between carbohydrates and proteins are hydrogen bonds that are formed between polar sugar groups and polar amino acid side chains in the binding cleft (Bourne *et al.* 1993). Protein-bound water molecules sometimes act as bridges between these two binding partners (Bundle and Young 1992). Apart from polar groups, carbohydrates also expose hydrophobic groups such as the hydrophobic face of hexopyranose rings and the methyl moiety of the amido and acetamido sugars (Sharon 2006). These groups

interact with aromatic amino acids through van der Waals bonds (stacking) (Breyer and Matthews 2001) and hydrophobic interactions with aliphatic amino acid side chains (Sharon 2006).

Lectins can tightly attach to carbohydrate structures by recognising very short oligosaccharide structures. These bonds are mostly weak due to the limited surface area of the bound ligand, but lead to strong interactions with a dissociation constant in the μM range by simultaneous formation of multiple such bonds (Mammen *et al.* 1998, Collins and Paulson 2004). The carbohydrate binding sites of lectins provide a dense network of multiple hydrogen bonds and aromatic stacking interactions (Wright and Kellogg 1996, Wälti *et al.* 2008), which allow the specific and tight attachment to short oligosaccharide residues as decorations on larger structures, such as glycosylated proteins or glycolipids in the cellular membrane (Sharon and Lis 2004, Vasta 2009). Hydrogen bonding between the carbohydrate and lectin often involves water molecules as an extension of the protein surface and can be as strong as hydrogen bonding between amino acid side chains and their substrate (Watson *et al.* 1994). In addition to water, lectins sometimes contain metal ions, e.g. Mn^{2+} and Ca^{2+} in legume lectins (e.g. *Erythrina corallodendron* lectin, Shaanan *et al.* 1991), which can stabilise the structure or may directly coordinate with the sugar ligand as in the calcium-dependent collectins (Håkansson and Reid 2000).

A second group of carbohydrate-binding proteins are carbohydrate-specific antibodies, which, with the exception of genetically engineered variants (Roberts *et al.* 1990), are devoid of metal ion cofactors. Lectins, as opposed to antibodies, bind carbohydrates mainly through unspecific but strong stacking interactions, acting between aromatic amino acid side chains and the hydrophobic sides of monosaccharide units (Bundle and Young 1992, Rose *et al.* 1993, Sharon 2006). In addition, hydrogen bonds formed between polar groups of the antibody binding site and carbohydrate increase the binding strength and specificity (Bundle and Young 1992).

The binding sites of carbohydrate-active enzymes are highly diverse, but a few characteristics can be seen that are distinct from other types of carbohydrate binding proteins (Davies *et al.* 2005). Carbohydrate-active enzymes contain only a single binding site and bind usually a rather large part of the substrate at several subsites through hydrogen bonds and non-polar van der Waals interactions (aromatic stacking) (MacGregor *et al.* 2001, Mikami *et al.* 1993). Generally the carbohydrate binding site is seen as a linear array of different subsites, each of which accommodates a single monosaccharide residue. The cleavage of the gly-

cosidic bond occurs (by definition) between the carbohydrate units bound at the subsites -1 and +1. The neighbouring subsites towards the reducing end of the bound carbohydrate are given positive numbers and the subsites at the non-reducing end are given negative numbers starting from the cleavage site (e.g. -5, -4, -3, -2, -1, +1, +2 and +3). Glycoside hydrolases hydrolyse a carbohydrate substrate by exposing the glycosidic bond located between subsites -1 and +1 to an acidic or basic environment, distorting the monosaccharide unit at the -1 subsite into a boat conformation and twisting the oligosaccharide chain at the enzyme's active site (van Aalten *et al.* 2001, Vyas 1991). Under these conditions, the glycosidic oxygen is readily protonated, which is energetically unfavourable when the carbohydrate ligand is solvated and unstrained (Tews *et al*. 1997, van Aalten *et al*. 2001). The hydrolysis of the glycosidic bond that is catalysed by glycoside hydrolases either inverts or retains the anomeric configuration of the carbohydrate in the -1 site (Koshland 1953, Schülein 2000). Retention of the stereochemistry proceeds through reactions where a covalent intermediate or, alternatively, an oxocarbonium species is formed (Hehre 1999). For GH-18 chitinases, a retaining mechanism called substrate-assisted catalysis has been proposed for hydrolysis of GlcNAc-containing substrates. Here, catalysis involves participation of the acetamido-function of a GlcNAc residue at the -1 position (Figure 6) (Dall'Acqua and Carter 2000).

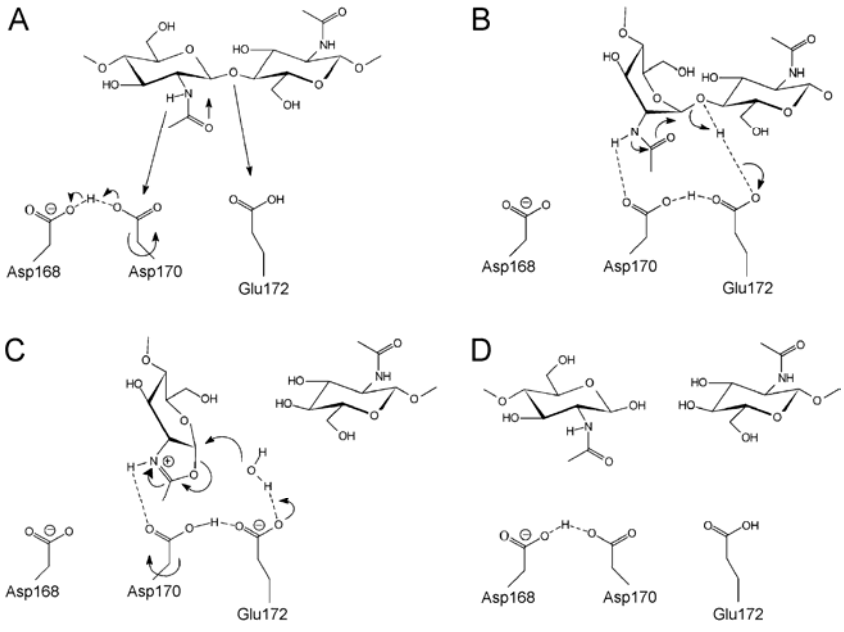


Figure 6. Putative catalytic mechanism of chitin hydrolysis by *T. harzianum* Chit42. The process proceeds via four steps: (A) substrate binding, (B) substrate distortion and electrophilic attack of the glycosidic bond through the acidic residue (Glu172), (C) oxazolium ion formation, leaving group departure and approach of a water molecule which hydrolyses the oxazolium intermediate and (D) release of products with retained conformation at the generated anomeric carbon. Modified with permission from the publisher from (Vaaje-Kolstad *et al.* 2004) and based on the mechanism proposed for GH-18 chitinases (Tews *et al.* 1997, van Aalten *et al.* 2001).

Among carbohydrate-active enzymes the glycoside hydrolases are best characterised and exhibit high structural diversity (Davies and Henrissat 1995, Davies *et al.* 2005). In addition to the catalytic module, some carbohydrate-active enzymes contain carbohydrate-binding modules (CBMs), which are contiguous amino acid sequences with a discrete fold that can bind carbohydrates and increase the catalytic activity of a carbohydrate-active enzyme on non-soluble substrates such as cellulose or chitin (Shoseyov *et al.* 2006). Like the carbohydrate active enzymes, CBMs are classified into different families based on sequence similarity (http://www.cazy.org/fam/acc_CBM.html). Some CBMs bind specifically to crystalline glycan substrates, whereas others bind preferentially to less ordered carbohydrates. For some CBMs, a more disruptive function has been assigned (Boraston *et al.* 2004). Besides being a component of polysaccharide-degrading enzymes, individual CBM-like proteins have been described

which bind to crystalline polysaccharides but lack a catalytic module (Vaaje-Kolstad *et al.* 2005, Vaaje-Kolstad *et al.* 2009). These proteins resemble lectins but may still be differentiated from these by their function in assisting catalysis rather than playing a role in recognition and binding of glycan-carrying structures (Sharon and Lis 2004). Carbohydrate binding in lectins and enzymes may require in some cases the presence of a metal ion (e.g. Ca^{2+} or Mg^{2+}), which can interact with hydroxyl groups of the carbohydrate and histidine (or lysine) groups of the protein (Lavie *et al.* 1994).

Processive glycoside hydrolases acting on crystalline substrates hydrolyse polymeric carbohydrates through successive cleavage of oligosaccharides from the polymer without release of the shortened polymer (processive action). Aromatic residues are reported to be important for chitinase processivity as they form a flexible and hydrophobic sheath in long substrate binding channels, which allows sliding of a bound carbohydrate towards the active site (Breyer and Matthews 2001, Meyer and Schulz 1997, Stern and Jedrzejas 2008).

1.4 Modification of protein properties using directed evolution and rational protein design

Structure–function relationships of proteins including e.g. the details of a catalytic mechanism and substrate binding can be studied by rational design (Boer *et al.* 2004, Branco *et al.* 2008). In addition to rational approaches, directed evolution methods including random mutagenesis and/or gene shuffling can be used for altering the protein properties. This technique has received increasing attention during recent decades for optimisation of enzymes for industrial applications. Here, harsh conditions are often used for which biocatalysators with modified properties are desired, including e.g. altered specific activity, end-product inhibition, pH behaviour, thermostability and substrate binding specificity (Bornscheuer and Pohl 2001). The main advantage of directed evolution over rational protein design is that in this method mutation and selection are used for obtaining enzymes with a desired function and a detailed understanding of the complex relationship between sequence, structure and function, which is often unattainable, is not in fact required (Bloom *et al.* 2005). Glycoside hydrolases (see section 1.2.1), for example, were turned into glycosynthases by removing the hydrolytic activity through replacement of the nucleophilic active site residue with another residue unable to perform the same function (Blanchard *et al.* 2007, Hancock *et al.* 2006).

Evaluation of the mutations introduced by both techniques, rational protein design and directed evolution, requires a functional gene expression system in a host organism, such as a bacterium or a yeast, and an analysis method on the basis of which the best mutants can be selected for one or more possible further rounds of mutagenesis (Bornscheuer and Pohl 2001). In addition, rational protein design requires knowledge of the protein structure, either in the form of a crystal structure or a structural model, whereas directed evolution approaches do not require such information although it may be helpful in targeting e.g. the random mutagenesis to a certain area in the protein structure (Turner 2009, Bloom *et al.* 2005). For performing directed evolution, the introduction of random mutations into a certain DNA is often carried out using PCR (Fromant *et al.* 1995). Alternatively, it can be performed by more traditional methods such as DNA exposure to UV radiation or hydroxylamine. UV radiation leads to dimerisation of pyrimidine residues (Setlow and Carrier 1966) and hydroxylamine converts cytosine residues to uracil groups through deamination (Brown and Phillips 1965). These methods produce a large number of different mutated DNA molecules, called a mutant library and ideally containing 10^5–10^9 variants of the original DNA, depending on the screening system (Bloom *et al.* 2005). A high mutation rate is usually avoided, as beneficial mutations are likely to be masked by additional deleterious mutations. Rather a gradual improvement of the enzyme is commonly preferred, which is designed to identify single beneficial mutations by transforming expression organisms with plasmids harbouring the mutated gene and screening for altered enzymatic properties in a high-throughput technique. After completion of this first mutagenesis round, beneficial mutations are either further optimised by saturation mutagenesis, combined with additional new mutations using further rounds of mutagenesis, or combined with other beneficial mutations using shuffling, where fragments of different variants (of the same gene) or related gene sequences are exchanged leading to mosaic genes with new properties (Stemmer 1994). An efficient way to obtain biocatalysts with new substrate specificities is to screen initially for mutants with a broadened substrate spectrum, then mutate these “generalists” further and finally select mutants from this library with an increased specificity for the substrate of interest (Tracewell and Arnold 2009). Examples of studies in which this technique was successfully applied are the conversion of a cytochrome P450 fatty acid hydroxylase into a propane hydroxylase (Fasan *et al.* 2007), the generation of a stereoselective D-amino acid dehydrogenase from a meso-diaminopimelate D-

dehydrogenase (Vedha-Peters *et al.* 2006) and the substrate specificity shift of a galactose oxidase towards 1-phenylethanol (Escalettes and Turner 2008).

1.5 Methods for measuring protein–carbohydrate interactions

Several different techniques are currently available for characterising protein–carbohydrate interactions, including isothermal titration calorimetry (ITC) (Cederkvist *et al.* 2007), surface plasmon resonance (SPR) (Duverger 2003, Linman *et al.* 2008), NMR (Jimenez-Barbero *et al.* 2006), the quartz crystal microbalance (QCM) gravitation method (Zhang *et al.* 2006), circular dichroism (CD) and fluorescence spectroscopy (Hilaire *et al.* 1994, Laurent *et al.* 2008), analytical ultracentrifugation (Asensio *et al.* 2000), mass spectrometry (Soya *et al.* 2009), crystallography (Hashimoto 2006) and atomic force microscopy (AFM) (Ratto *et al.* 2004). Of these methods, AFM and SPR are particularly sensitive (McMahon and Präfcke) and were used during this thesis for determining affinities of WGA lectin and Chit42 variants for chitinoligosaccharide-related compounds. One limitation of both techniques is that at least one partner of a pair of interacting molecules must be immobilised, which imposes steric constraints on the modified molecule and might interfere with the studied interaction. When ligand modification is critical, ITC (Leavitt and Freire 2001) could, among other techniques, be considered. ITC can detect binding affinities with high sensitivity (down to 10–11 M), but requires a comparably large amount of sample (19.2 mg of a 40 kDa protein with a 10 μM affinity; McMahon and Präfcke).

1.5.1 Glycan presentation in self-assembled monolayers

Many biological recognition and signalling events based on protein–carbohydrate interactions take place at surfaces such as membranes or cell walls (Sharon and Lis 2004) and can therefore be represented by surface-bound two-dimensional carbohydrate arrays. Self-assembled monolayers (SAMs) can be used to present carbohydrates in a well-defined and at the same time, flexible manner, as their density and environment can be adjusted by blending with functional molecules, which integrate into the layer (Love *et al.* 2005). Prominent techniques available for studying biological surface-based adsorption processes are SPR, AFM, scanning tunneling microscopy, reflection absorption infrared

spectroscopy, surface-enhanced Raman spectroscopy, the QCM gravitation method, cyclic voltammetry and amperometry (Ferretti *et al*. 2000).

SAMs can be obtained by direct self-assembly of a dissolved amphiphile on the substrate or by the Langmuir-Blodgett method (Langmuir 1920, Blodgett 1935), in which a pre-assembled film, which has formed at an air–water interface, is transferred onto a solid substrate. Among the different possible chemistries which can lead to SAM formation, organosulfur-based SAMs on noble metals are best characterised with respect to stability and physicochemical properties (Love *et al*. 2005, Ulman 1996). The SAM layer adsorption process relies on hydrophobic interactions between alkane groups for which a minimum length of eleven carbon atoms has been postulated in order to obtain stable and tight SAMs (Templeton *et al.* 1998). In studies dealing with SAMs, noble and coinage metals are commonly used as a substrate for adsorption of organosulfur compounds (Love *et al*. 2005). This reaction proceeds through oxidative addition of the thiol group to the noble metal, which is followed by reductive elimination of hydrogen (Ulman 1996). This reaction results in a thiolate chemisorbed to the metal; gold is frequently chosen as it is, among other beneficial properties, easy to pattern, inert to most chemicals and compatible with versatile analytical techniques such as SPR spectroscopy and QCM (Love *et al.* 2005). During this reaction no gold oxide is formed and it is believed that molecular hydrogen might be released, although the details of the nature of the gold–sulfur bond remain controversial (Love *et al*. 2005). Based on this, the reaction is assumed to proceed as shown in Scheme 1.

Scheme 1. Suggested mechanism for the formation of SAMs from alkanethiols on gold

$$R\text{–}S\text{–}H + Au^{0}_{n} \rightarrow R\text{–}S^{-}\,Au^{+}\cdot Au^{0}_{n-1} + \tfrac{1}{2}H_2$$

This reaction is the first step of SAM layer formation on gold, which is completed within a few minutes and followed by re-arrangement of the organosulfurs on the substrate surface to form a dense layer (Ulman 1996). At submonolayer coverages, which correspond to the early adsorption, striped phases can be seen, which are a parallel array of linearly arranged alkanethiol molecules with their hydrophobic parts lying flat on the gold pointing to the end of another alkyl group, lying on the gold (Yang *et al.* 2007). This is believed to be an intermediate state preceding the upright arrangement which is typical for full coverage. In the upright arrangement, the carbon chain is tilted with respect to the surface normal by an angle of 28º and rotated by 53º with respect to the CCC

bond plane relative to the plane of the surface normal and the tilted chain (Laibinis *et al*. 1991). The chains inside a high-coverage SAM adopt a configuration termed as a ($\sqrt{3}\times\sqrt{3}$)R30º overlayer, where neighbouring alkane chains are twisted by 90º relatively to each other (Poirier 1997). The resulting monolayers usually contain irregularities, which are defects at grain boundaries, exposed chains and defects at gold steps and vacancy islands (Love *et al*. 2005). The frequency of these defects can be minimised by careful preparation of a flat and clean gold support, which will increase the reproducibility of the data obtained during measurements at these surfaces.

1.5.2 Atomic force microscopy for surface imaging and binding strength determination

The first AFM was developed by Binnig *et al.* in 1986 (Binnig *et al.* 1986), making it possible to observe biological structures in a liquid environment (Weisenhorn *et al*. 1989, Drake *et al*. 1989). This was an important step, as it offered the possibility to study the structure and dynamics of biomolecules (Shao *et al.* 1996, Engel *et al.* 1997). By contrast, other imaging techniques with similar resolution such as scanning tunneling microscopy (STM), scanning electron microscopy and transmission electron microscopy required drying of samples and exposure to strong radiation (Miles *et al.* 2003) which causes sample denaturation. AFM and STM are classified as types of scanning probe microscopy (SPM), which includes all techniques that use a physical probe for imaging a sample during a scanning process. Biological molecules with varying sizes have been studied by AFM, such as linear DNA and whole cells (Alessandrini and Facci 2005), which demonstrates the critical dimensions of a sample of 0.1 nm to 100 μM in width and 0.1 nm to 10 μM in height. For the investigation of larger objects, complementary imaging techniques such as optical and electron microscopy are available (Figure 7). Apart from mapping the surface topography, AFM can also be used to measure the viscoelastic and chemical properties, dynamic changes and intra- and intermolecular interactions of a given sample (Barattin and Voyer 2008, Ulcinas *et al.* 2007).

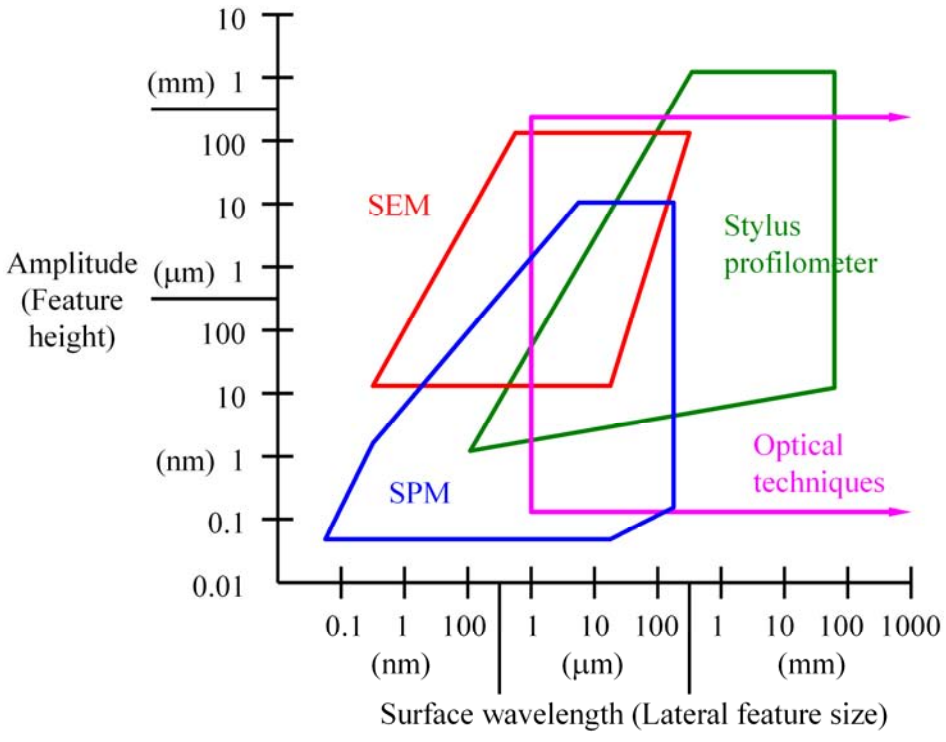


Figure 7. Dimensional measurement ranges of commonly used imaging techniques. Reproduced with permission from Jim Flach (Veeco Instrumens – Europe).

AFM Imaging

The AFM apparatus is designed to create a topographical image of a sample surface by scanning it with a sharp tip (made from Si or Si_3N_4) mounted on a flexible cantilever from which a laser beam is reflected (Alessandrini and Facci 2005). The reflected laser projects the movements of the cantilever onto the split photodiode detector as multiplied amplitude and thereby functions as an optical lever. The detector converts the beam movement into an electrical signal that is referenced inside the controller electronics, against a setpoint value which corresponds to a predefined cantilever deflection. The setpoint is used to adjust the cantilever deflection to a reference value by vertical movement of the sample. During scanning, the sample is moved continuously back and forth by the width of the image in the X-direction (fast scan axis) while it is moved upwards in the Y-direction (slow scan axis) by the length of one pixel after completion of each X-movement cycle. This movement sequence is executed until the sample has

scanned the bottom line of the imaged area. During this process, the tip, which is located at the end of a flexible cantilever (see Figure 8), scans the sample surface and is bent up or moves back down when it encounters elevations or depressions (elevation contours). In response, the vertical position of the sample is adjusted to maintain the cantilever. The performed vertical movements of the sample are recorded for each pixel of the imaged area to create a complete topographical image of the scanned area. One drawback of this technique is that the tip is constantly in contact with the sample and in the case of soft samples (such as biological materials) might carve into the sample during the scanning procedure. A way to eliminate the lateral forces responsible for this surface alteration is a method called "tapping mode" (also known as intermittent contact mode) (Zhong *et al*. 1993), in which the lateral forces exhibited by the tip during scanning are eliminated. Here, the tip is vibrated near its resonance frequency and touches the sample surface just at the lower position of its vibrational movement. The tip–sample interaction is sensed by the dampening of the tip vibration and controlled by the movement of the Z-scanner (see Figure 8).

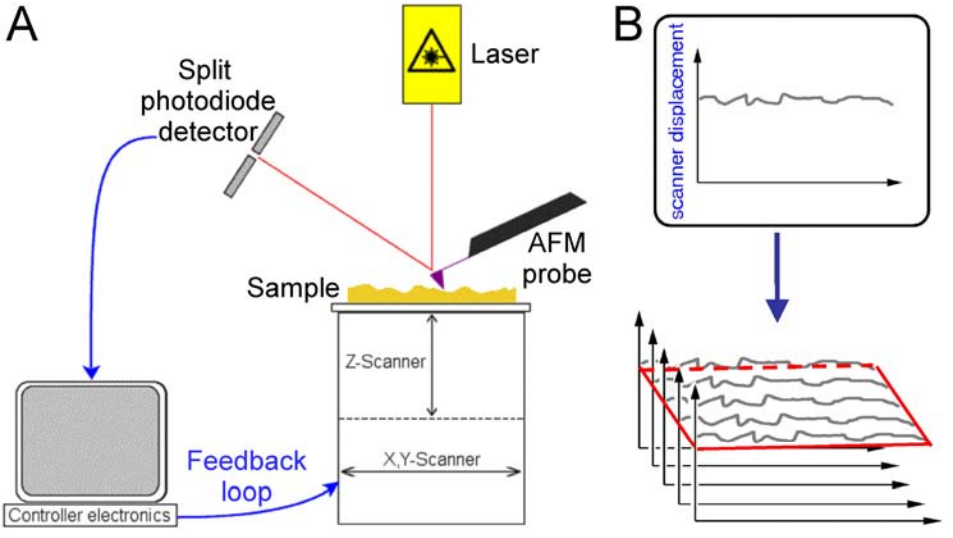


Figure 8. A) Schematic drawing of the setup of an AFM. The sample is located on a piezo-electric scanner which moves the sample in the X-, Y- and Z-directions according to voltage changes from a controller. The sample's topography is sensed by a probe consisting of a flexible cantilever and a sharp tip. The feedback loop maintains cantilever deflection (contact mode) or oscillation (tapping mode). (B) The scanner displacement recording of all scan lines is joined to create a topographical image of the sample surface.

AFM Force Spectroscopy

AFM can be used to measure intra- and intermolecular forces with a method called force spectroscopy (Rief and Grubmüller 2002, Butt *et al.* 2005). This technique can provide quantitative information about the strength of biological interactions. In force spectroscopy experiments, the sample is probed at a fixed spot on the surface. The measurement is performed as an approach−retraction cycle which starts with the tip being located some 100 nm above the sample. From this point, the sample is moved towards the tip until the tip touches the surface and the cantilever is bent upwards until the deflection corresponds to a preset loading force. After reaching the maximum upward deflection, the sample is lowered until the tip detaches from the probed surface and the cantilever returns to a relaxed state (Figure 9). During the downward movement attractive or repulsive interactions between the tip and the surface can be detected as delayed or early detachment of the tip.

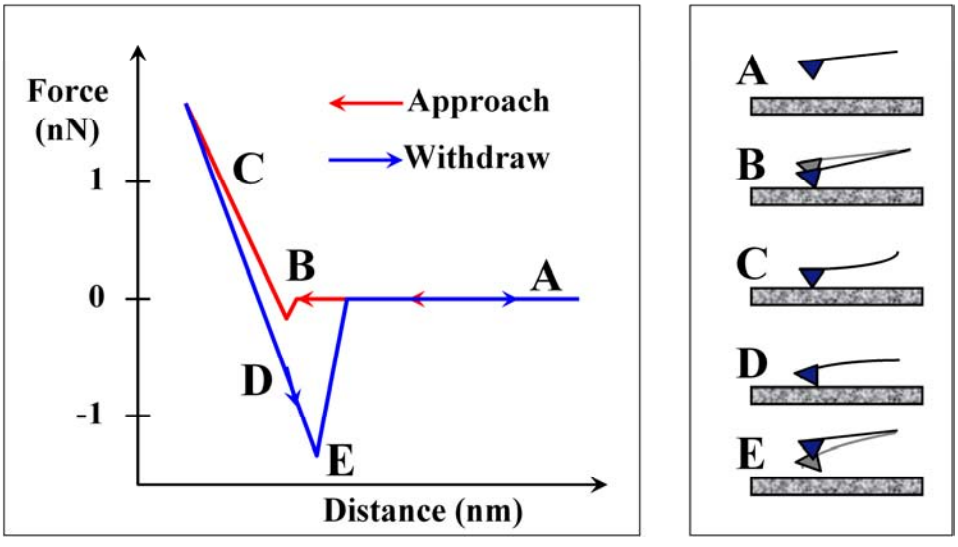


Figure 9. Scheme of an AFM force curve where the force exhibited on the AFM cantilever during an approach–retraction cycle is displayed in relation to the distance of vertical sample movements. The conditions represented are (A) No interaction at large separation; (B) Snap into contact close to the surface; (C) Tip contacts surface = constant compliance region; (D) Adhesion maintains contact during retraction and (E) Cantilever release. Figure redrawn with permission from Arja Paananen.

The total force exhibited at the tip during the process is monitored using the laser reflection from the sensing element – the cantilever of the probe – and can be quantified by determination of the cantilever deflection. This deflection can be converted into a force by using Hooke's law $F = kx$, where F is the force in nN acting on the cantilever, x is the cantilever deflection in nm and k is the spring constant in nN/nm. The spring constant is usually determined separately for each cantilever used, as it is common for the actual flexibility to deviate significantly from the supplier's specifications. A simple method for this determination is the thermal method in which the thermal oscillation is measured in order to deduce the spring constant (Hutter and Bechhoefer 1993). AFM force measurements are usually performed in an aqueous solution in order to prevent interfering capillary forces, and tips are functionalised with one of the molecules of interest (Barattin and Voyer 2008). For measuring the interaction between the probed surface and the molecule attached to the tip, it is important to choose an attachment chemistry which results in stronger bonds than those exerted by the probed interaction. Conjugation chemistries suitable for studying biological interactions are therefore either covalent (e.g. amide, glutaraldehyde and S–Au → withstands up to ~1.4 nN (Grandbois *et al*. 1999)) or strong and non-covalent (e.g. Ni^{2+}–NTA and biotin–avidin → withstands up to 200 pN (Merkel *et al*. 1999)). Commonly, the ligand is coupled to the tip through PEG-based linkers, which gives the ligand higher mobility and access to the receptors on the probed surface. During a typical AFM force spectroscopy experiment, hundreds of approach–retraction cycles are performed on different areas of the sample, with and without interaction and inhibition (Hinterdorfer and Dufrene 2006). The recorded forces are presented in histograms in order to obtain representative estimates of the acting forces and differentiate forces related to specific and non-specific binding events, of which the latter are caused by non-specific adhesion.

1.5.3 Quartz crystal microbalance with dissipation monitoring

The detailed characterisation of self-made carbohydrate-modified surfaces is a prerequisite for the correct interpretation of adsorption events, observed at modified sensors. An appropriate tool for this analysis is the quartz crystal microbalance with dissipation monitoring (QCM-D). This device can be used to determine the time scale of the initial functionalisation (e.g. SAM-coating with neoglycoconjugates as performed in the present study) and to characterise the

formed layer in terms of adsorbed mass, thickness, density, viscosity, or storage modulus (Marx 2003, Dixon 2008, Kanazawa and Cho 2009). When the molecular weight of the adsorbed molecule is known, the packing density of adhered molecules can be calculated, which can be compared to published data on reference layers. The sensing device in the QCM-D is a 0.3 mm thin quartz disk onto which two gold electrodes are deposited. The disk can be oscillated through application of an alternating electrical field, at a resonance frequency dependent on the total oscillating mass of the disk. An adsorbed rigid layer will follow the crystal oscillation and thereby lead to resonance frequency changes with little dampening. In such a case, the adsorbed mass can be calculated using the Sauerbrey equation $\Delta m = C \bullet (\Delta f/n)$, where n is the overtone number, Δf is the measured resonance frequency change and C is the sensitivity constant of the quartz crystal (Sauerbrey 1959). However the, viscoelastic properties of soft layers cannot be quantified with this technique when high frictional energy losses occur inside them characterised by a dissipation factor higher than 10^{-7} Hz^{-1} (QCM device manual) or by a resonant frequency change that is not proportional to the harmonic number of the resonant frequency (1, 3, 5, 7, 9, etc.) (Kanazawa and Cho 2009). In addition to monitoring of monolayer adsorption, the QCM-D technique can also be used to measure lectin–carbohydrate association on SAMs and to determine the lectin binding affinity for the immobilised ligand (Zhang *et al.* 2006).

1.5.4 Surface plasmon resonance

The measurement of binding properties of proteins differs from other types of protein analysis in that no reaction products are released during binding which could be detected spectrometrically. Therefore, most methods designed to measure binding processes, such as SPR, require that one interaction partner is immobilised on a sensor surface (Schuck 1997). In SPR binding measurements, solute adsorption to an SPR sensor is sensed through electromagnetic waves (plasmons) which originate from electrons that oscillate in unison at the surface of a metal and interact with their proximal environment through an evanescent wave field (effective penetration depth is 20% of the wavelength of incident light, typically ~150 nm) (Homola 2008). In SPR devices, a gold-coated glass sensor is optically coupled to a prism to reflect incident monochromatic light under conditions of total internal reflection, which results in a characteristic intensity dip at a certain reflective angle (SPR angle, Figure 10). One partner of

the interaction pair is immobilised onto the sensor, which is often covered with a hydrogel (Gedig 2008). The layer thickness is critical for the measurement, as a change in solute concentration is effectively sensed in a limited space above the gold surface. Within this range, changes in the refractive index can be detected as changes of the SPR angle and displayed as a sensogram (see Figure 10).

In an SPR interaction experiment, one interaction partner of a studied molecule pair is introduced in solution into a flow cell which contains the second interaction partner immobilised on the sensor surface (Schuck 1997). During the injection of the soluble interaction partner binding is monitored as change of SPR angle, from which the apparent rate constants of the association and dissociation of the protein–carbohydrate pair can be deduced. The detection limit of this method is dependent on the mass density per area obtained by adsorption of a certain ligand, which in turn depends on its molecular weight and the density of binding sites provided by the immobilisation of the first interaction partner. In general, detection of binding of ligands with a molecular weight <1000 g/mole by SPR is difficult because these molecules must be injected at high concentrations in order to allow their detection, leading to an apparent loss of specific binding (Becatti *et al.* 2005). On the other hand, adhesion of large particles such as cells cannot be accurately monitored by SPR as the evanescent wave does not effectively sense mass which is located further away from the surface than the effective penetration depth of the evanescent wave field (Homola 2008).

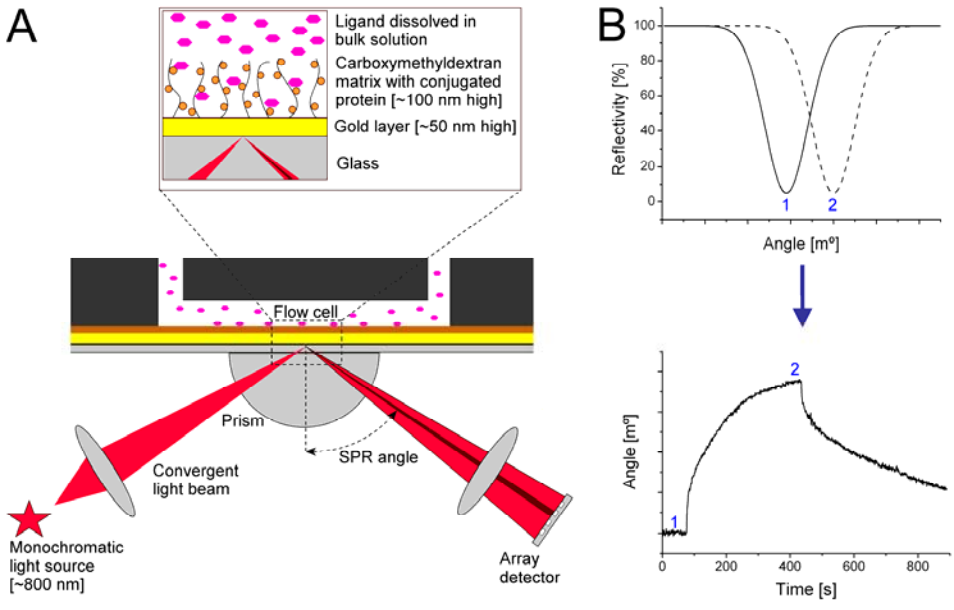


Figure 10. Detection of ligand binding inside an SPR apparatus (BIAcore type). (**A**) A convergent light beam with a wavelength of 800 nm is directed towards the gold-coated surface of an optically coupled (through prism) glass sensor from where it is reflected under conditions of total internal reflection. Plasmons are generated at the gold surface and absorb energy from the incident light at a specific incident angle which can be detected in the reflected light beam as an intensity drop at a specific angle (SPR angle). Solute concentration changes within the close environment of the gold surface (up to 150 nm) affect the plasmon energy absorption and result in a changed SPR angle. Ligand adsorption to hydrogel-coupled proteins at the sensor surface can be monitored with this system. (**B**) SPR angles measured at a sensor before and after ligand binding (1 and 2, respectively) are different. For determination of thermodynamic parameters the time course of SPR angle change is displayed in a sensogram (bottom).

SPR Data Analysis

The scientific value of performing SPR binding experiments is the determination of kinetic and/or thermodynamic parameters for an association between different molecules. This is usually done by fitting the measured association and dissociation profile to a theoretical model, which provides an estimate of the kinetic parameters of the monitored interaction such as the on rate (k_{on}), the off rate (k_{off}) and the affinity constant (K_a) as (k_{on}/k_{off}) (Schuck 1997). The simplest model describing the association of two molecules at a single binding site is the Langmuir binding model (see Scheme 2).

Scheme 2. Bimolecular binding equilibrium according to the Langmuir model

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB$$

The Langmuir binding model is valid when the dissolved analyte (*A*) is monovalent and homogeneous, the immobilised ligand (*B*) is homogenous and all binding events are independent (Myszka 1997, Schuck 1997). According to this model, the kinetics of the complex formation can be described by the following equation:

$$\frac{d[AB]}{dt} = k_{on} \cdot [A] \cdot [B] - k_{off} \cdot [AB] \qquad (1)$$

The change of complex concentration [*AB*] is proportional to the response change in an SPR measurement, which can therefore be described by the following equations (O'Shannessy *et al.* 1993):

Association: $$R(t) = \frac{k_{on} \cdot [A] \cdot R_{Eq,\max}}{k_{on} \cdot [A] + k_{off}} \cdot \left(1 - e^{-\left(k_{on} \cdot [A] + k_{off}\right) t}\right) \qquad (2)$$

Dissociation: $$R(t) = R_{eq} \cdot e^{-k_{off} \cdot t} \qquad (3)$$

The association and dissociation rates are commonly obtained by non-linear least squares curve fitting of the experimental data to the Langmuir model (Duverger *et al.* 2003). The response change during the dissociation is a simple exponential decay, the shape of which is only dependent on the dissociation rate and the initial response. However, the equation describing the association is more complex, requiring the association rate, analyte concentration and maximum equilibrium response ($R_{Eq,max}$) to be determined, which can be performed using the BIAevaluation software delivered with the BIAcore SPR apparatus. Fitting the association phase is therefore preceded by fitting the dissociation data in order to determine the dissociation constant. For Langmuir type systems, the highest changes in equilibrium response are seen at concentrations around the affinity constant ($K_d = 1/K_a$), at which 50% of the binding sites are saturated (Figure 11) (Schuck 1997). Varying the concentration around this value is therefore most useful for characterising binding processes by SPR. It should be noted that the Langmuir model is only correct if the concentration of free ligand is very similar to the concentration of total ligand, i.e. if the concentration of immobilised binding partner is much lower than the concentration of dissolved ligand. If this is

not the case, a quadratic binding model must be applied, which is for example observed in isothermal titration calorimetry experiments as a sigmoidal.

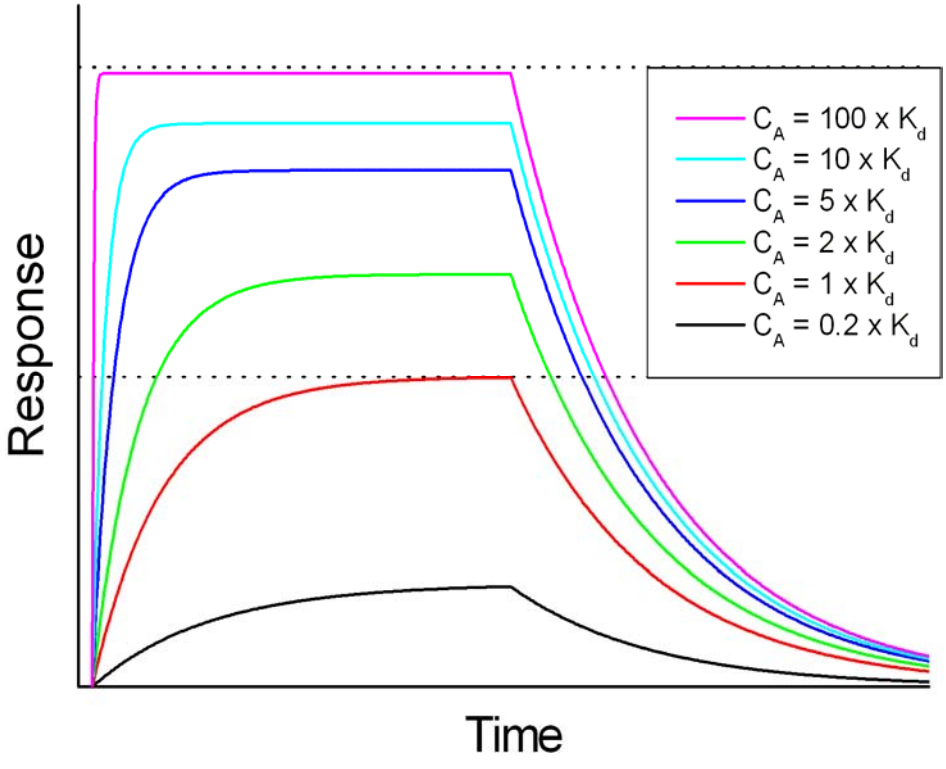


Figure 11. Sensograms predicted by the Langmuir model at different analyte concentrations (C_A). Equal association and dissociation rates were assumed and the maximum and half-maximum equilibrium responses are marked with dotted lines (top and bottom, respectively). It can be seen that the most dramatic changes are seen when the analyte concentration is varied around the dissociation constant (K_d) and that the equilibrium state is delayed at low concentrations.

As can be seen from the association equation, a variation of the on-rate measured with a Langmuir-type system will not have an effect on the shape of the recorded sensogram when the analyte concentration is matched to the affinity constant as $K_d = k_{off}/k_{on}$. Off-rate changes, however, have a high influence on the course of the association and dissociation. In systems with high off-rates this leads to rapid association and dissociation, which cannot be fitted to the Langmuir model due to the limited time resolution of the SPR apparatus (rate of data acquisition). In such cases, the dissociation constant is determined by equilib-

rium analysis, where the data is analysed as Langmuir binding isotherms by plotting the equilibrium SPR response against the solute concentration (Huber and Mueller 2006). This data fitting can be performed with common mathematical tools such as the computer programs Origin (Microcal, part of GE Healthcare) or Sigma Plot (Systat Software Inc.) and is based on the law of mass which defines K_d as follows:

$$K_d = \frac{[A]\cdot[B]}{[AB]} \tag{4}$$

Through rearrangement and replacement of [*B*] with ([*AB*]$_{max}$ – [*AB*]) this equation can be transformed into:

$$\frac{[AB]}{[A]} = -\frac{1}{K_d}\cdot[AB] + \frac{[AB]_{max}}{K_d} \tag{5}$$

Langmuir binding isotherms for the determination of K_d and $R_{Eq,max}$ can be obtained by fitting the experimental data using non-linear regression to a modified version of this equation in which [*AB*] is substituted with R_{Eq}:

$$R_{Eq} = \frac{[A]\cdot R_{Eq,max}}{[A] + K_d} \tag{6}$$

1.6 Aims of the present study

The aim of this thesis was to apply two different methods for characterisation of protein–carbohydrate interactions, namely atomic force microscopy (AFM) and surface plasmon resonance (SPR). The goal was also to take advantage of neoglycoconjugates that form self-assembled monolayers (SAMs) on gold and had been developed by a collaborator. These neoglycoconjugate SAMs present glycan structures at their surface and have been shown to be useful for studying biological recognition events. Furthermore, this thesis is concerned with the investigation of the carbohydrate binding properties of *Trichoderma harzianum* chitinase (Chit42) and the engineering of this chitinase towards binding of more complex oligosaccharides, thus creating enzymes with new substrate specificities and neolectins which bind medically important carbohydrates.

2. Materials and Methods

2.1 Methods used in published experiments

The methods used in the published experiments are briefly described here. A more detailed description of the techniques can be found in the Introduction and in the corresponding publications (see Appendix III).

2.1.1 Preparation of neoglycoconjugate SAMs

Self-assembled monolayers (SAMs) of neoglycoconjugates on gold surfaces were used in Publication I and unpublished experiments (see sections 3.3.2 and 3.3.3) to study multivalent lectin binding. The alkanethiols that were used in this experiment contain a thiol function, which oxidises at gold surfaces to form a thiolate. This function adsorbs strongly (but not covalently) at gold surfaces (see section 1.5.1). In addition, these alkanethiols interact strongly with each other through van der Waals interactions with undecanyl cores of other alkanethiols, resulting in highly organised and densely packed monolayers. At the terminus opposite to the thiol function, the used alkanethiols carry various polar functions which are included in order to either prevent non-specific adhesion (-OH, -tEG) or lead to carbohydrate-specific adsorption (-$(GlcNAc)_{1–4}$). These alkanethiols are water insoluble and were therefore dissolved in DMSO or ethanol at millimolar concentrations for SAM formation.

2.1.2 Quartz crystal microbalance with dissipation monitoring

The adsorption of alkanethiols onto gold was studied using a quartz crystal microbalance (QCM) (see Publication I). This technique allows monitoring of adsorption processes through change of the resonant frequency, as it is very sen-

sitive to mass adsorption. When SAM formation was studied, the measured mass density could be compared with other published data of SAMs with respect to packing density. Additionally, the change of mass was used to quantify the changes in crystal oscillation dampening (dissipation) upon formation of a SAM layer, which could be used to determine the stiffness of the formed layer. More details of the QCM method can be found in the Introduction (see section 1.5.3).

2.1.3 Functionalisation of AFM probes

WGA lectin was attached to PEG-tethered carboxyl functions of AFM-probes for the AFM force spectroscopy measurements presented in Publication I. This was done by firstly activating the carboxyl functions of the AFM probe tip in a solution of EDC and sulfo-NHS, followed by incubating the cantilever in a drop of lectin solution. This procedure was performed using a self-made apparatus, in which the AFM probes are placed onto a parafilm-coated plastic box with the cantilevers of all probes pointing in the same direction and the tips pointing upwards. The probes are held in place by a flat metal piece which is coated with parafilm and touches the probes only at the end opposite to the one carrying the cantilever. The metal piece is pressed onto the probes by a rubber band that is attached to the end of the metal and spanned around the plastic box. Incubation of the cantilevers in the reaction solution was performed under a humidified atmosphere in a sealed container.

2.1.4 Fluorescent microscopy with antibody-labelled AFM probes

In Publication I, the result of the coating of an AFM probe tip with WGA was investigated by fluorescent microscopy. This was done by incubating a WGA-coated cantilever and an uncoated cantilever firstly in a solution containing WGA-specific antibody, followed by incubation in a solution with a fluorescently labelled antibody, which was specific against the primary antibody. The WGA-coverage of the cantilevers could be seen as fluorescence from the secondary antibody under a fluorescence microscope.

2.1.5 Atomic force microscopy imaging and force spectroscopy

Atomic force microscopy (AFM) was used in Publication I to image the topography of the neoglycoconjugate-coated gold surface and measure the binding

force acting between WGA lectin attached to an AFM probe and the neoglycoconjugate monolayer. The latter technique was also used in unpublished experiments to measure unbinding of WGA from polymeric chitin (see section 3.2.2). In AFM, a tip mounted to a flexible cantilever is used to probe the surface by scanning an area (imaging) or performing approach–retraction cycles at the sample surface (force spectroscopy). Both techniques require control of the distance between the tip and the sample surface. This is achieved through measuring the cantilever deflection or vibration through a laser beam reflected from the top side of the cantilever and using this signal to control a piezo scanner, which adjusts the vertical position of the sample. A more detailed description of both techniques can be found in the Introduction (see section 1.5.2).

2.1.6 Surface plasmon resonance

Surface plasmon resonance (SPR) was used in the experiments of both publications and in the unpublished experiments (see sections 3.3.2 and 3.3.3) to study binding processes related to protein–carbohydrate interactions. This method is based on the use of electromagnetic waves (called plasmons) at the gold surface of an SPR sensor, which can be used to monitor adsorption and desorption processes as a change of refractive index in the proximity of the gold layer (within 150 nm). The equilibrium responses measured at different analyte concentrations were used to calculate the binding specificity of Chit42 variants and the GlcNAc-specific reference lectin for various conjugated and soluble carbohydrates. In inhibition experiments, the basis of the observed adhesion was investigated, e.g. non-specific or carbohydrate-specific WGA–SAM interactions.

2.1.7 Gene mutagenesis

In Publication II, mutations were introduced into the Chit42 gene by PCR using site-directed mutagenesis (see Appendix I). This was done by including oligonucleotides containing the desired nucleotide sequences in the PCR reaction mixture in order to create Chit42 gene variants coding for Chit42 protein variants with a modified C-terminus (added oligo His-Tag and a Gly-Ala-Cys-Thr sequence) and alterations in the substrate binding cleft (see Publication II).

2.1.8 Heterologous production of Chit42 variants in *E. coli*

The Chit42 variants discussed in Publication II and the unpublished results were produced in *E. coli* strain TOP10. Prior to expression, the bacterial cells were transformed with a pBAD-gIII construct which contained the Chit42 gene (Figure 12). The produced Chit42 protein was directed into the periplasmic compartment of the bacterial host. This led to the processing of the N-terminal gIII-signal sequence during passage through the inner cellular membrane. The cell cultivation was performed at 30ºC in shake flasks and recombinant protein production was induced by addition of arabinose to the growth medium. After completion of the protein production, the cells were harvested by centrifugation, washed and the periplasmic protein fraction was isolated by osmotic shocking, using concentrated sucrose solution.

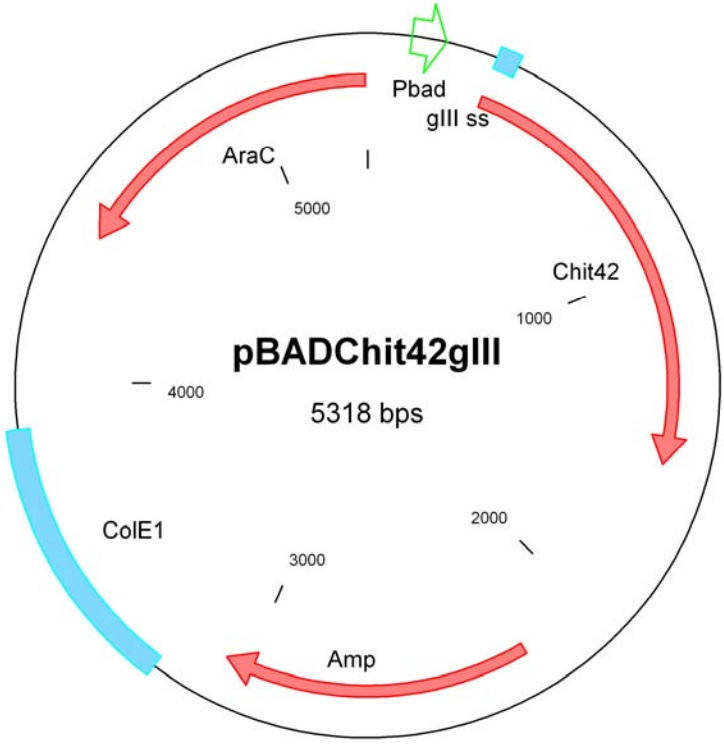


Figure 12. Plasmid map of the construct pBADChit42gIII used for recombinant expression of mutated Chit42 genes in E. coli TOP10; AraC: arabinose-dependent regulator protein of Pbad promotor; Amp: Gene conferring ampicillin resistance; Chit42: Chit42-enconding gene.

2.1.9 Protein purification

The Chit42 variants described in Publication II were purified to a single band in an SDS-PAGE gel. The purification was carried out using agarose matrices containing immobilised nickel ions to which Chit42-variants with a C-terminal His_8-tag adsorbed. Purified protein was eluted from the affinity matrix using an imidazole gradient (0.8–200 mM).

2.1.10 Circular dichroism spectroscopy

In Publication II, the effects of amino acid sequence alterations in Chit42 on its overall structure and functionality were analysed by circular dichroism (CD) spectroscopy (Kelly *et al.* 2005). By this method changes in the secondary protein structure can be detected as the difference in absorbance of right- and left-circularly polarised light. Differential absorption of both light species is encountered with compounds containing chiral chromophores and chirally disposed chromophores in a three-dimensional structure (e.g. amidic bonds in an α-helix).

2.2 Methods used in unpublished experiments

2.2.1 AFM force measurements with WGA-modified AFM probes on chitin beads

Chitin beads (New England Biolabs, MA) were immobilised on a glass disk by first applying a thin layer of UV-reactive glue (Type: NOA81, Norland Products Inc, NJ) and then dispersing dried chitin beads over the glue by flipping a plastic stick with adhered polymer. The glue was hardened by UV-irradiation (λ = 320–500 nm) for 10 s at an intensity of 10 W/cm^2 (absolute mode) using an Omni-Cure Series2000 UV-curing device (EXFO Life Sciences and Industrial Division, Canada). The immobilised beads were rehydrated by incubating them for 1 h at room temperature in sterile-filtered 10 mM HEPES buffer (pH 7.4) containing 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnSO_4$. An AFM-probe was prepared by coating with WGA lectin at a concentration of 0.24 mg/ml using EDC/sulfo-NHS cross-linking chemistry (Figure 13A). Then, the coated AFM probe tip was positioned on the center of an immobilised chitin bead (Figure 13B) and the AFM force spectroscopy measurement was started by performing approach–retraction cycles in HEPES buffer and in the presence of the binding

competitors such as free chitintetraose (100 µM and 1.0 mM) and soluble WGA lectin (0.24 mg/ml). During the AFM measurement a maximum loading force of 70 pN and loading rate of 4.8 nN/s were applied and at the end of each approach phase the tip was incubated for 1 ms at the surface before retraction.

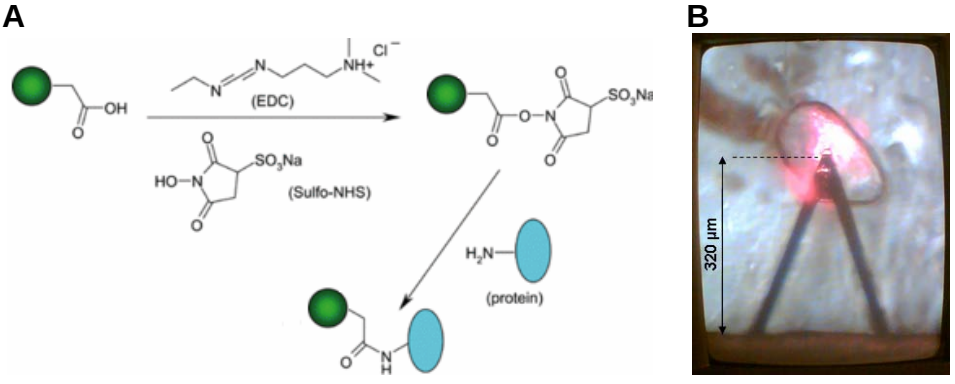


Figure 13. Functionalisation and alignment of WGA-modified probe on chitin bead. (**A**) Schematic presentation of activation of carboxyl groups at the probe by EDC and sulfo-NHS and successive protein conjugation resulting in formation of a peptide bond; (**B**) Microscopic image of how the cantilever is positioned above a chitin bead during the performed AFM force measurements.

2.2.2 SPR binding measurements on alkanethiol-based SAM containing GlcN-(GlcNAc)$_4$

A gold-coated SPR sensor was modified with an SAM of N^{I},N^{II},N^{III},N^{IV}-tetra-acetyl-chitopentaose-neoglycoconjugate (obtained from S. Cottaz *et al.*, CERMAV Grenoble, France) in a laminar air flow cabinet. In detail, this was done by covering the gold face overnight with 100 µl DMSO containing the neoglycoconjugate at a concentration of 1 mM. Afterwards, unreacted neoglyco-conjugate was rinsed off with DMSO and the sensor was cleaned with ddH_2O and dried before insertion into the SPR apparatus (BIAcore 1000, GE Health-care, Sweden). A reference surface was produced by covering a gold sensor for 2 h with ethanol containing 11-hydroxy-1-undecanethiol at a concentration of 5 mM. During this procedure, fresh ethanol had to be added frequently (about every 10 min) in order to compensate for evaporation of solvent. After coating, the surface was cleaned with pure ethanol and docked into the SPR apparatus. The SPR binding measurements were performed at a flow rate of 40 µl/min in

10 mM HEPES buffer (pH 7.4, sterile-filtered) containing 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MnSO_4$ and 0.005% surfactant P20 (GE Healthcare, Sweden).

2.2.3 SPR binding measurements on tEG-containing neoglycoconjugate SAMs

Preparation of an SPR sensor coated with a tEG-containing SAM was performed using a two step procedure. Firstly, an SAM of carboxyl-terminated tetraethylene glycol alkanethiols was formed by covering the gold surface of the sensor inside a laminar air flow cabinet for 3 h with the organosulfur compound dissolved in ethanol at a concentration of 5 mM. During this step, ethanol was added frequently in order to prevent complete evaporation of the solvent. This step was completed by rinsing the sensor with ethanol to remove unreacted solvent. The cleaned sensor was docked into the SPR apparatus (BIAcore 1000, GE Healthcare, Sweden) for the second coating step. Here, the carboxyl groups of each flow cell (FC) were first activated by injection of EDC and NHS and then conjugated to different amine ligands. The four FCs were modified with ethanolamine as a reference (FC 1) and with the di-, tri- and tetrasaccharide conjugate (FC 2, 3 and 4, respectively). The FC modification was completed by deactivation of unreacted carboxyl groups in FC 2, 3 and 4 by injection of a 1 M ethanolamine solution. For measuring lectin binding, the lectins were added to all four flow cells at a flow rate of 40 µl/min dissolved in sterile-filtered 10 mM HEPES buffer (pH 7.4) containing 0.15 M NaCl and 0.005% (w/v) surfactant P20. The used lectin concentrations were 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 µM (WGA) and 0.1, 2, 5 and 10 µM (Chit42 variant -E172Q-His_8-GACT). Sensograms were normalised by subtracting a corresponding sensogram of the reference cell and a sensogram recorded in the sample cell with buffer.

3. Results and Discussion

3.1 Contributions of co-authors to the published experiments

All experiments presented in the two publications (see Appendix III) with the exception of the synthesis of GlcNAc-neoglycoconjugates (Publication I) were carried out by myself. The neoglycoconjugate synthesis was performed by Isabel García under supervision of Prof. Soledad Penadés, who also composed the corresponding method description which can be found in Publication I. The co-authors Drs. Arja Paananen and Harry Boer contributed to the presented experiments by introducing me to the methodology of AFM imaging and force spectroscopy (Dr. Arja Paananen) and CD spectroscopy as well as recombinant protein production (Dr. Harry Boer). My supervisors Drs. Anu Koivula, Arja Paananen and Harry Boer provided me with advice on the presented experiments and participated in the preparation of both manuscripts. Prof. Sylvain Cottaz is included in the authors list to acknowledge his contribution of several neoglycoconjugates that have been used in the SPR binding experiments which are presented in Publication II.

3.2 Strength of WGA–oligosaccharide interactions at different chitinous substrates

3.2.1 Strength and specificity of WGA adsorption on GlcNAc-neoglycoconjugate SAMs

The WGA binding site presents a rather complex system as each WGA molecule possesses multiple and heterogeneous binding sites (see section 1.2.3.1) which may participate differently in binding to carbohydrate ligands. This interaction

was studied in Publication I essentially by AFM and SPR, providing estimates of the most-likely unbinding force, affinity/avidity, and minimum concentration of free oligosaccharide that inhibits the WGA–GlcNAc-neoglycoconjugate SAM interaction.

It was seen to be important that the affinity of the interaction that was probed in an AFM force spectroscopy setup (Figure 14A) should exceed a certain strength, because only binding of WGA could be resolved in AFM force spectrograms as weak unbinding events at an unbinding force of 47 ± 15 pN (Figures 14B–D), whereas no such interaction was detectable with an AFM probe with attached Chit42. The reason for this difference in binding strength was believed to be due to an avidity effect that was further studied in the SPR experiments (see section 3.3.1). One conclusion of this thesis is that non-specific adhesion on alkanethiol-based SAMs can mask specific binding events and must therefore be minimised. The nature of the observed binding events was therefore investigated by the addition of soluble chitintetraose, which inhibited the unbinding event at forces of 47 pN ± 15 pN and less frequently also at 120 ± 20 pN in a concentration-dependent manner. This indicated that the observed interaction at 47 ± 15 pN was due to specific WGA recognition of the monosaccharide that was presented on the SAM. It was further demonstrated that even binding of multiple WGA molecules or binding sites resulting in bond rupture forces of 120 ± 20 pN can be resolved in this setup. In order to obtain the displayed data the experimental parameters such as lectin density on the AFM probe, cantilever approach and surface preparation had to be optimised, which required recording of several thousands of force curves. In conclusion, the AFM force spectroscopy method offers important insight into the molecular interactions during binding but requires significant preparative efforts which should be considered before such measurements are undertaken.

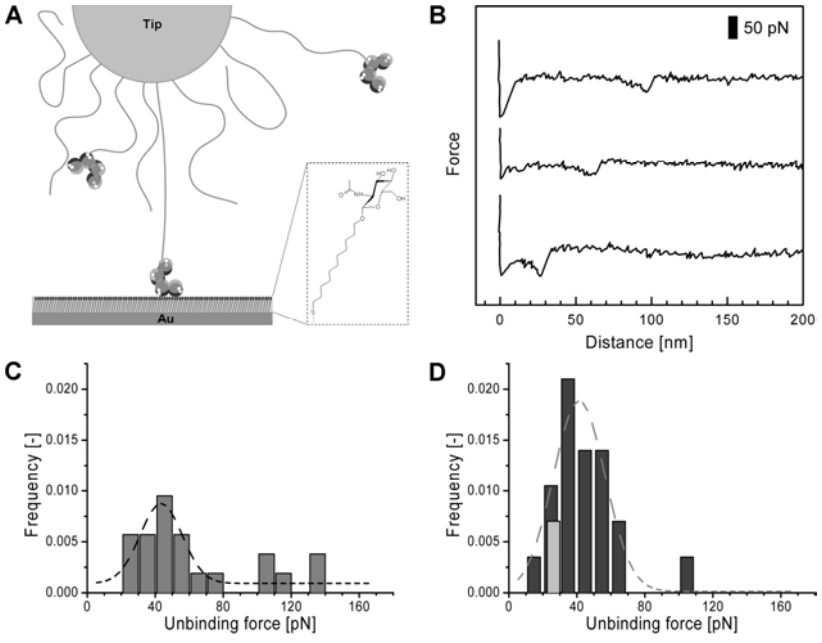


Figure 14. AFM force measurements on a neoglycoconjugate-SAM surface with a WGA-modified probe at pH 7.4. (A) A schematic presentation of the neoglycoconjugate-coated gold surface and the AFM tip to which WGA dimers are attached through PEG linkers. WGA binding sites are indicated as white squares and the structure of the neoglycoconjugate is displayed in the inset. (B) Retraction phases of three typical force spectrograms showing the tip–sample surface separations at which proposed specific unbinding occurred at forces of around 40 pN. (C) A histogram of the frequency of unbinding events measured in the buffer. (D) A histogram of the frequency of unbinding events measured in the presence of 100 µM (dark gray) and 1 mM chitintetraose (light gray). The histograms are based on datasets comprising 526 (in C) and 285 (in D, both measurement sets) individual spectrograms. The dotted lines represent Gaussian fits from which the most likely unbinding forces were determined. Figure adapted from Publication I.

3.2.2 Significance of carbohydrate ligand length for WGA binding

Lectins bind carbohydrates typically in a multivalent fashion where each binding site usually interacts with a single monosaccharide or a short oligosaccharide stretch. The plant lectin WGA is a dimer which contains eight putative substrate binding sites (section 1.2.3.1, Wright 1992). It is still, however, debated how many functional binding sites exist in dimeric WGA. As pointed out above and in Publication I, WGA bound specifically to GlcNAc residues on immobilised neoglycoconjugate SAMs, which suggested multivalency of the binding. The

WGA unbinding forces were tested further in AFM force spectroscopy experiments using polymeric chitin in the form of chitin beads (New England Biolabs, MA) immobilised on a glass disk (see section 2.2.1) and by probing a bead with a WGA-modified AFM probe (Figure 15).

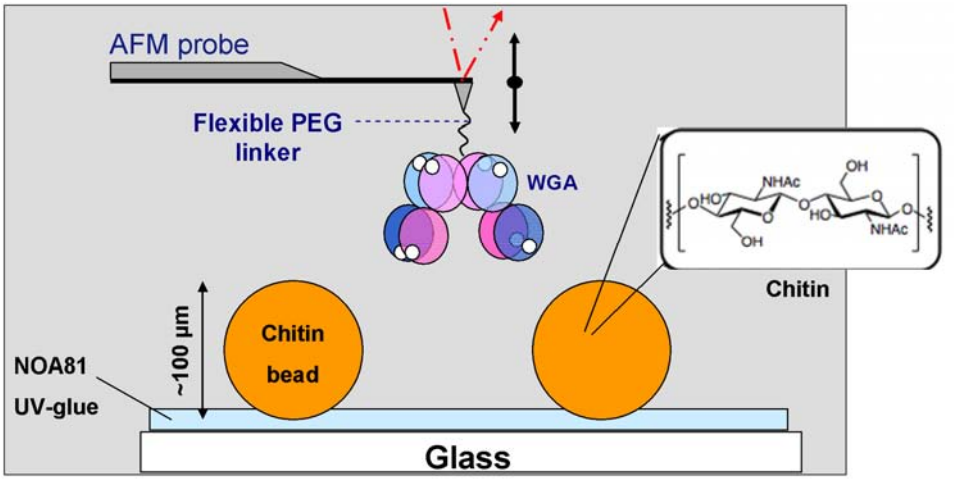


Figure 15. Setup and glycans used in recent carbohydrate binding measurements. Glycans were used in recent protein–carbohydrate interaction studies: polymeric chitin beads (~100 µM diam.). Chitin beads were attached to a glass support using a UV-sensitive glue and probed with a WGA-modified AFM probe.

In the absence of a competitor, complex force profiles were recorded which contained multiple peaks representing weak and strong adhesion over a wide range of tip–sample separation (Figure 16). In the presence of a competitor, 100 µM $(GlcNAc)_4$, clearer unbinding events could be seen and force profiles indicated sequential detachment of the lectin-modified tip from different parts of the polymer at an unbinding force of about 250 pN. The profiles suggested effective inhibition and GlcNAc-specificity of the previous detected interactions. No unbinding events with tether-stretching were observed in the presence of WGA (competitor) solution, also supporting the specificity of the binding events observed with and without chitintetraose.

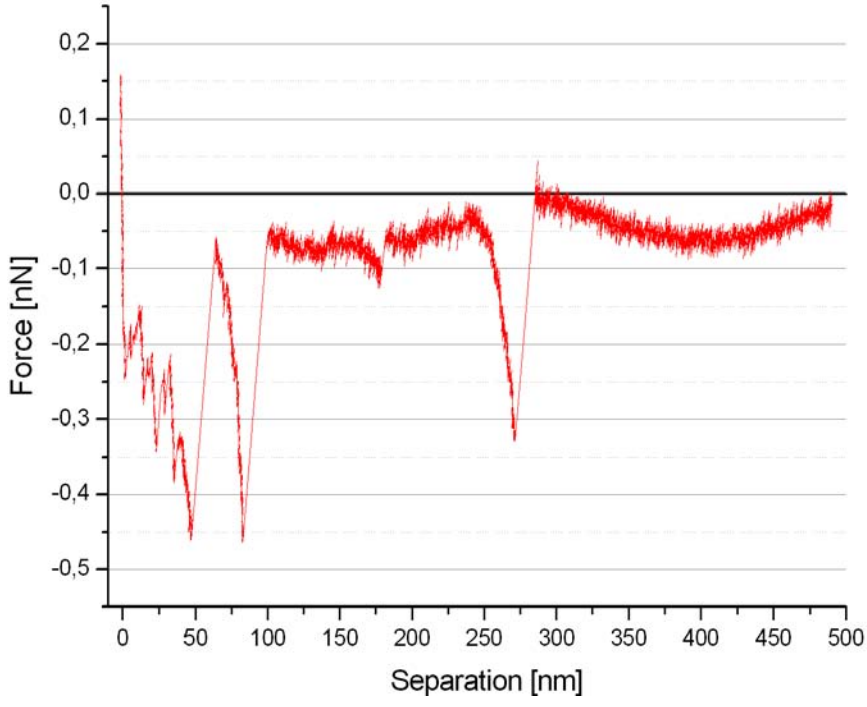


Figure 16. Typical force spectrogram obtained with a WGA-modified AFM probe (k = 0.024 N/m) on a chitin bead in HEPES buffer (pH 7.4). Multiple unbinding events can be seen at different rupture forces which are preceded by tether-stretching.

The multiple unbinding events observed at around 250 pN in the presence of 100 µM $GlcNAc_4$ on the polymeric substrate are in line with the measurements performed on a neoglycoconjugate SAM, where a force of around 47 pN (see above and Publication I) was found to be characteristic for the interaction with a monosaccharide. The five-fold increase of the binding force by the participation of additional WGA binding subsites is rather low when compared to previously performed microcalorimetry studies, which showed that WGA bound GlcNAc with a 30-fold lower affinity than $(GlcNAc)_3$ (Bains *et al.* 1992). Although both measurements indicate tighter binding as a result of increased substrate length, identification of a direct correlation of affinity with unbinding forces is difficult. This is partly due to the fact that the affinity is constant at ambient conditions, whereas the unbinding force determined in AFM experiments increases linearly with the logarithm of the applied loading rate (Evans and Ritchie 1997).

3.3 Importance of multivalent ligand presentation for specific (neo)lectin binding

3.3.1 Comparison of WGA binding to soluble and SAM-coupled chitinous ligands

In Publication I, SPR measurements were conducted in order to determine the affinity of WGA for different chitinoligosaccharides and to characterise the WGA binding to GlcNAc-neoglycoconjugate SAMs. The simplest interaction that can be realised with lectins containing several binding sites is binding of dissolved carbohydrate ligands, as each binding site interacts independently with a ligand. Binding data recorded in such a system can be used to determine the affinities of the binding sites using a simple 1:1 interaction model. In the case of chitinoligosaccharide binding to WGA, this could only be done using steady-state analysis as the oligosaccharide association and dissociation was found to be a very rapid process. Data analysis was performed by fitting isotherms defined by a bimodal (Langmuir) interaction model to equilibrium response levels obtained at different oligosaccharide concentrations (Figure 17). The deduced dissociation constants (K_d) were 160 μM for $GlcNAc_2$, 45 μM for $GlcNAc_3$, 47 μM for both $GlcNAc_4$ and $GlcNAc_5$, and suggested the presence of three subsites in each binding site of WGA.

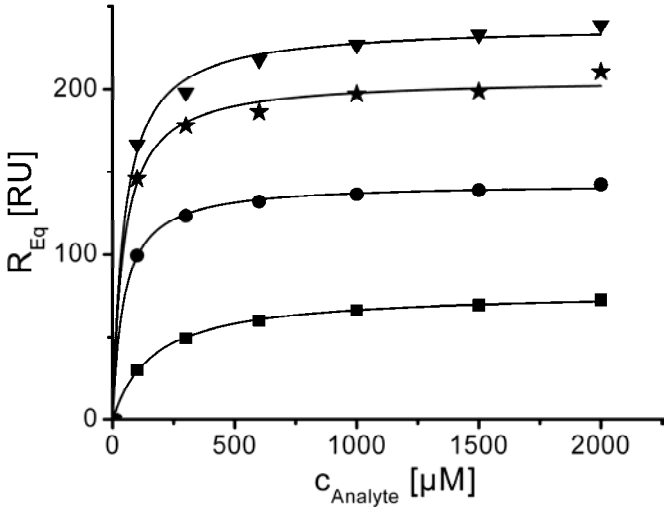


Figure 17. Analysis of the WGA binding to soluble chitinoligosaccharides at pH 7.4 by SPR. Equilibrium binding curves are shown for the binding of the dissolved saccharides $(GlcNAc)_2$ (square), $(GlcNAc)_3$ (circle), $(GlcNAc)_4$ (star), and $(GlcNAc)_5$ (triangle) to the WGA-modified sensor surface. The data were obtained from the response at equilibrium (REq) for the applied concentration of chitinoligosaccharides (0.1, 0.3, 0.6, 1.0, 1.5, and 2.0 mM in each case). The calculated dissociation constants were 165, 45, 44, and 47 µM for $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$, respectively. Adapted from Publication I with permission from the publisher.

In order to compare multivalent and monovalent binding, soluble WGA lectin was added to GlcNAc-neoglycoconjugate SAMs, which should show avidity-driven binding under simultaneous participation of several WGA binding sites. The resulting binding is therefore more complex than the previously described interaction and cannot be analysed using the Langmuir model as it describes monovalent binding of a homogeneous ligand pair. In the experiments of Publication I, clear WGA binding to the SAM could be seen (Figures 18A and 18B), which was strongest on SAMs that were entirely composed of GlcNAc-exposing neoglycoconjugates (Figures 18A and 18C). In a similar manner as performed with the AFM force spectroscopy experiments, the specificity of the observed interaction was investigated by addition of free ligand as a competitor. Here, complete inhibition of WGA binding to the SAM was observed at around 50 µM (Figure 18D). This observation indicated that the measured binding was related to interaction of the lectin carbohydrate binding sites with the monosaccharide function rather than to non-specific adhesion.

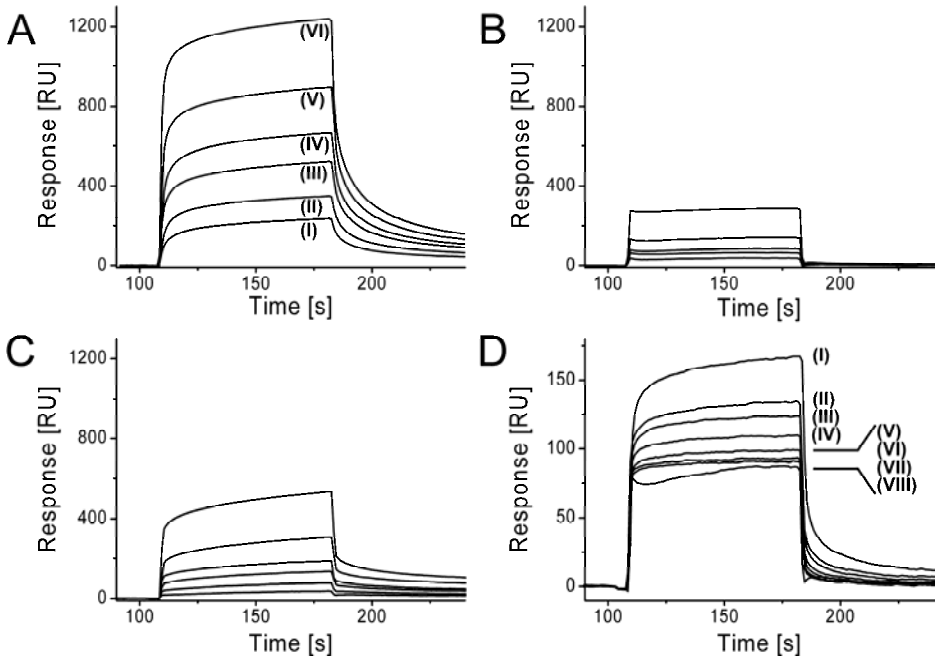


Figure 18. Binding of WGA to the SAM surfaces measured with SPR at pH 7.4. Sensograms showing WGA solutions injected at six different concentrations of 0.05, 0.1, 0.2, 0.3, 0.5, and 1.0 mg/ml (I–VI) onto a GlcNAc-neoglycoconjugate-SAM surface (A), on a reference surface functionalized with a hydroxylated 11-thioundecanyl linker (B), and on a surface coated with a 1:10 dilution of neoglycoconjugate and linker (same order of injected solutions I–VI in all three graphs) (C). (D) Inhibition of WGA (0.3 mg/ml) binding to a GlcNAc-neoglycoconjugate SAM in the presence of seven different concentrations of soluble chitintetraose, a competitor. The upper seven sensograms represent experiments in which the oligosaccharide was present at concentrations of 5.0, 12, 18, 30, 42, 53, and 65 μM (I–VII). The response change due to the refractive index of the injected WGA solution (VIII) was measured on a linker SAM surface, for which no binding of WGA is detected. Adapted from Publication I with permission from the publisher.

SPR data analysis is not limited to systems of 1:1 interactions but may also be used to quantify binding strength of bivalent interactions. The sensograms recorded with WGA on GlcNAc-neoglycoconjugate SAMs were therefore subjected to model fitting in order to clarify whether the bivalent or monovalent interaction model can be used to characterise the studied interaction system. This analysis showed that neither of these two models was able to predict the concentration-dependent association and dissociation observed with WGA (see Figure 19). This indicated that the multivalent lectin–ligand interactions are strong but complex and cannot readily be quantified.

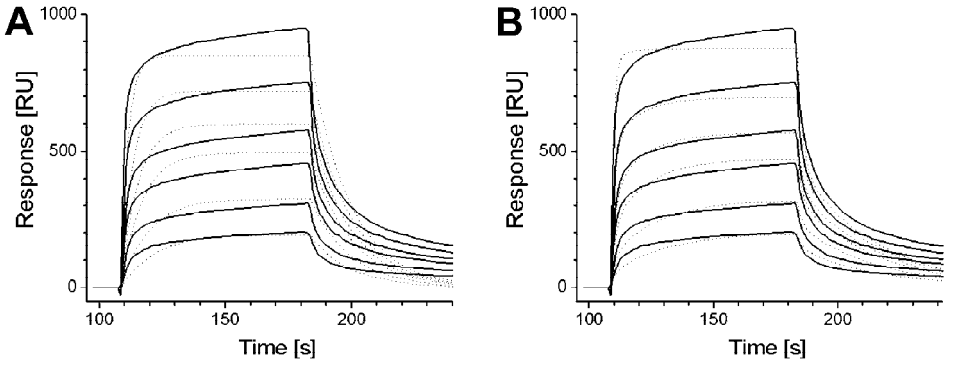


Figure 19: SPR data fitting of WGA binding to the neoglycoconjugate-SAM using two different binding models. The sensograms shown in Figure 18A have been fitted to monovalent (Langmuir) (A) and bivalent binding models (B). The time intervals used for fitting the association and dissociation were 111–144 s and 196–216 s, respectively. Injection start and end were set as 109 and 184 s, respectively. Before data fitting, the reference curves were subtracted from the sample curves. Statistical analysis showed that both models were inadequate to describe the experimental data (solid lines), as indicated by the obtained closeness of the fit ($\chi 2$) of 2.44×10^3 (A) and 572 (B) and the deviation of the fitted sensograms (dashed lines) from the measured data. Adapted from Publication I with permission from the publisher.

The importance of multivalency during WGA binding could be demonstrated in SPR binding experiments which showed that lectin did not bind to soluble GlcNAc within the available concentration range (up to 5 mM), but strongly interacted with the GlcNAc-neoglycoconjugate SAMs. It was thereby shown that multivalent binding including several binding sites can increase the binding strength significantly beyond the affinity of the individual binding sites for a certain ligand. This finding can be useful for the development of high-affinity neolectins binding specifically to short ligands through cooperative binding involving several subsites.

3.3.2 Chit42 binding to alkanethiol-based SAM containing GlcN-$(GlcNAc)_4$

As seen for WGA, the length of the oligosaccharide can have a great effect on the binding affinity. In the case of the *T. harzianum* chitinase (Chit42), a single binding cleft provides interactions for a polymeric substrate at seven putative subsites (see section 1.2.1.1 and Figure 4). The binding affinities measured with small chitinous carbohydrates such as GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$ were

very low (below the detection limit of 5 mM; Publication II) and longer chitinoligosaccharides were therefore chosen for studying the carbohydrate binding properties of the catalytically inactive Chit42 variant D170A/E172Q-His_8-GACT.

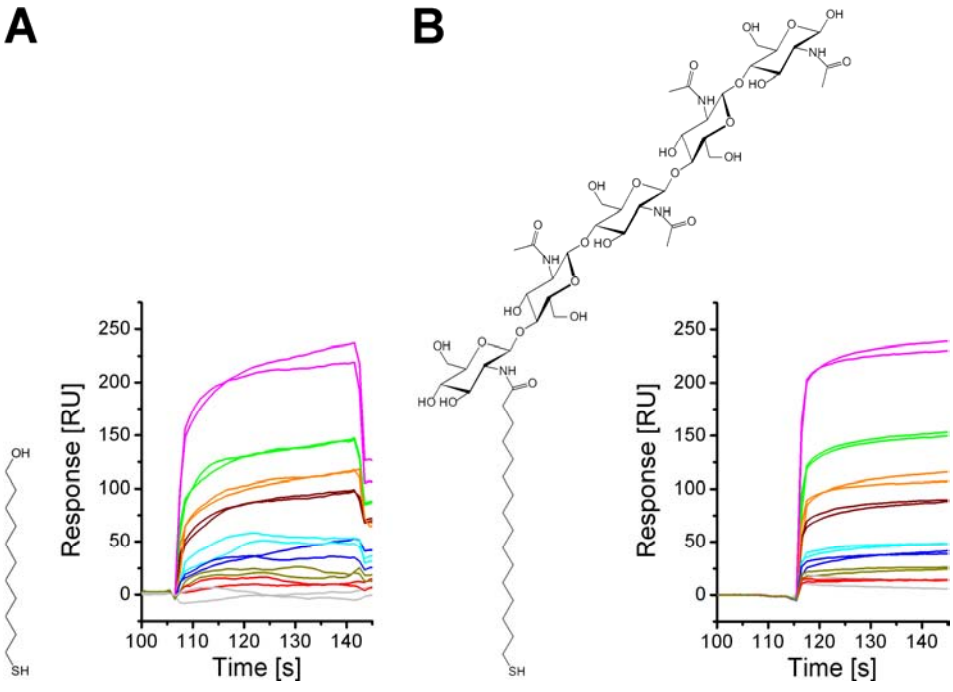


Figure 20. Non-specific adhesion of Chit42 to alkanethiol-based SAMs. (A) SPR signal changes measured with a sensor coated with 11-hydroxy-1-undecanethiol (chemical structure given on the left) upon injection of buffer (gray) and the Chit42 variant D170A/E172Q-His_8-GACT at concentrations of 0.02 (red), 0.04 (olive), 0.07 (blue), 0.1 (cyan), 0.2 (brown), 0.28 (orange), 0.4 (green) and 0.7 mg/ml (magenta); (B) SPR signal changes measured with a sensor coated with N^{I},N^{II},N^{III},N^{IV}-tetra-acetyl-chitopentaose-neoglycoconjugate (chemical structure given on the left). The depicted partial sensograms were recorded with the same solutions as described in (A).

Through collaboration with S. Cottaz *et al.* (CERMAV Grenoble, France), a pentasaccharide-containing neoglycoconjugate was obtained that exposes a chitintetraose to the solution when it is immobilised as a substrate-supported SAM. In detail, the neoglycoconjugate is N-terminally deacetylated chitinpentaose coupled to hexadecanethiol from its non-reducing end (Figure 20). This compound is soluble in DMSO and forms SAMs on gold in a millimolar solu-

tion. During SPR measurements with Chit42 on a SAM of unconjugated 11-hydroxy-1-undecanethiol (see section 2.2.2), high non-specific adhesion was observed (Figure 20A), which was believed to be due to hydrophobic interaction with the underlying linker. Similar adhesion was seen with the pentasaccharide-containing SAM, and detection of specific adhesion to the conjugated chitinoligosaccharide was virtually impossible (Figure 20B). In order to improve the sensitivity of this method, addition of salt or polyhydric compounds such as glycerol of polyethylene glycol could be considered, as they increase protein stability (Gekko and Timasheff 1981) and might therefore promote specific adhesion.

3.3.3 Effect of tEG linkers on non-specific adhesion to neoglycoconjugate SAMs

Simple alkanethiol-based SAMs were seen to be prone to non-specific adhesion of Chit42, and new ways to present carbohydrates on sensor surfaces were therefore sought. Corresponding to this, during a meeting of the Marie Curie Research Training Network "GlycoGold" (see Appendix II) Prof. Soledad Penadés suggested the use of tetraethylene glycol (tEG)-containing SAMs which were used in her laboratory previously in carbohydrate–carbohydrate interaction experiments (Hernaiz *et al.* 2002) and could prevent non-specific protein adhesion (Ostuni *et al.* 1999). A series of amino-functionalised neoglycoconjugates was obtained from M. Lahmann *et al.* (Bangor University, U.K.), which comprised conjugates to chitinbiose, -triose and -tetraose (Figure 21). For immobilisation of these compounds, a carboxy-terminated linker was required which was provided by S. Penadés *et al.* (CIC biomaGUNE and CIBER-BBN, San Sebastian, Spain). This linker is soluble in ethanol and forms SAMs on gold in a similar fashion as the neoglycoconjugates discussed previously. The linker contains a hydrophilic tEG function which will render the resulting sensor surface resistant to non-specific protein adhesion. The attachment of the linker was done outside the BIAcore device as the compound was soluble in ethanol, which is incompatible with the SPR apparatus. The linker-covered sensor was rinsed with solvent and docked to the SPR device in order to immobilise the glycoconjugates. The procedure could be performed inside the SPR apparatus due to the water solubility of the neoglycoconjugates. This strategy is advantageous for studying binding of lectins as the ligand attachment can be monitored through a change of the refrac-

tive index and four different modifications can be studied in parallel, using the four flow cells for optimal referencing (Possible with models BIAcore2000 and BIAcore3000).

The responses obtained from the four flow cells indicated that non-specific adhesion at the reference cell was low compared to the mass adhesion detected in the carbohydrate-containing flow cells. This was a significant improvement compared to the tEG-free SAM and this assay format can be useful as it offers the possibility of comparing simultaneously the effects of oligosaccharide length and multivalency. This method could complement solution-based methods such as ITC where more protein sample is required and only a single interaction pair can be tested at a time.

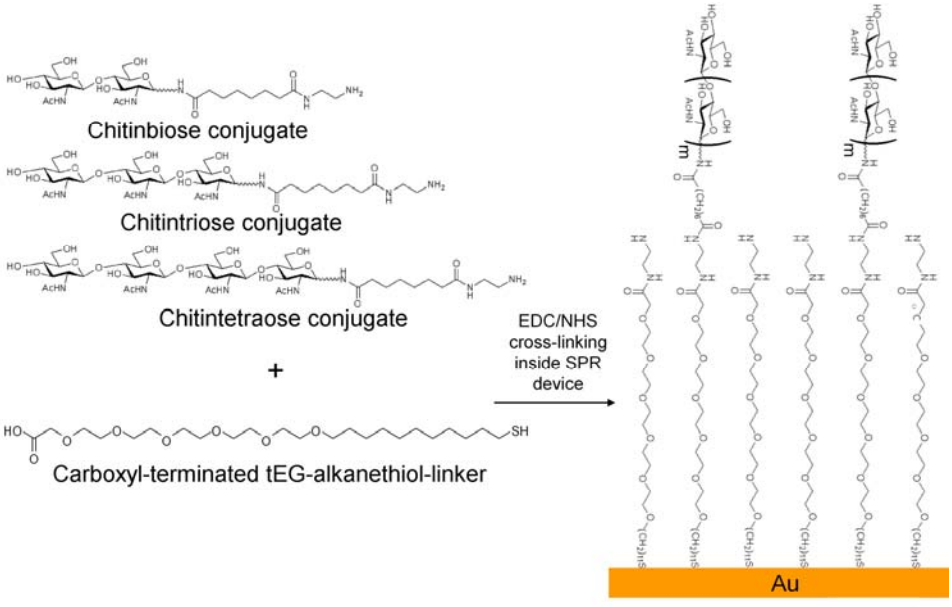


Figure 21. tEG-Based neoglycoconjugate SAM formation using EDC/NHS coupling, Reaction scheme of chitinoligosaccharide neoglycoconjugate SAMs (m = 1, 2 or 3) prepared by carbodiimide coupling on gold-covered SPR sensors.

3.4 Creation of (neo)lectins based on glycoside hydrolases

3.4.1 Characterisation and engineering of the Chit42 binding site

The three-dimensional structural model of Chit42 in complex with an oligosaccharide (Boer *et al*. 2004) suggested that chitin is bound in the substrate cleft through hydrogen bonds and aromatic stacking (Figure 22). Based on this complex structure, amino acids were substituted that were suggested either to participate in binding in the GlcNAc units at subsites -3, -2 or +2 or to be important for the catalysis. This was done in order to determine the role of these amino acids in binding and also to identify positions resulting in the highest changes in binding affinity or selectivity.

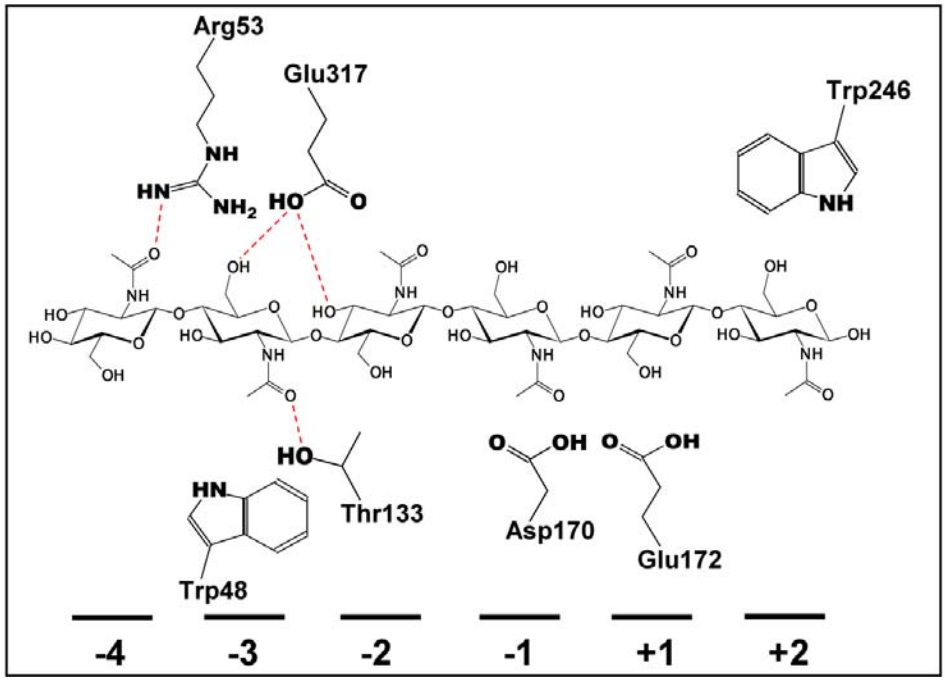


Figure 22. Location of the mutated amino acid residues in relation to a bound substrate ($(GlcNAc)_6$) occupying binding sites -4 → +2 is displayed, also including the proposed hydrogen bonds. Modified from Publication II with permission from the publisher.

Hydrogen-bond forming residues at subsites -3 and -2 were modified as several chitinoligosaccharide-resembling substances were available that were likely to bind to these sites with their non-chitinous parts. The aromatic amino acids in subsites -3 and +2 were exchanged against alanine in order to determine the preferred binding mode of short oligosaccharides, i.e. to which subsites they preferably bind. The mutants were expressed in *E. coli* TOP10 at 30ºC, extracted from the periplasmic fraction and purified by Ni-ion affinity chromatography. For all variants, except for the Arg53Leu, this procedure yielded concentrated and highly pure protein solutions (c = 1.4–5.1 mg/ml) which were suitable for studying carbohydrate binding by SPR. Based on the homology model it was concluded that the aliphatic part of the arginine side chain was important for the structural integrity of the protein and could not be compensated by the shorter and more bulky side chain of leucine.

The binding affinity of the different Chit42 variants towards the three oligosaccharides Gal-$(GlcNAc)_4$, GlcN-$(GlcNAc)_4$, Gal-$(GlcNAc)_2$-Me-umbelliferone, and the chitinoligosaccharides $(GlcNAc)_{4–6}$ was determined by immobilising the variants on SPR sensors and adding of the dissolved ligands. Here, characteristic association and dissociation kinetics were observable in which step-shaped sensograms indicated rapid dissociation of the Chit42–ligand complex and curved sensograms were indicative of binding and unbinding at a lower rate (Figure 23). For Gal-$(GlcNAc)_2$-Me-umbelliferone, no saturation was observed within the available concentration range (up to ~500 μM), which indicated that this ligand is bound at a much lower affinity than the remaining compounds (Table 3). The inhibitor allosamidin, on the other hand, saturated the binding site of wt Chit42 already at nanomolar concentrations, indicating high-affinity binding (Figure 23). This demonstrates the importance of steric complementarity of the solvated ligand and binding site (Boraston *et al.* 2004), which is lowest in the case of the umbelliferyl compound and consequently resulted in the lowest detectable affinity.

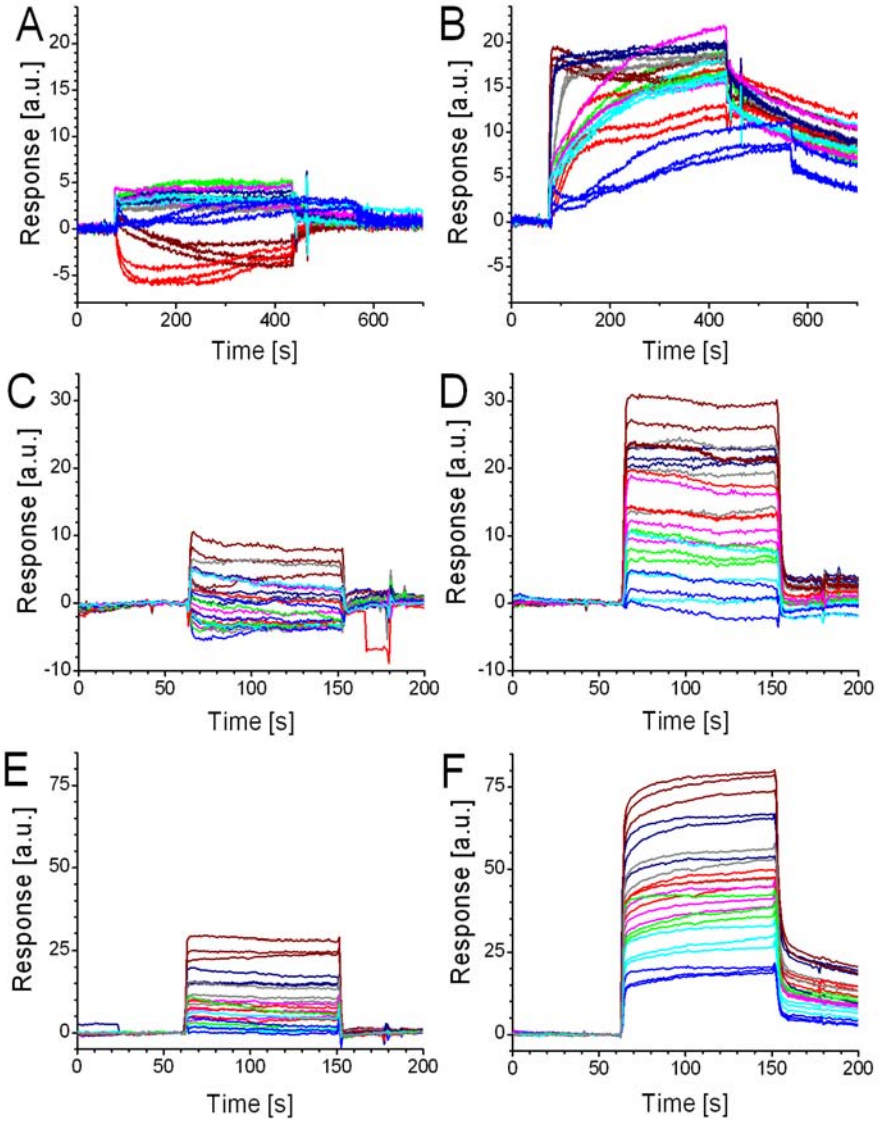


Figure 23. Examples of sensograms recorded with different Chit42 mutant–oligosaccharide combinations. (A and B) Reference and Chit42-His_8-GACT (immobilization level (IL) = 2862 RU) with allosamidin at 3 nM (blue), 6 nM (cyan), 8 nM (green), 10 nM (magenta), 30 nM (red), 50 nM (gray), 200 nM (dark blue), and 500 nM (brown); (C and D) reference and Chit42-E172Q-His_8-GACT (IL = 3137 RU) with $(GlcNAc)_5$ at 1 µM (blue), 2.5 µM (cyan), 5 µM (green), 10 µM (magenta), 25 µM (red), 40 µM (gray), 150 µM (dark blue), and 200 µM (brown) and (E and F) reference and T133Q/E172Q-His_8-GACT (IL = 3864 RU) with Gal*β*-4(GlcNAc)4 at 5 µM (blue), 10 µM (cyan), 20 µM (green), 30 µM (magenta), 50 µM (red), 100 µM (gray), 150 µM (dark blue), and 250 µM (brown). Adapted from Publication II with with permission from the publisher.

Table 3. Dissociation constants for the binding of chitinoligosaccharides to His_8-GACT-tagged Chit42 wt and variants determined by SPR at pH 6.5 and 25ºC. The affinities were determined by fitting a Langmuir model to equilibrium responses at different ligand concentrations as triplicate measurements. The given error is an estimate obtained through the models. (Adapted from Publication II with permission from the publisher.)

		Ligand affinity/K_d (μM)						
Chit42 mutant	Altered subsite[a]	$(GlcNAc)_4$	$(GlcNAc)_5$	$(GlcNAc)_6$	$(GlcNAc)_8$	Galβ-4$(GlcNAc)_4$	GlcNβ-4$(GlcNAc)_4$	Allosamidin
E172Q	−1/+1	12 ± 3	6.2 ± 0.5	0.29 ± 0.03	0.26 ± 0.05	13 ± 3	2.7 ± 0.4	10 ± 4
W48A/E172Q	−3	120 ± 16	199 ± 142	87 ± 11	n.d.[b]	167 ± 41	422 ± 71	n.d.
E317A/E172Q	−3/−2	48 ± 4	37 ± 6	1.6 ± 0.3	n.d.	59 ± 8	17 ± 3	n.d.
T133D/E172Q	−3/−2	25 ± 2	30 ± 4	2.7 ± 0.2	n.d.	39 ± 5	3.0 ± 0.3	n.d.
T133N/E172Q	−3/−2	7.5 ± 0.6	6.0 ± 0.5	0.34 ± 0.08	n.d.	8.3 ± 1.6	2.1 ± 0.4	n.d.
T133Q/E172Q	−3/−2	21 ± 3	30 ± 3	1.7 ± 0.2	n.d.	28 ± 2	10 ± 4	n.d.
D170A/E172Q	−1/+1	12 ± 3	2.9 ± 0.4	0.10 ± 0.02	0.19 ± 0.05	14 ± 4	2.0 ± 0.3	23 ± 6
W246A/E172Q	+2	326 ± 79	381 ± 129	22 ± 3	n.d.	548 ± 110	138 ± 17	n.d.
wt	–	n.d.	n.d.	n.d.	(n.d.)[c]	n.d.	n.d.	0.003 ± 0.001

[a]Referring to position of the amino acid substitution, other than the E172Q in the double mutants.
[b]n.d. = not determined.
[c](n.d.): Could not be determined for wt/octaose apparently due to hydrolysis.

A comparison of the affinities measured with the different Chit42 variants shows that the modifications of the active site could clearly be sensed as a change of inhibitor affinity. Furthermore, it was shown that the probed stacking interactions were affecting binding of all compounds similarly by decreasing the affinity below 15% of that of the variant with both aromats present (Figure 24). Substitutions of threonine at position 133 were seen to be most effective for changing the binding specificity. Here, a substitution with aspartic acid retained the affinity of the inactivated wt Chit42 for GlcN-$(GlcNAc)_4$ and decreased at the same time the affinity for the other tested ligands by 50 – 90%. This particular mutation creates a counter charge at subsite -3 for the basic glucosamyl-residue of GlcN-$(GlcNAc)_4$. The resulting change in binding selectivity indicates that carbohydrate binding sites can be engineered towards binding of basic or acidic oligosaccharide ligands through introduction of charged residues. This type of attractive interaction is well known for other glycoside hydrolases that act on charged substrates. An example is the hyaluronate lyase which achieves tight but fluid binding of its anionic substrate through basic residues in its binding site (Breyer and Matthews 2001). It is noteworthy that the attractive interactions created by this modification did not require a strict alignment of the participating atoms, which is important for the introduction of hydrogen bonds. This difficulty exemplifies why rational protein design is often limited by the understanding of the molecular basis for a desired function due to the complexity of atomic interactions within a given protein (Arnold 2001).

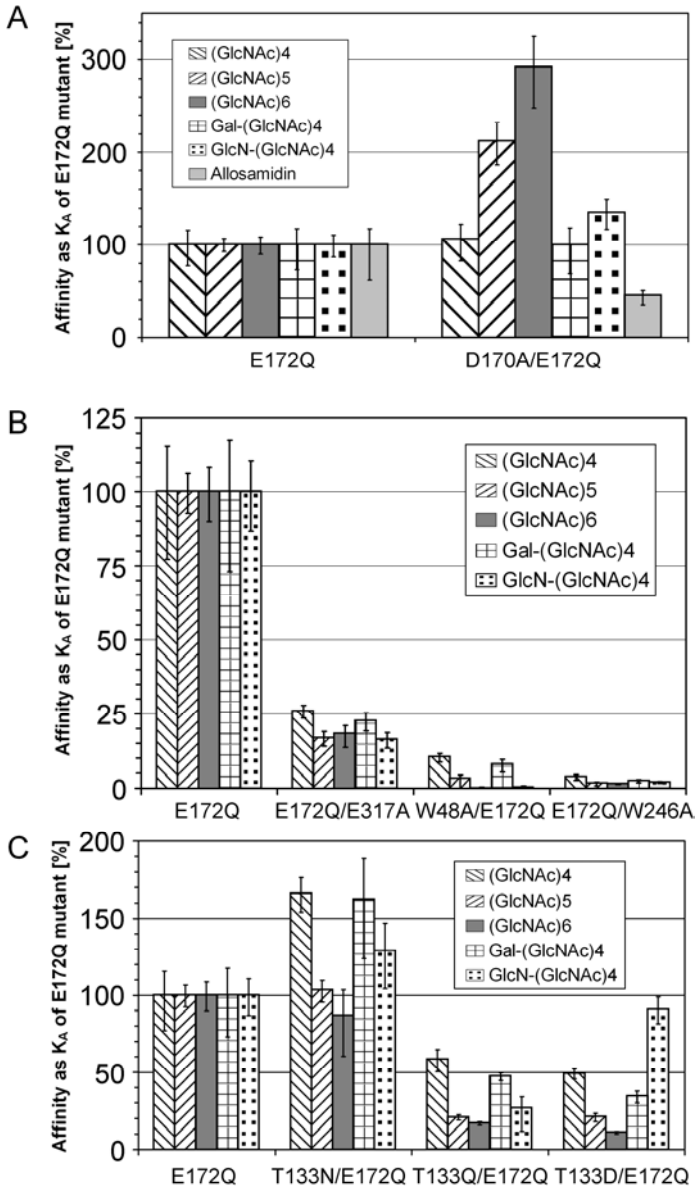


Figure 24. Relative affinities of Chit42 binding-site mutants compared to the reference protein Chit42-E172Q-His_8-GACT. The affinities were determined as K_d values by fitting a Langmuir model to REq-values at different ligand concentrations as triplicate measurements. The given error is an estimate obtained through the model. Relative affinities of the mutant D170A located between subsites -1/+1 (A), of W48A at subsite -3 and W246A at +2, as well as of the E317A located between subsites -2/-3 (B), and of T133N, T133Q, and T133D mutants located between subsites -2/-3 (C). Adapted from Publication II with permission from the publisher.

Based on the presented Chit42 variants, neolectins with higher affinities could be created by strengthening the lectin character of the neolectin binding site. For this, it might be helpful to compare the binding sites of lectins and glycoside-hydrolase-associated CBMs in order to understand the determinants of lectin-type binding. Similarly to lectins, CBMs bind their ligands in a conformation closely resembling the solvated state that results in a minimisation of the energetic penalty paid upon binding (Boraston *et al.* 2004). However, these two classes of carbohydrate-binding proteins differ in the number of hydrogen bonds formed during carbohydrate binding. In lectins about 3.4 hydrogen bonds are formed per 100 $Å^2$ of buried polar surface area, whereas in CBMs it is estimated that only about two hydrogen bonds are involved in ligand binding per 100 $Å^2$ of buried polar surface area (Boraston *et al.* 2004). This indicates that hydrolysis-associated binding involves fewer hydrogen bonds than lectin binding and suggests that the lectin character of neolectins could be strengthened by the introduction of additional hydrogen-forming residues into the carbohydrate binding cleft. Additionally, binding affinity could be increased (as shown in Publication II) by releasing strain of the substrate bound in the active site of the Chit42 wt.

3.4.2 Structural similarities between Chit42 and other chitinases

Among chitinases, *Serratia marcescens* chitinase ChiA and ChiB belong to the best studied enzymes. Both enzymes share similar sequence identity with Chit42 (see Table 2) but process chitin from different ends (ChiB from the non-reducing end and ChiA from the reducing end). Chi42 processes chitinous substrates from the same end as ChiA, which is therefore considered as a suitable reference for structure–function correlation. Despite the low sequence identity of 28%, overall structural consistency of the Chit42 and ChiA structures can be seen (see Figure 25). When comparing the two structures, it is apparent that the greatest deviations are present at the N-terminus, which, in the case of ChiA, contained an additional chitin-binding domain and two consecutive α-helix sequences between the second β-sheet and α-helix of the ChiA $(\beta\alpha)_8$-barrel. The latter structure shields the bound substrate against the solvent and might provide more interactions in the binding cleft when compared to Chit42. As can be seen from Figure 4, in the Chit42 model a double β-sheet is inserted into the $(\beta\alpha)_8$-barrel at the same position where the unique double α-helix structure is found in ChiA (between the second β-sheet and α-helix). However, the corresponding double β-sheet in Chit42 faces away from the substrate binding cleft and allows the

substrate more conformational freedom when compared to ChiA. It is probable that the additional domain in Chit42 has a similar role for enzyme stability, catalytic activity and/or specificity as that shown for ChiA (Zees *et al*. 2009).

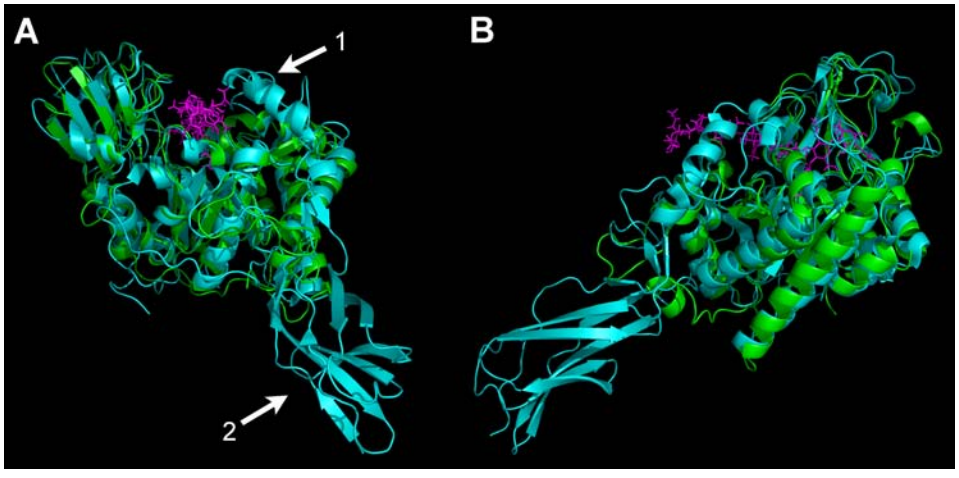


Figure 25. Structural alignment of the homology model of *Trichoderma harzianum* chitinase ChiA (green) and *Serratia marcescens* chitinase ChiA (cyan) in complex with the substrate chitinoctaose (magenta). The ChiA structure was determined by Papanikolau *et al.* (Papanikolau *et al.* 2001) (PDB-ID 1EHN). (A) View along the substrate axis with ChiA specific structural features marked: (1) V-shaped double *α*-helix insertion between second *β*-sheet and *α*-helix of the $(\beta\alpha)_8$-barrel and (2) N-terminal chitin binding domain. (B) View after a 90º leftward rotation. The high degree of structural identity is visible. Image created using PyMol (DeLano 2002).

4. Conclusions

This thesis investigated the interactions between proteins and carbohydrates and the strategies for modifying the ligand specificity of proteins through rational protein design. The study also examined the effect of multivalent binding on the binding affinity of lectins. The main research techniques were SPR and AFM, which were used to compare the affinity of modified Chit42 proteins and native lectin for various carbohydrates and resolve to the binding force acting between a single protein and GlcNAc-exposing SAM and, alternatively, polymeric microscopic substrates.

The SAM-related work of this thesis shows that this surface modification can easily be prepared and that it resembles biological membranes. Furthermore, it was shown that it is compatible with several techniques suitable for studying adhesion to surfaces, such as AFM, SPR, QCM and electron microscopy. Moreover, comparisons of proteins binding to both dissolved and SAM-immobilised chitinous carbohydrates provided evidence for the multivalent WGA–chitinoligosaccharide interactions. SPR experiments with soluble chitinoligosaccharides provided proof for the existence of three monosaccharide-binding subsites within a WGA binding site. This aspect of WGA function remained elusive until today, although a substantial number of experiments aiming at its characterisation have been published. In the case of Chit42, high non-specific adhesion was found on the SAMs used for the detection of WGA binding, which could be prevented by the use of tEG-containing SAMs, thus extending the usability of the SAM technology from high affinity multivalent lectin systems to low affinity monovalent carbohydrate-degrading enzymes. It has thereby been shown that neoglycoconjugate SAMs can mimic the two-dimensional glycan arrays on living cells, are easy to prepare and can be adjusted to match the requirements of different carbohydrate-binding proteins such as ligand density and hydrophobicity of the SAM support, e.g. aglycan parts of the neoglycoconjugate can be ac-

cessed by by the interacting protein. The presented experiments have shown that non-specific protein adhesion to neoglycoconjugates limits the sensitivity of the presented SPR-based lectin binding assay and that masking of solution-accessible hydrophobic neoglycoconjugates can significantly lower background adhesion.

In addition to the binding experiments on neoglycoconjugate SAMs, AFM force spectroscopy experiments were performed with WGA on polymeric chitin. These provided a useful reference for the SAM-based experiments as they provided an estimate of the maximum unbinding force that can be obtained with this particular lectin and of the number of WGA molecules which can simultaneously bind to a limited area of chitin polymer. Further experiments would be necessary to clarify how polysaccharide chains in the chitin beads are packed and how they are accessed by the lectin. This information could be compared to results obtained with the neoglycoconjugate SAMs to further elucidate the differences in binding to crystalline and non-crystalline polysaccharides.

Mutagenesis experiments aiming at modifying the substrate specificity of *T. harzianum* Chit42 verified the roles of some conserved amino acid residues involved in the substrate binding and catalysis of the fungal chitinase. Moreover, it was demonstrated that the binding specificity of Chit42 could be broadened or changed towards non-chitinoligosaccharides. The latter was done by introduction of a counter charge into the substrate binding channel for interaction with an amine-containing oligosaccharide. It was also shown that hydrophobic interactions can increase binding affinity significantly while having little effect on the ligand specificity. On the other hand, removal or introduction of hydrogen bond-forming residues had a clear effect on binding selectivity while changing the binding strength only to a minor extent. A broadening of the substrate binding preference towards galactosylated chitintetraose could be shown by exchange of a hydrogen-bond forming residue. These results demonstrate that the long binding site groove of Chit42 offers possibilities for creating binding proteins (neolectins) or active hydrolases with novel substrate specificity towards e.g. medically significant oligosaccharides. Finally, it was demonstrated that, despite the remote relatedness of Chit42 and the bacterial chitinase *Serratia marcescens* ChiA, the structures of both enzymes resemble each other closely. This finding suggests that structural elements of one enzyme, such as the chitin binding domain of ChiA, could be added to structurally related chitinases, such as Chit42, in order to confer new functions (e.g. increased substrate affinity) to the acceptor enzyme. This idea is supported by previous studies during which a chi-

tin-binding domain from the *Nicotiana tabacum* ChiA chitinase and a cellulose-binding domain of the *Trichoderma reesei* cellobiohydrolase II were fused to Chit42 yielding chimeras with increased hydrolytic activity on insoluble substrates (Limon *et al.* 2001). The inactive Chit42 variants (neolectins) could be used as sensing elements in carbohydrate biosensors useful for monitoring medical conditions that are characterised by reorganisation of cell-surface-bound glycans. Further experiments could aim at an increase of binding affinity by decreasing the energetic penalty paid upon substrate binding through a release of strain of the substrate which is bound in wt Chit42 in a twisted conformation. When the binding affinities of WGA and the inactivated Chit42 variant E172Q for soluble chitinoligosaccharides are compared it can be seen that WGA binds the dimer and trimer with an affinity of 165 and 45 μM, respectively, (see section 3.2.1 and Publication I) whereas no binding of these oligosaccharides can be detected with the chitinase variant (see section 3.4.1 and Publication II). If the chitinoligosaccharide length is increased to the tetramer, the oligosaccharide is bound by Chit42 with an affinity of 12.4 μM whereas the WGA affinity remained close to that measured for the trisaccharide. When even longer oligosaccharides are used, submicromolar affinities were measured with Chit42. This shows that the chitinase is designed to interact preferably with longer carbohydrates, whereas WGA provides the maximum number of interactions already for short oligosaccharides that are bound multivalently if present as dendrimeric structures.

In conclusion, the knowledge gained during this thesis can be used for the creation of novel carbohydrate binding proteins with modified ligand specificity and/or affinity and deepens the understanding of oligosaccharide binding to chitinases, which has previously been addressed in only very few published studies.

References

Alessandrini, A. and Facci, P. 2005. "AFM: A versatile tool in biophysics", Meas. Sci. Technol. 16, pp. R65–R92.

Araujo, A.C.G., Padron, T.S. and De Souza, W. 1993. "Cytochemical localization of carbohydrate residues in microfilariae of Wuchereria bancrofti and Brugia malayi", J. Histochem. Cytochem. 41, pp. 571–578.

Arnold, F.H. 2001. "Combinatorial and computational challenges for biocatalyst design", Nature 409, pp. 253–257.

Aronson, M., Medalia, O. and Schori, L. 1979. "Prevention of colonization of the urinary tract of mice with Escherichia coli by blocking of bacterial adherence with methyl α-D-mannopyranoside", J. Infect. Dis. 139, pp. 329–332.

Asensio, J.L., Canada, F.J., Siebert, H.C., Laynez, J., Poveda, A., Nieto, P.M. Soedjanaamadja, U.M., Gabius, H.J. and JimeneZ-Barbero, J. 2000. "Structural basis for chitin recognition by defense proteins: GlcNAc residues are bound in a multivalent fashion by extended binding sites in hevein domains", Chem. Biol. 7, pp. 529–543.

Bains, G., Lee, R.T., Lee, Y.C. and Freire, E. 1992. "Microcalorimetric study of wheat germ agglutinin binding to N-acetylglucosamine and its oligomers", Biochemistry 31, pp. 12624–12628.

Baker, A.A., Helbert W., Sugiyama, J. and Miles, M.J. 1997. "High-resolution atomic force microscopy of native Valonia cellulose I microcrystals", J. Struct. Biol. 119, pp. 129–138.

Baker, A.A., Helbert, W., Sugiyama, J. and Miles, M.J. 2000. "New insight into cellulose structure by atomic force microscopy shows the I alpha crystal phase at near-atomic resolution", Biophys. J. 79, pp. 1139–1145.

Barattin, R. and Voyer, N. 2008. "Chemical modifications of AFM tips for the study of molecular recognition events", Chem. Commun. (Camb.) 13, pp. 1513–1532.

Becatti, D., Halkes, K.M., Batema, G.D., Guillena, G., Carvalho de Souza, A., van Koten, G. and Kamerling, J.P. 2005. "SPR studies of carbohydrate-protein interactions: Signal enhancement of low-molecular-mass analytes by organoplatinum(II)-labeling", Chembiochem 6, pp. 1196–1203.

Binnig, G., Quate, C.F. and Gerber, C. 1986. "Atomic force microscope", Phys. Rev. Lett. 56, pp. 930–933.

Blanchard, S., Cottaz, S., Coutinho, P.M., Patkar, S., Vind, J., Boer, H., Koivula, A., Driguez, H. and Armand, S. 2007. "Mutation of fungal endoglucanases into glycosynthases and characterization of their acceptor substrate specificity", J. Mol. Catal., B Enzym. 44, pp. 106–116.

Blodgett, K.B. 1935. "Films built by depositing successive monomolecular layers on a solid surface", J. Am. Chem. Soc. 57, pp. 1007–1022.

Bloom, J.D., Meyer, M.M., Meinhold, P., Otey, C.R., MacMillan, D. and Arnold, F.H. 2005. "Evolving strategies for enzyme engineering", Curr. Opin. Struct. Biol. 15, pp. 447–452.

Boer, H., Munck, N., Natunen, J., Wohlfahrt, G., Söderlund, H., Renkonen, O. and Koivula, A. 2004. "Differential recognition of animal type β4-galactosylated and -fucosylated chito-oligosaccharides by two family 18 chitinases from Trichoderma harzianum", Glycobiology 14, pp. 1303–1313.

Boer, H., Simolin, H., Cottaz, S., Söderlund, H. and Koivula, A. 2007. "Heterologous expression and site-directed mutagenesis studies of two Trichoderma harzianum chitinases, Chit33 and Chit42, in Escherichia coli", Protein Expr. Purif. 51, pp. 216–226.

Boraston, A.B., Bolam, D.N., Gilbert, H.J. and Davies, G.J. 2004. "Carbohydrate-binding modules: Fine-tuning polysaccharide recognition", Biochem. J. 382, pp. 769–781.

Bornscheuer, U.T. and Pohl, M. 2001. "Improved biocatalysts by directed evolution and rational protein design", Curr. Opin. Chem. Biol. 5, pp. 137–143.

Bourne, Y., van Tilbeurgh, H. and Cambillau, C. 1993. "Protein–carbohydrate interactions", Curr. Opin. Struct. Biol. 3, pp. 681–686.

Boyd, W.C. and Shapleigh, E. 1954. "Specific precipitating activity of plant agglutinins (lectins)", Science 119, pp. 419.

Branco, R.J.F., Seifert, A., Budde, M., Urlacher, V.B., Ramos, M.J. and Pleiss, J. 2008. "Anchoring effects in a wide binding pocket: The molecular basis of regioselectivity in engineered cytochrome P450 monooxygenase from B. megaterium", Proteins 73, pp. 597–607.

Breyer, W.A. and Matthews, B.W. 2001. "A structural basis for processivity", Protein Sci. 10, pp. 1699–1711.

Brown, D.M. and Phillips, J.H. 1965. "Mechanisms of the mutagenic action of hydroxylamine", J. Mol. Biol. 11, pp. 663–671.

Buleon, A., Colonna, P., Planchot, V. and Ball, S. 1998. "Starch granules: Structure and biosynthesis", Int. J. Biol. Macromol. 23, pp. 85–112.

Bundle, D.R. and Young, N.M. 1992. "Carbohydrate–protein interactions in antibodies and lectins", Curr. Opin. Struct. Biol. 2, pp. 666–673.

Butt, H.J., Cappella, B. and Kappl, M. 2005. "Force measurements with the atomic force microscope: technique, interpretation and applications", Surf. Sci. Rep. 59, pp. 1–152.

Cantarel, B.L., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V. and Henrissat, B. 2009. "The Carbohydrate-Active enZymes database (CAZy): An expert resource for glycogenomics", Nucleic Acids Res. 37, pp. D233–238.

Cederkvist, F.H, Saua, S.F., Karlsen, V., Sakuda, S., Eijsink, V.G.H. and Sorlie, M. 2007. "Thermodynamic analysis of allosamidin binding to a family 18 chitinase", Biochemistry 46, pp. 12347–12354.

Collins, B.E. and Paulson J.C. 2004. "Cell surface biology mediated by low affinity multivalent protein–glycan interactions", Curr. Opin. Chem. Biol. 8, pp. 617–625.

Cosgrove, D.J. 2005. "Growth of the plant cell wall", Nat. Rev. Mol. Cell Biol. 6, pp. 850–861.

Dahiya, N., Tewari, R. and Hoondal, G.S. 2006. "Biotechnological aspects of chitinolytic enzymes: A review", Appl. Microbiol. Biotechnol. 71, pp. 773–782.

Dall'Acqua, W. and Carter, P. 2000. "Substrate-assisted catalysis: Molecular basis and biological significance", Protein Sci. 9, pp. 1–9.

Davies, G.J., Gloster, T.M. and Henrissat, B. 2005. "Recent structural insights into the expanding world of carbohydrate-active enzymes", Curr. Opin. Struct. Biol. 15, pp. 637–645.

Davies, G. and Henrissat, B. 1995. "Structures and mechanisms of glycosyl hydrolases", Structure 3, pp. 853–859.

Davis, A.P. and Wareham, R.S. 1999. "Carbohydrate recognition through noncovalent interactions: A challenge for biomimetic and supramolecular chemistry", Angew. Chem. Int. Ed. Engl. 38, pp. 2978–2996.

de la Cruz, J., Hidalgo-Gallego, A., Lore, J.M., Benitez, T., Pintor-Toro, J.A. and Llobell, A. 1992. "Isolation and characterization of three chitinases from Trichoderma harzianum", Eur. J. Biochem. 206, pp. 859–867.

DeLano, W.L. 2002. The PyMOL Molecular Graphics System, http://www.pymol.org edn, DeLano Scientific, Palo Alto, CA, USA.

Dixon, C.M. 2008. "Quartz crystal microbalance with dissipation monitoring: enabling real-time characterization of biological materials and their interactions", J. Biomol. Tech. 19, pp. 151–158.

Drake, B., Prater, C.B., Weisenhorn, A.L., Gould, S.A.C., Albrecht, T.R., Quate, C.F., Cannell, D.S., Hansma, H.G. and Hansma, P.K. 1989. "Imaging crystals, polymers, and processes in water with the atomic force microscope", Science 243, pp. 1586–1589.

Duverger, E., Frison, N., Roche, A.-C. and Monsigny, M. 2003. "Carbohydrate–lectin interactions assessed by surface plasmon resonance", Biochimie 85, pp. 167–179.

Dwek, R.A. 1996. "Glycobiology: Toward understanding the function of sugars", Chem. Rev. 96, pp. 683–720.

Engel, A., Schoenenberger, C.A. and Müller D.J. 1997. "High resolution imaging of native biological sample surfaces using scanning probe microscopy", Curr. Opin. Struct. Biol. 7, pp. 279–284.

Escalettes, F. and Turner, N.J. 2008. "Directed evolution of galactose oxidase: Generation of enantioselective secondary alcohol oxidases", Chembiochem 9, pp. 857–860.

Esko, J.D., Doering, T.L. and Raetz, C.R.H. 2008. "Eubacteria and archea." In: Essentials of Glycobiology, 2nd edn, Cold Spring Harbor Laboratory Press. Pp. 293–320.

Evans, E. and Ritchie, K. 1997. "Dynamic strength of molecular adhesion bonds", Biophys. J. 72, pp. 1541–1555.

Fasan, R, Chen, M.M., Crook, N.C. and Arnold, F.H. 2007. "Engineering alkane-hydroxylating cytochrome P450(BM3) exhibiting nativelike catalytic properties", Angew. Chem. Int. Ed. 46, pp. 8414–8418.

Ferguson, M.A.J., Kinoshita, T. and Hart, G.W. 2008. "Glycosylphosphatidylinositol anchors." In: Essentials of Glycobiology, 2nd edn, Cold Spring Harbor Laboratory Press. Pp. 143–162.

Ferretti, S., Paynter, S., Russell, D.A., Sapsford, K.E. and Richardson, D.J. 2000. "Self-assembled monolayers: A versatile tool for the formulation of bio-surfaces", Trends Analyt. Chem. 19, pp. 530–540.

Freeze, H.H. and Elbein, A.D. 2008. "Glycosylation precursors." In: Essentials of Glycobiology, 2nd edn, Cold Spring Harbor Laboratory Press. Pp. 47–62.

Fromant, M., Blanquet, S. and Plateau, P. 1995. "Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction", Anal. Biochem. 224, pp. 347–353.

Gabius, H.-J., Siebert, H.-C., André, S., Jiménez-Barbero, J. and Rüdiger, H. 2004. "Chemical biology of the sugar code", Chembiochem 5, pp. 740–764.

Gedig, E.T. 2008. "Surface chemistry in SPR technology." In: Handbook of Surface Plasmon Resonance, RSC Publishing, Cambridge. Pp. 173–220.

Gekko, K. and Timasheff, S.N. 1981. "Mechanism of protein stabilization by glycerol: Preferential hydration in glycerol–water mixtures", Biochemistry 20, pp. 4667–4676.

Gottschalk, A. 1952. "Carbohydrate residue of a urine mucoprotein inhibiting influenza virus haemagglutination", Nature 170, pp. 662–663.

Grandbois, M., Beyer, M., Rief, M., Clausen-Schaumann, H. and Gaub, H.E. 1999. "How strong is a covalent bond", Science 283, pp. 1727–1730.

Hancock, S.M., Vaughan, M.D. and Withers, S.G. 2006. "Engineering of glycosidases and glycosyltransferases", Curr. Opin. Chem. Biol. 10, pp. 509–519.

Hart, G.W. and Akimoto, Y. 2008. "The O-GlcNAc Modification." In: Essentials of Glycobiology, 2nd edn, Cold Spring Harbor Laboratory Press. Pp. 263–280.

Hascall, V. and Esko, J.D. 2008. "Hyaluronan." In: Essentials of Glycobiology, 2nd edn, Cold Spring Harbor Laboratory Press. Pp. 219–228.

Hashimoto, H. 2006. "Recent structural studies of carbohydrate-binding modules", Cell. Mol. Life Sci. 63, pp. 2954–2967.

Hayes, C.K., Klemsdal, S., Lorito, M., Di Pietro, A., Peterbauer, C., Nakas, J.P., Tronsmo, A. and Harman, G.E. 1994. "Isolation and sequence of an endochitinase-encoding gene from a cDNA library of Trichoderma harzianum", Gene 138, pp. 143–148.

Hehre, E.J. 1999. "A fresh understanding of the stereochemical behavior of glycosylases: Structural distinction of 'inverting' (2-MCO-type) versus 'retaining' (1-MCO- types) enzymes", Adv. Carbohydr. Chem. Biochem. 55, pp. 265–310.

Hennig, M., Jansonius, J.N., Terwisscha van Scheltinga, A.C., Dijkstra, B.W. and Schlesier, B. 1995. "Crystal structure of concanavalin B at 1.65 Å resolution. An “inactivated” chitinase from seeds of Canavalia ensiformis", J. Mol. Biol. 254, pp. 237–246.

Hennig, M., Schlesier, B., Pfeffer, S. and Hohne, W.E. 1990. " Narbonin, a 2 S globulin from Vicia narbonensis L. Crystallization and preliminary crystallographic data ", J. Mol. Biol. 215, pp. 339–340.

Hernaiz, M.J., de la Fuente, M.J., Barrientos, A.G. and Penades, S. 2002. "A model system mimicking glycosphingolipid clusters to quantify carbohydrate self-interactions by surface plasmon resonance", Angew. Chem. Int. Ed. 41, pp. 1554–1557.

Hilaire, P.M.S, Boyd, M.K. and Toone, E.J. 1994. "Interaction of the shiga-like toxin type 1 B-subunit with its carbohydrate receptor", Biochemistry 33, pp. 14452–14463.

Hinterdorfer, P. and Dufrene, Y.F. 2006. "Detection and localization of single molecular recognition events using atomic force microscopy", Nat. Methods 3, pp. 347–355.

Hoflack, B. and Kornfeld, S. 1985. "Lysosomal enzyme binding to mouse P388D1 macrophage membranes lacking the 215-kDa mannose 6-phosphate receptor: Evidence for the existence of a second mannose 6-phosphate receptor", Proc. Natl. Acad. Sci. U.S.A. 82. Pp. 4428–4432.

Hollis, T., Monzingo, A.F., Bortone, K., Ernst, S., Cox, R. and Robertus, J.D. 2000. "The X-ray structure of a chitinase from the pathogenic fungus Coccidioides immitis", Protein Sci. 9, pp. 544–551.

Homola, J. 2008. "Surface plasmon resonance for detection of chemical and biological species", Chem. Rev. 108, pp. 462–493.

Huber, W. and Mueller, F. 2006. "Biomolecular interaction analysis in drug discovery using surface plasmon resonance technology", Curr. Pharm. Des. 12, pp. 3999–4021.

Hutter, J.L. and Bechhoefer, J. 1993. "Calibration of atomic-force microscope tips", Rev. Sci. Instrum. 64, pp. 1868–1873.

Håkansson, K. and Reid, K.B.M. 2000. "Collectin structure: A review", Protein Sci. 9, pp. 1607–1617.

Jimenez-Barbero, J. Asensio, J.L., Cuevas, G., Canales, A., Fernandez-Alonso, M.C. and Canada, F.J. 2006. "Conformational insights on the molecular recognition processes of carbohydrate molecules by proteins and enzymes: A 3D view by using NMR", Biocatal. Biotransformation 24, pp. 13–22.

Kanazawa, K. and Cho, N.J. 2009. "Quartz crystal microbalance as a sensor to characterize macromolecular assembly dynamics", J. Sens., Article ID 824947.

Kelly, S.M., Jess, T.J. and Price, N.C. 2005. "How to study proteins by circular dichroism", Biochim. Biophys. Acta 1751, pp. 119–139.

Klemm, D., Heublein, B., Fink, H.P. and Bohn, A. 2005. "Cellulose: Fascinating biopolymer and sustainable raw material", Angew. Chem. Int. Ed. 44, pp. 3358–3393.

Koshland, D.E. 1953. "Stereochemistry and the mechanism of enzymatic reactions", Biol. Rev. 28, pp. 416–436.

Laibinis, P.E., Whitesides, G.M., Allara, D.L., Tao, Y.-T., Parikh, A.N. and Nuzzo, R.G. 1991. "Comparison of the structures and wetting properties of self-assembled monolayers of N-alkanethiols on the coinage metal surfaces, Cu, Ag, Au", J. Am. Chem. Soc. 113, pp. 7152–7167.

Langmuir, I. 1920. "The mechanism of the surface phenomena of flotation", Trans. Faraday Soc. 15, pp. 62–74.

Laurent, N., Voglmeir, J. and Flitsch, S.L. 2008. "Glycoarrays - Tools for determining protein-carbohydrate interactions and glycoenzyme specificity", Chem. Commun. (Camb.) 37, pp. 4400–4412.

Lavie, A., Allen, K.N., Petsko, G.A. and Ringe, D. 1994. "X-ray crystallographic structures of D-xylose isomerase-substrate complexes position the substrate and provide evidence for metal movement during catalysis", Biochemistry 33, pp. 5469–5480.

Leavitt, S. and Freire, E. 2001. "Direct measurement of protein binding energetics by isothermal titration calorimetry", Curr. Opin. Struct. Biol. 11, pp. 560–566.

Lehane, M.J. 1997. "Peritrophic matrix structure and function", Annu. Rev. Entomol. 42, pp. 525–550.

Limon, M.C., Margolles-Clark, E., Benitez, T. and Penttilä, M. 2001. "Addition of substrate-binding domains increases substrate-binding capacity and specific activity of a chitinase from Trichoderma harzianum", FEMS Microbiol. Lett. 198, pp. 57–63.

Linman, M.J., Taylor, J.D., Yu, H., Chen, X. and Cheng, Q. 2008. "Surface plasmon resonance study of protein–carbohydrate interactions using biotinylated sialosides", Anal. Chem. 80, pp. 4007–4013.

Love, J.C., Estroff, L.A., Kriebel, J.K., Nuzzo, R.G. and Whitesides, G.M. 2005. "Self-assembled monolayers of thiolates on metals as a form of nanotechnology", Chem. Rev. 105, pp. 1103–1169.

Lynd, L.R., Weimer, P.J., van Zyl, W.H. and Pretorius, I.S. 2002. "Microbial cellulose utilization: Fundamentals and biotechnology", Microbiol. Mol. Biol. Rev. 66, pp. 506–577.

MacGregor, E.A., Janecek, S. and Svensson, B. 2001. "Relationship of sequence and structure to specificity in the α-amylase family of enzymes", Biochim. Biophys. Acta 1546, pp. 1–20.

Madigan, M.T., Martinko, J.M. and Parker, J. 2001. Brock Mikrobiologie, 9th edn, Spektrum, Akademischer Verlag, Heidelberg.

Mammen, M., Choi, S.K. and Whitesides, G.M. 1998. "Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors", Angew. Chem. Int. Ed. 37, pp. 2754–2794.

Marx, K.A. 2003. "Quartz crystal microbalance: A useful tool for studying thin polymer films and complex biomolecular systems at the solution–surface interface", Biomacromolecules 4, pp. 1099–1120.

McMahon H. and Präfcke G. Content published online at http://www2.mrc-lmb.cam.ac.uk/groups/hmm/techniqs/ITC.html# and http://www2.mrc-lmb.cam.ac.uk/groups/hmm/techniqs/ITC/Calorimetry%20Tutorial.ppt.htm (sites accessed at 15.9.2009).

Merkel, R., Nassoy, P., Leung, A., Ritchie, K. and Evans, E. 1999. "Energy landscapes of receptor–ligand bonds explored with dynamic force spectroscopy", Nature 397, pp. 50–53.

Meyer, J.E.W. and Schulz, G.E. 1997. "Energy profile of maltooligosaccharide permeation through maltoporin as derived from the structure and from a statistical analysis of saccharide–protein interactions", Protein Sci. 6, pp. 1084–1091.

Mikami, B., Hehre, E.J., Sato, M., Katsube, Y., Hirose, M., Morita, Y. and Sacchettini, J.C. 1993. "The 2.0-Å resolution structure of soybean β-amylase complexed with α-cyclodextrin", Biochemistry 32, pp. 6836–6845.

Miles, M., Antognozzi, M., Haschke, H., Hobbs, J., Humphris, A. and McMaster, T. 2003. "Tour de force microscopy", Materials today 6, pp. 30–37.

Mitchell, S.W. 1860. "Researches upon the venom of the rattlesnake", Smithson. Contr. Know. 12, pp. 89–90.

Monsigny, M., Sene, C., Obrenovitch, A., Roche, A.C., Delmotte, F., and Boschetti, E. 1979. "Properties of succinylated wheat-germ agglutinin", Eur. J. Biochem. 98, pp. 39–45.

Muraki, M., Ishimura, M. and Harata, K. 2002. "Interactions of wheat-germ agglutinin with GlcNAcβ1,6Gal sequence", Biochim. Biophys. Acta 1569, pp. 10–20.

Myszka, D.G. 1997. "Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors", Curr. Opin. Biotechnol. 8, pp. 50–57.

Nagata, Y. and Burger, M.M. 1974. "Wheat germ agglutinin. Molecular characteristics and specificity for sugar binding", J. Biol. Chem. 249, pp. 3116–3122.

Nielsen, J.E. and Borchert, T.V. 2000. "Protein engineering of bacterial α-amylases", Biochim. Biophys. Acta 1543, pp. 253–274.

O'Shannessy, D.J., Brigham-Burke, M., Soneson, K.K., Hensley, P. and Brooks, I. 1993. "Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: Use of nonlinear least squares analysis methods", Anal. Biochem. 212, pp. 457–468.

Ostuni, E., Yan, L. and Whitesides, G.M. 1999. "The interaction of proteins and cells with self-assembled monolayers of alkanethiolates on gold and silver", Colloids Surf. B Biointerfaces 15, pp. 3–30.

Papanikolau, Y., Prag, G., Tavlas, G., Vorgias, C.E., Oppenheim, A.B. and Petratos, K. 2001. "High resolution structural analyses of mutant Chitinase A complexes with substrates provide new insight into the mechanism of catalysis", Biochemistry 40, pp. 11338–11343.

Patenaude, S.I., MacKenzie, C.R., Bilous, D., To, R.J., Ryan, S.E., Young, N.M. and Evans, S.V. 1998. "Production, crystallization and diffraction to atomic resolution of an antibody Fv specific for the blood-group A oligosaccharide antigen", Acta Cryst. D54, pp. 1456–1459.

Poirier, G.E. 1997. "Characterization of organosulfur molecular monolayers on Au(111) using scanning tunneling microscopy", Chemical Rev. 97, pp. 1117–1127.

Rao, V., Guan, C. and Van Roey, P. 1995. "Crystal structure of endo-1-N-acetylglucosaminidase H at 1.9 Å resolution: Active-site geometry and substrate recognition", Structure 3, pp. 449–457.

Ratto, T.V., Langry, K.C., Rudd, R.E., Balhorn, R.L., Allen, M.J. and McElfresh, M.W. 2004. "Force spectroscopy of the double-tethered concanavalin-A mannose bond", Biophys. J. 86, pp. 2430–2437.

Rief, M. and Grubmüller, H. 2002. "Force spectroscopy of single biomolecules", Chemphyschem 3, pp. 255–261.

Rinaudo, M. 2006. "Chitin and chitosan: Properties and applications", Prog. Polym. Sci. 31, pp. 603–632.

Rini, J.M. 1995. "Lectin structure", Annu. Rev. Biophys. Biomol. Struct. 24, pp. 551–577.

Roberts, V.A., Iverson, B.L., Iverson, S.A., Benkovic, S.J., Lerner, R.A., Getzoff, E.D. and Tainer, J.A. 1990. "Antibody remodeling: A general solution to the design of a metal-coordination site in an antibody binding pocket", Proc. Natl. Acad. Sci. USA 87. Pp. 6654–6658.

Robertus, J.D. and Monzingo, A.F. 1999. "The structure and action of chitinases", EXS 87, pp. 125–135.

Rose, D.R., Przybylska, M., To, R.J., Kayden, C.S., Oomen, R.P., Vorberg, E., Young, N.M. and Bundle, D.R. 1993. "Crystal structure to 2.45 Å resolution of a monoclonal Fab specific for the Brucella A cell wall polysaccharide antigen", Protein Sci. 2, pp. 1106–1113.

Rudall, K.M. 1963. "The chitin/protein complexes of insect cuticles", Adv. Insect Physiol. 1, pp. 257–313.

Sauerbrey, G. 1959. "Verwendung von Schwingquarzen zur Wägung dünner Schichten und zur Mikrowägung", Z. Phys. 155, pp. 206–222.

Scheffers, D.J. and Pinho, M.G. 2005. "Bacterial cell wall synthesis: New insights from localization studies", Microbiol. Mol. Biol. Rev. 69, pp. 585–607.

Schnaar, R.L., Suzuki, A. and Stanley, P. 2008. "Glycosphingolipids." In: Essentials of Glycobiology, 2nd edn, Cold Spring Harbor Laboratory Press. Pp. 129–142.

Schuck, P. 1997. "Use of surface plasmon resonance to probe the equilibrium and dynamic aspects of interactions between biological macromolecules", Annu. Rev. Biophys. Biomol. Struct. 26, pp. 541–566.

Schülein, M. 2000. "Protein engineering of cellulases", Biochim. Biophys. Acta 1543, pp. 239–252.

Seidl, V. 2008. "Chitinases of filamentous fungi: A large group of diverse proteins with multiple physiological functions", Fungal. Biol. Rev. 22, pp. 36–42.

Setlow, R.B. and Carrier, W.L. 1966. "Pyrimidine dimers in ultraviolet-irradiated DNA's", J. Mol. Biol. 17, pp. 237–254.

Seto, N.O.L. and Evans, S.V. 2000. "Specificity in protein–carbohydrate recognition", Curr. Org. Chem. 4, pp. 411–427.

Shaanan, B., Lis, H. and Sharon, N. 1991. "Structure of a legume lectin with an ordered N-linked carbohydrate in complex with lactose", Science 254, pp. 862–866.

Shao, Z., Mou, J., Czajkowsky, D. M., Yang, J., and Yuan, J.J. 1996. "Biological atomic force microscopy: What is achieved and what is needed", Adv. Physics 45, pp. 1–86.

Sharon, N. 2006. "Atomic basis of protein–carbohydrate interactions: An overview." In: Protein–Carbohydrate Interactions in Infectious Diseases, RSC Publishing, Cambridge. Pp. 1–5.

Sharon, N. and Lis, H. 2004. "History of lectins: From hemagglutinins to biological recognition molecules", Glycobiology 14, pp. 53R–62R.

Shoseyov, O., Shani, Z. and Levy, I. 2006. "Carbohydrate binding modules: Biochemical properties and novel applications", Microbiol. Mol. Biol. Rev. 70, pp. 283–295.

Singh, R.S., Tiwary, A.K. & Kennedy, J.F. 1999, "Lectins: Sources, activities, and applications", Crit. Rev. Biotechnol. 19, pp. 145–178.

Sinha, S., Gupta1, G., Vijayan, M. and Surolia, A. 2007. "Subunit assembly of plant lectins", Curr. Opin. Struct. Biol. 17, pp. 498–505.

Soya, N., Shoemaker, G.K., Palcic, M.M. and Klassen, J.S. 2009. "Comparative study of substrate and product binding to the human ABO(H) blood group glycosyltransferases", Glycobiology 19, pp. 1224–1234.

Stanley, N.F. 2006, "Agars." In: Food Polysaccharides and Their Applications, CRC Press, Boca Raton. Pp. 217–238.

Stanley, P. and Cummings, R.D. 2008. "Structures common to different glycans." In: Essentials of Glycobiology, 2nd edn, Cold Spring Harbor Laboratory Press. Pp. 175–198.

Stemmer, W.P.C. 1994. "Rapid evolution of a protein in vitro by DNA shuffling", Nature 370, pp. 389–391.

Stern, R. and Jedrzejas, M.J. 2008. "Carbohydrate polymers at the center of life's origins: The importance of molecular processivity", Chem. Rev. 108, pp. 5061–5085.

Stryer, L. 1999. Biochemie, 1st corrected re-print of the 4th edn, Spektrum, Akademischer Verlag, Heidelberg.

Templeton, A.C., Hostetler, M.J., Kraft, C.T. and Murray, R.W. 1998. "Reactivity of monolayer-protected gold cluster molecules: Steric effects", J. Am. Chem. Soc. 120, pp. 1906–1911.

Terwisscha van Scheltinga, A.C., Kalk, K.H., Beintema, J.J. and Dijkstra, B.W. 1994. "Crystal structures of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with an inhibitor ", Structure 2, pp. 1181–1189.

Tews, I., Terwisscha van Scheltinga, A.C., Perrakis, A., Wilson, K.S. and Dijkstra, B.W. 1997. "Substrate-assisted catalysis unifies two families of chitinolytic enzymes", J. Am. Chem. Soc. 119, pp. 7954–7959.

Toone, E.J. 1994. "Structure and energetics of protein–carbohydrate complexes", Curr. Opin. Struct. Biol. 4, pp. 719–728.

Tracewell, C.A. and Arnold, F.A. 2009. "Directed enzyme evolution: Climbing fitness peaks one amino acid at a time", Curr. Opin. Chem. Biol. 13, pp. 3–9.

Turner, N.J. 2009. "Directed evolution drives the next generation of biocatalysts", Nat. Chem. Biol. 5, pp. 567–573.

Ulcinas, A., Butler, M.F., Heppenstall-Butler, M., Singleton, S. and Miles, M.J. 2007. "Direct observation of spherulitic growth stages of CaCO3 in a poly(acrylic acid)-chitosan system: In situ SPM study", J. Cryst. Growth 307, pp. 378–385.

Ulman, A. 1996. "Formation and structure of self-assembled monolayers", Chemical Rev. 96, pp. 1533–1554.

Vaaje-Kolstad, G., Bunaes, A.C., Mathiesen,G. and Eijsink, V.G.H. 2009. "The chitinolytic system of Lactococcus lactis ssp. lactis comprises a nonprocessive chitinase and a chitin-binding protein that promotes the degradation of α- and β-chitin", FEBS J. 276, pp. 2402–2415.

Vaaje-Kolstad, G., Horn, S.J., Van Aalten, D.M.F., Synstad, B. and Eijsink, V.G.H. 2005. "The non-catalytic chitin-binding protein CBP21 from Serratia marcescens is essential for chitin degradation", J. Biol. Chem. 280, pp. 28492–28497.

Vaaje-Kolstad, G., Houston, D.R., Rao, F.V., Peter, M.G., Synstad, B., Van Aalten, D.M.F. and Eijsink, V.G.H. 2004. "Structure of the D142N mutant of the family 18 chitinase ChiB from Serratia marcescens and its complex with allosamidin", Biochim. Biophys. Acta 1696, pp. 103–111.

van Aalten, D.M.F., Komander, D., Synstad, B., Gåseidnes, S., Peter, M.G. and Eijsink, V.G.H. 2001. "Structural insights into the catalytic mechanism of a family 18 exo-chitinase", Proc. Natl. Acad. Sci. U.S.A. 98. Pp. 8979–8984.

Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W. and Etzler, E.E. (Eds.). 2008. "Essentials of Glycobiology", 2nd edn, Cold Spring Harbor Laboratory Press.

Varki, A. and Lowe, J.B. 2008. "Biological roles of glycans." In: Essentials of Glycobiology, 2nd edn, Cold Spring Harbor Laboratory Press. Pp. 75–88.

Varki, A. and Sharon, N. 2008. "Historical background and overview." In: Essentials of Glycobiology, 2nd edn, Cold Spring Harbor Laboratory Press. Pp. 1–22.

Vasta, G.R. 2009. "Roles of galectins in infection", Nat. Rev. Microbiol. 7, pp. 424–438.

Vedha-Peters, K., Gunawardana, M., Rozzell, J.D. and Novick, S.J. 2006. "Creation of a broad-range and highly stereoselective D-amino acid dehydrogenase for the one-step synthesis of D-amino acids", J. Am. Chem. Soc. 128, pp. 10923–10929.

Vyas, N.K. 1991. "Atomic features of protein–carbohydrate interactions", Curr. Opin. Struct. Biol. 1, pp. 732–740.

Wälti, M.A., Walser, P.J., Thore, S., Grünler, A., Bednar, M., Künzler, M. and Aebi1, M. 2008. "Structural basis for chitotetraose coordination by CGL3, a novel galectin-related protein from Coprinopsis cinerea", J. Mol. Biol. 379, pp. 146–159.

Watson, K.A., Mitchell, E.P., Johnson, L.N., Jong Chan Son, Bichard, C.J.F., Orchard, M.G., Fleet, G.W.J., Oikonomakos, N.G., Leonidas, D.D., Kontou, M. and Papageorgioui, A. 1994. "Design of inhibitors of glycogen phosphorylase: A study of α- and β-C-glucosides and 1-thio-β-D-glucose compounds", Biochemistry 33, pp. 5745–5758.

Weisenhorn, A.L., Hansma, P.K., Albrecht, T.R. and Quate, C.F. 1989. "Forces in atomic force microscopy in air and water", Appl. Phys. Lett. 54, pp. 2651–2653.

Wright, C.S. 1980. "Crystallographic elucidation of the saccharide binding mode in wheat germ agglutinin and its biological significance", J. Mol. Biol. 141, pp. 267–291.

Wright, C.S. 1987. "Refinement of the crystal structure of wheat germ agglutinin isolectin 2 at 1.8 Å resolution", J. Mol. Biol. 194, pp. 501–529.

Wright, C.S. 1992. "Crystal structure of a wheat germ agglutinin/glycophorin–sialoglycopeptide receptor complex. Structural basis for cooperative lectin–cell binding", J. Biol. Chem. 267, pp. 14345–14352.

Wright, C.S. and Kellogg, G.E. 1996. "Differences in hydropathic properties of ligand binding at four independent sites in wheat germ agglutinin–oligosaccharide crystal complexes", Protein Sci. 5, pp. 1466–1476.

Wright, C.S. and Raikhel, N. 1989. "Sequence variability in three wheat germ agglutinin isolectins: Products of multiple genes in polyploid wheat ", J. Mol. Evol. 28, pp. 327–336.

Xie, Z.P., Staehelin, C., Vierheilig, H., Wiemken, A., Jabbouri, S., Broughton, W.J., Vogeli-Lange, R. and Boller, T. 1995. "Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans", Plant Physiol. 108, pp. 1519–1525.

Yang, Y.C., Chang, T.Y. and Lee, Y.L. 2007. "Adsorption behavior of 11-mercapto-1-undecanol on Au(111) electrode in an electrochemical system", J. Phys. Chem. C 111, pp. 4014–4020.

Zakariassen, H., Aam, B.B., Horn, S.J., Vårum, K.M., Sørlie, M. and Eijsink, V.G.H. 2009. "Aromatic residues in the catalytic center of chitinase A from Serratia marcescens affect processivity, enzyme activity, and biomass converting efficiency", J. Biol. Chem. 284, pp. 10610–10617.

Zees, A.C., Pyrpassopoulos, S. and Vorgias, C.E. 2009. "Insights into the role of the (α + β) insertion in the TIM-barrel catalytic domain, regarding the stability and the enzymatic activity of Chitinase A from Serratia marcescens", Biochim. Biophys. Acta 1794, pp. 23–31.

Zhang, Y., Luo, S., Tang, Y., Yu, L., Hou, K.Y., Cheng, J.P., Zeng, X. and Wang, P.G. 2006. "Carbohydrate-protein interactions by “clicked” carbohydrate self-assembled monolayers", Anal. Chem. 78, pp. 2001–2008.

Zhong, Q., Inniss, D., Kjoller, K. and Elings, V.B. 1993. "Fractured polymer/silica fiber surface studied by tapping mode atomic force microscopy", Surf. Sci. 290, pp. L688–L692.

Zugenmaier, P. 2001. "Conformation and packing of various crystalline cellulose fibers", Prog. Polym. Sci. 26, pp. 1341–1417.

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

Series title, number and report code of publication

VTT Publications 735
VTT-PUBS-735

Author(s)
Michael Lienemann

Title

Characterisation and engineering of protein–carbohydrate interactions

Abstract

Protein−carbohydrate interactions play a crucial role in many biological processes such as cell−cell recognition and receptor−ligand interactions and catalysis. This thesis explores the possibilities of engineering the protein–carbohydrate interactions between carbohydrate-binding proteins and their ligands. Protein–carbohydrate interactions were characterised using different techniques, such as atomic force microscopy (AFM) and surface plasmon resonance (SPR).

Two different chitinoligosaccharide-binding proteins were used in the presented experiments, e.g. the plant lectin wheat germ agglutinin (WGA) and a neolectin created by inactivation of the fungal chitin-degrading enzyme Chit42. The structures of WGA and Chit42, which were available as X-ray diffraction data and a homology model, respectively, revealed differences between their binding site architectures. The interaction of monomeric and polymeric *N*-acetyl-D-glucosamine (GlcNAc) with WGA, that normally binds chitinoligosaccharides on cell surfaces, was probed in nanomechanical force measurements using AFM. These measurements aimed at determining the effect of ligand length and binding site clustering (multivalent binding) on binding strength between the protein and the carbohydrate ligand. Here, the GlcNAc ligand was presented at the surface of a planar self-assembled monolayer (SAM) of neoglycoconjugates and in a polymeric form as chitin beads. In adsorption measurements using the quartz crystal microbalance (QCM) it was shown that alkanethiols adsorb rapidly to a gold surface covering it completely within a few minutes as a SAM. In AFM force measurements, GlcNAc-specific binding events were detected with a WGA-modified probe on a GlcNAc-neoglycoconjugate SAM at bond rupture forces of 47 ± 15 pN. When this experiment was repeated on a polymeric substrate, multiple times higher forces were detectable. This indicated a high increase of affinity with additional binding subsites which were able to interact with the chitinous ligand. SPR measurements confirmed that WGA has higher affinity towards the immobilized GlcNAc-neoglycoconjugate SAM than towards the soluble free monosaccharide providing evidence of a significant affinity increase as a result of binding site cooperativity. Furthermore, two different SAM formats have been tested for their suitability for studying protein–carbohydrate interactions. Here, non-specific adhesion was identified as a critical factor that was mainly related to the hydrophobic parts of the neoglycoconjugates and could be attenuated by the introduction of a tetraethylene glycol spacer into the neoglycoconjugate. The binding measurements on neoglycoconjugate SAMs demonstrated that this type of carbohydrate presentation is a useful reference for interactions on naturally occurring two-dimensional glycan arrays and demonstrated that the minimization of non-specific adhesion of proteins is often required in order to obtain meaningful binding data.

Apart from WGA–carbohydrate interactions this thesis deals also with the carbohydrate binding cleft of the glycoside hydrolase *Trichoderma harzianum* chitinase Chit42, which solubilises as wild-type form polymeric crystalline chitin (composed of *β*-4 linked GlcNAc units). Nine different catalytically inactive Chit42 variants having amino acid alterations along the binding site cleft (at sugar-binding subsites -4 to +2) were created. These Chit42 variants were characterised with regard to their affinity towards chitinous and non-chitinous oligosaccharides by SPR. As a result, hydrogen bonding between subsites -2/-3 and particularly stacking interactions by tryptophanes at subsites -3 and +2 were seen to be very important for the carbohydrate binding. The exchange of the corresponding amino acids did not cause a change of binding specificity, however the selective binding of GlcNβ-4$(GlcNAc)_4$ could be improved by providing a counter charge through an amino acid substitution at subsite -3, replacing threonine with aspartic acid. In addition the introduction of glutamine and particularly an asparagine residue at this position seemed to broaden the substrate preference towards Gal*β*-4$(GlcNAc)_4$ and was thereby implicated as a binding groove hot spot for creating binding proteins, or hydrolytic enzymes with novel substrate specificity towards e.g. medically related oligosaccharides. The analysis of the Chit42 variants with modified active sites showed how the binding selectivity and affinity of neolectins can be engineered and may thereby function as a model for further neolectins and glycoside hydrolases with new ligand and substrate specificities, respectively.

ISBN
978-951-38-7391-2 (soft back ed.)
978-951-38-7392-9 (URL: http://www.vtt.fi/publications/index.jsp)

Series title and ISSN
VTT Publications
1235-0621 (soft back ed.)
1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)

Project number

Date
May 2010

Language
English, German abstr.

Pages
90 p. + app. 30 p.

Name of project

Commissioned by

Keywords
chitinase, mutagenesis, protein–carbohydrate interaction, Trichoderma harzianum, surface plasmon resonance, self-assembled monolayer, AFM force spectroscopy, wheat germ agglutinin

Publisher
VTT Technical Research Centre of Finland
P.O. Box 1000, FI-02044 VTT, Finland
Phone internat. +358 20 722 4520
Fax +358 20 722 4374

Seriennname, Nummer und Kode

VTT Publications 735
VTT-PUBS-735

Autoren
Michael Lienemann

Titel

Charakterisierung und Engineering von Protein–Kohlenhydrat-Wechselwirkungen

Zusammenfassung

Protein−Kohlenhydrat-Wechselwirkungen sind von entscheidender Bedeutung in zahlreichen biologischen Prozessen, wie der gegenseitigen Erkennung von Zellen und Rezeptor–Ligand-Wechselwirkungen sowie bei der Katalyse. In dieser Doktorarbeit werden die Möglichkeiten erforscht Protein–Kohlenhydrat-Wechselwirkungen zwischen kohlenhydratbindenden Proteinen und ihren Liganden mittels Protein Engineering gezielt zu manipulieren. Die betrachteten Wechselwirkungen wurden mit Hilfe von verschiedenen Techniken zur Messung von Bindungsprozessen wie Rasterkraftmikroskopie (AFM) und Oberflächenplasmonresonanz (SPR) charakterisiert.

In den dargestellten Experimenten wurden zwei Chitinoligosaccharid-bindende Proteine verwendet um die Wechselwirkungen zwischen Proteinen und Kohlenhydraten zu charakterisieren. Hierbei handelte es sich um Weizenkeimagglutinin (WGA) und ein Neolektin, welches durch Inaktivierung des chitinabbauenden fungalen Enzyms Chit42 erzeugt wurde. Bei einem Vergleich der Strukturen von WGA und Chit42, welche auf Röntgenstrahlstreuungsmessungen beziehungsweise auf einem Homologiemodell basieren, wurde deutlich, dass sich beide Proteine im Aufbau ihrer Bindungstaschen unterscheiden. Mit Hilfe von nanomechanischer AFM Kraftmessungen wurden die Wechselwirkungen zwischen monomerem sowie polymerem *N*-Acetyl-D-Glukosamin (GlcNAc) und WGA untersucht, welches normalerweise an auf Zelloberflächen befindliche Chitinoligosaccharide bindet. Das Ziel dieser Messungen war die Bestimmung des Einflusses von Ligandenlänge und Bindungstaschenzusammenlagerung auf die Bindungsstärke zwischen dem Protein und dem Kohlenhydratliganden. Hierbei wurde der GlcNAc-Ligand als Monomer an der Oberfläche einer planaren selbstangeordneten Monoschicht (SAM) von Neoglykokonjugaten und in polymerer Form als Chitinkügelchen eingesetzt. Mittels Adsorptionsmessungen mit der Schwingquarzmikrowaage (QCM) wurde gezeigt, dass Alkanthiole sehr schnell an eine Goldoberfläche binden und diese innerhalb von wenigen Minuten vollständig als SAM bedecken. In den dargestellten AFM-Kraftspektroskopieexperimenten wurden mit Hilfe einer WGA-modifizierten Sonde GlcNAc-spezifische Bindungsereignisse bei einer Bindungsabrisskraft von 47 ± 15 pN an einer GlcNAc-Neoglykokonjugat-SAM aufgezeichnet. Während einer Wiederholung dieses Experiments auf einem Polymersubstrat wurden Kräfte gemessen, welche die auf der GlcNAc-Neoglykokonjugat-SAM gemessene Bindungsabrisskraft um ein Vielfaches überstiegen. Dies wies auf einen starken Anstieg der Bindungsaffinität unter Beteiligung zusätzlicher Bereiche der Lektinbindungstaschen hin, die mit dem Chitinliganden in Wechselwirkung treten konnten. SPR-Messungen zeigten, dass WGA eine höhere Affinität zu den auf Neoglykokonjugat-SAMs immobilisierten GlcNAc-Monosacchariden als zum gelösten Kohlenhydrat hatte was einen bedeutenden Affinitätszuwachs aufgrund von Bindungstaschenkooperativität anzeigte. Zusätzlich hierzu wurden zwei weitere SAM-Formate auf ihre Eignung zur Untersuchung von Protein–Kohlenhydrat-Wechselwirkungen hin untersucht. Hierbei wurde nicht-spezifische Adhäsion als kritischer Faktor identifiziert, die hauptsächlich auf die hydrophoben Teile der Neoglykokonjugate zurückzuführen war und durch die Einführung von Tetraethylenglykolelementen abgemildert werden konnte. In den Bindungsstudien mit Neoglykokonjugat-SAMs konnte gezeigt werden, dass diese Art von Kohlenhydratpräsentation eine nützliche Referenz für Wechselwirkungen auf natürlich auftretenden zweidimensionalen Glykananordnungen darstellt. Außerdem wurde demonstriert, dass bei dieser Art von Experimenten eine Minimierung von nicht-spezifischer Proteinadhäsion von Proteinen nötig ist um aussagekräftige Bindungsdaten zu erhalten.

Neben WGA–Kohlenhydrat-Wechselwirkungen wird in dieser Doktorarbeit auch die Substratbindungstasche der Glykosylhydrolase *Trichoderma harzianum* Chitinase Chit42 untersucht, welche in ihrer unveränderten Form polymeres kristallines Chitin (aufgebaut aus *β*-4-verknüpften GlcNAc-Einheiten) solubilisiert. Neun verschiedene katalytisch inaktive Chit42-Varianten wurden hergestellt, die Modifikationen entlang der Substratbindungstasche zwischen den kohlenhydratbindenden Bindungstaschenuntereinheiten -4 und +2 trugen. Diese Chit42-Varianten wurden durch Bestimmung ihrer Affinität zu chitinartigen und nicht-chitinartigen Oligosacchariden mittels SPR charakterisiert. Hierbei wurde gezeigt, dass Wasserstoffbrückenbindungen und aromatische Wechselwirkungen, die von polaren Gruppen in Bindungstaschenuntereinheiten -3 und -2 bzw. von Tryptophanresten in Bindungstaschenuntereinheiten -3 and +2 ausgehen entscheidend zur Bindung des Kohlenhydratliganden beitragen. Der Austausch der betreffenden Aminosäuren hatte keine Veränderung der Bindungsspezifität zur Folge. Eine weitere Veränderung der Bindungstaschenuntereinheit -3 durch einen Austausch von Threonin gegen die saure Aminosäure Asparaginsäure führte jedoch zu einer Erhöhung der Bindungsselektivität für das Disaccharid GlcNβ-4(GlcNAc)$_4$, was auf eine Paarung entgegengesetzter Ladungen zurückgeführt wurde. Außerdem konnte eine Erweiterung des Substratspektrums zu Galβ-4(GlcNAc)$_4$ durch den Austausch des Threonins mit Glutamin und Asparagin erreicht werden. Diese Region in der Substratbindungstasche wurde somit als “Hot spot” für die Erzeugung von Kohlenhydratbindungsporteinen oder Glycosidhydrolasen mit neuartigen Ligand- bzw. Substratspezifitäten für z.B. medizinisch relevante Oligosaccharide identifiziert. Die Untersuchung der Chit42 Varianten mit modifizierter Bindungstasche hat gezeigt wie die Bindungsselektivität und Affinität von (Neo-)Lektinen gezielt verändert werden kann und könnte daher als Vorlage in der Entwicklung weiterer Neolektine und Glykosylhydrolasen mit neuen Ligand- bzw. Substratspezifitäten dienen.

ISBN
978-951-38-7391-2 (ungebunden)
978-951-38-7392-9 (URL: http://www.vtt.fi/publications/index.jsp)

Serientitel und ISSN	Nummer des Projektes
VTT Publications 1235-0621 (ungebunden) 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)	

Datum	Sprache	Seiten
Mai 2010	Englisch, Deutsch Zusammenfassung	90 S. + App. 30 S.

Name des Projektes	Auftragsgeber

Stichwörter	Herausgeber
chitinase, mutagenesis, protein−carbohydrate interaction, Trichoderma harzianum, surface plasmon resonance, self-assembled monolayer, AFM force spectroscopy, wheat germ agglutinin	VTT Technical Research Centre of Finland Postfach 1000, FI-02044 VTT, Finnland Tel. internat. +358 20 722 4520 Fax +358 20 722 4374

VTT Publications

ISBN 978-951-38-7391-2 (soft back ed.)
ISSN 1235-0621 (soft back ed.)
ISBN 978-951-38-7392-9 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)

University of Stuttgart
Germany