CHARACTERIZATION OF THE MULTI-MODULAR FAMILY 20-β-*N*-ACETYLHEXOSAMINIDASE (CHITOBIASE) FROM *SERRATIA MARCESCENS*

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Abstract

The aim of this study was to clone and characterize the *N*-acetylhexosaminidase (Chitobiase) from the soil bacterium *Serratia marcescens*. This enzyme is part of the chitinolytic machinery of *S. marcescens,* a Gram-negative bacterium known for its efficient degradation of chitin in nature. Research on understanding how this machinery works will provide knowledge that may be useful in the production of several chitin-derived compounds, and, thus, for utilization of the abundant resource that chitin represents. The knowledge of chitin degradation can also be used for better understanding the process of degradation of other carbohydrates, such as cellulose, which also represents a great resource, as it has potential in replacing fossil fuels.

After successful cloning, expression and purification of the enzyme, kinetic analyses of its properties were performed with both artificial and natural substrates. The kinetic parameters calculated for the artificial and natural substrate were similar: K_M = 52 +/- 3.545 μM (4-MU-GlcNAc), K_M = 55 +/- 9.17 μM ($GlcNAc_2$) and K_{cat} =129 +/- 2.517s^{-1} (4-MU-GlcNAc), K_{cat} = 135 +/- 5.975 s^{-1} ($GlcNAc_2$), an observation that provides insight into the rate-limiting step in the catalytic mechanism of Chitobiase.

Experiments on degradation of β-chitin did not reveal any synergistic effects when combining chitinases and Chitobiase. The rate of β-chitin degradation by Chitobiase increased when incubated together with CBP21 (in presence of reduced glutathione), a recently discovered oxidohydrolytic enzyme that disrupts crystalline substrates.

To further understand the interplay between the various chitinolytic enzymes, the hypothesis that chitooligosaccharides bind to α- or β- chitin, thereby possibly inhibiting efficient chitin degradation by chitinases and possibly creating a role for Chitobiase, was tested. However, these experiments did not reveal any binding.

Pulldown experiments with the recombinant Chitobiase, and a preliminary secretome analysis of *S. marcescens* were performed to investigate possible interaction partners of Chitobiase as well as hitherto unknown contributors to the

chitinolytic machinery of *S. marcenscens.* The pulldown experiment resulted in cleavage of the recombinant Chitobiase, as proteolytic cleavage product of the enzyme were identified by MALDI-TOF MS. This was probably due to a protease in the bacterial extract/supernatant. The preliminary experiment on the secretome of *S.marcescens* should be optimized before making any conclusions of the secreted proteins, and by repeating this experiment with more optimized conditions could reveal important contributors to the efficient chitinolytic machinery of *S. Marcescens.*

Valuable knowledge concerning the *N*-acetylhexosaminidase (Chitobiase) from *S. marcescens* has been elucidated in this study, but there are still many questions concerning this large enzyme, especially with regard to the functions of its four distinct domain.

Sammendrag

Målet med denne studien var å klone og karakterisere N-acetylhexosaminidase (Chitobiase) fra *Serratia marcescens*. Dette enzymet er en del av det kitinolytiske maskineriet til jordbakterien *Serratia marcescens*, en Gram negativ bakterie kjent for sin effektive nedbrytning av kitin i naturen. Forskning basert på forståelse av hvordan dette maskineriet fungerer, vil bidra med kunnskap som kan brukes i produksjonen av nyttige forbindelser, og i utnyttelsen av kitin, som utgjør en stor ressurs. Denne kunnskapen kan også overføres til å forstå nedbrytning av andre karbohydrater, for eksempel cellulose, som også er en stor ressurs, særlig med tanke på dets potensiale i biodrivstoff industrien, hvor det kan erstatte fossile brennstoff.

Kinetiske analyser av enzymets aktivitet ble utført bade med kunstig og naturlig substrat. De kinetiske parametrene som ble kalkulert for det kunstige og det naturlige substratet var veldig like, og er interessante med tanke på å forstå hva som bestemmer hastigheten av reaksjonen som foregår: K_M = 52 +/- 3.545 μM (4-MU-GlcNAc), K_M = 55 +/- 9.17 μM ($GlcNAc_2$) og K_{cat} =129 +/- 2.517s^{-1} (4-MU-GlcNAc), K_{cat} = 135 +/- 5.975 s^{-1} ($GlcNAc_2$

Forsøk med nedbrytning av β-kitin viste ingen synergistiske effekter med kitinasene og Kitobiase, men nedbrytning av β-chitin med Kitobiase og CBP21 (og reduktant) økte nedbrytningsraten sammenliknet med nedbrytning med Chitobiase alene. CBP21 er et nylig oppdaget oksidohydrolytisk enzyme som bryter opp krystallinske substrater.

For å undersøke mulige interaksjonspartnere til Kitobiase og hittil ukjente bidragsytere til det kitinolytiske maskineriet til *Serratia marcenscens*, ble det foretatt "pulldown" forsøk med den rekombinante Kitobiase, og en innledende sekretomanalyse. "Pulldown" forsøket resulterte i kløyvning av den rekombinante Kitobiasen, proteolytiske produkter av enzymet ble identifisert ved hjelp av MALDI-TOF MS. Mest sannsynlig var den en protease i bakteriecelleekstraktet/supernatanten som kløyvet Kitobiasen. Eksperimentet som omhandlet sekretomanalyse av *S. Marcescens* bør optimaliseres før man si noe om sekretomet til bakterien. Et

optimalisert forsøk kan bidra med viktig kunnskap om mulige bidragsytere til det effektive kitinolytiske maskineriet til *S.marcescens.*

Denne studien har bidratt med verdifull kunnskap om *S.marcescens* Kitobiase, men det er fortsatt flere uløste spørsmål rundt dette store enzymet, særlig med tanke på funksjonen til dets fire distinkte domener.

Abbreviations:

α	Alpha
β	Beta
γ	Gamma
µ	Micro
µg	Microgram
µl	Microliter
µmol	Micromol
µm	Micrometer
µM	Micromolar
4-methylumbelliferyl	4-MU
ACN	Acetonitril
AmBic	Ammonium Bicarbonate
Arg (R)	Arginine
Asp (D)	Aspartic acid
BSA	Bovine serum albumin
°C	Degrees in Celsius
Da	Dalton
dH_2O	Sterile water (milliQ)
DTT	Dithiothreitol
g	Gram
Glu (E)	Glutamic acid
H	Hour
HPLC	High Performance Liquid Cromatography
IAA	Iodoacetamide
kDa	Kilodalton
L	Liter
M	Molar (mol/L)
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
m/z	Mass/charge
MeOH	Methanol
Mg	Milligram

min	Minute
Mm	Millimeter
mM	Millimolar
ml	Milliliter
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
nM	Nanomolar
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
TFA	Trifluoroacetic acid

Contents

1. Introduction

Chitin is an abundant polysaccharide with an estimated annual production of 10 gigatons (1.10^{13} kg), which represents a major renewable resource that is not yet utilized to its full potential (Muzzarelli 1999). In nature, microorganisms use chitin as a carbon and nitrogen source. Understanding the mechanisms of chitin degradation in natural environments can provide important knowledge that can be utilized for generation of chitin-derived products of human interest and benefit.

The Gram-negative soil bacterium *Serratia marcescens* is known for its ability to efficiently degrade and metabolize chitin (Monreal et al. 1969). When cultivated on chitin, *S.marcescens* secretes five chitin degrading enzymes: Chitinase A, Chitinase B, Chitinase C, CBP21 and Chitobiase (Toratani et al. 2008), (Horn et al. 2006), (Vaaje-Kolstad et al. 2010). The chitinases and CBP21 are involved in converting crystalline chitin to soluble short oligosaccharides, whereas Chitobiase converts the short soluble sugars to *N*-acetylglucosamine, which is taken up by the bacterium and further metabolized (Toratani, Shoji et al. 2008).

While the chitinases and CBP21 of *S. marcescens* are quite well studied, relatively little is known about the Chitobiase. The Chitobiase is a large protein consisting of four domains (Tews et al. 1996) and cleaves off N-acetylglucosamine (GlcNAc) from the non-reducing end of chitobiose ($GlcNAc_2$, dimer of chitin) and higher chitooligosaccharides (Drouillard et al. 1997). *Serratia marcescens* is only capable of metabolizing the *N*-acetylglucosamine monomer; therefore, the activity of Chitobiase is essential for the bacterium when growing on chitin. The chitinases produce some *N*-acetylglucosamine, but their main product is $GlcNAc_2$ (Horn, Sorbotten et al. 2006).

Even though the structure and the activity of Chitobiase have been characterized, there are still many questions concerning this large enzyme and its four distinct domains. Apart from the catalytic domain (domain III), the functions of these domains remain unsolved. It is interesting to investigate if these domains are involved in interactions with other, perhaps hitherto unknown parts of the chitinolytic

machinery, or may be involved in binding crystalline chitin. The kinetics of the enzyme's activity against its natural substrate has not been elucidated. Knowing these kinetics would allow comparison of Chitobiase activity against artificial and natural substrates which could provide insight into the rate-limiting step in the catalytic mechanism. Another important aspect to be elucidated concerns the contribution of Chitobiase to the degradation of crystalline chitin by the chitinases and CBP21. A more in–depth characterization of the properties and role of Chitobiase in chitin degradation may also provide a valuable contribution of an "enzyme toolbox" for generation of well defined, bioactive chitooligosaccharides.

1.1. Polysaccharides

Carbohydrates, or saccharides, are a diverse class of biological molecules, with several functions making them essential for all living organisms (Mathews 2000). The molecules fit the stoichiometric formula $(CH_2O)_n$, or are derivatives of such compounds. Monosaccharides are the most simple form of carbohydrates, and are small, monomeric molecules like e.g. glucose. The monomers can be linked together, to form chains of different lengths and composition; these longer chains are referred to as oligosaccharides (~3-20 sugar units) or polysaccharides (~more than 20 sugar units).

Regarded as a major energy source, carbohydrates play an important role in the metabolism of both plants and animals. In nature, the most important functions of carbohydrates are production and storage of energy, and they also play important roles as structural components. Starch and glycogen are examples of carbohydrates mainly involved in storage of energy, whereas cellulose and chitin are structural materials, e.g. chitin being an essential component of the cell wall of fungi. The specific and short oligosaccharides often play a role as cellular markers, helping the organism to differ between native and foreign cells.

The primary structure of polysaccharides is defined by the sequence of monomer units and the type of glycosidic linkages between them. In homopolysaccharides the polymer only comprises one kind of monomer unit (e. g cellulose, chitin), whereas heteropolysaccharides are polymers with two or more units involved. Structural

polysaccharides can form long and regular secondary structures, creating fibers and sheets.

1.2. Chitin and chitosan

After cellulose and hemicellulose, chitin is the most abundant biopolymer in nature, and is widely distributed in the kingdoms of organisms (Mathews 2000). The polysaccharide in chitin is a linear, insoluble homopolymer consisting of *N*-acetyl-β-D-glucosamine (GlcNAc). The basic structure of chitin is similar to that of cellulose, but with an acetylated amine group replacing the hydroxyl group on carbon 2 in the glucose units of cellulose. Both cellulose and chitin have some remarkable structural features, being insoluble and forming crystalline structure, due to the β-(1,4) linking of the sugar units. Chitin is a common constituent of cell walls in both yeast and fungi, and also an important structural component in the exoskeleton of insects and shells of crustaceans. As illustrated in Figure 1, the GlcNAc monomers are rotated 180° relative to each other in the chitin chain, with the disaccharide as the structural and functional unit (Aronson et al. 2003).

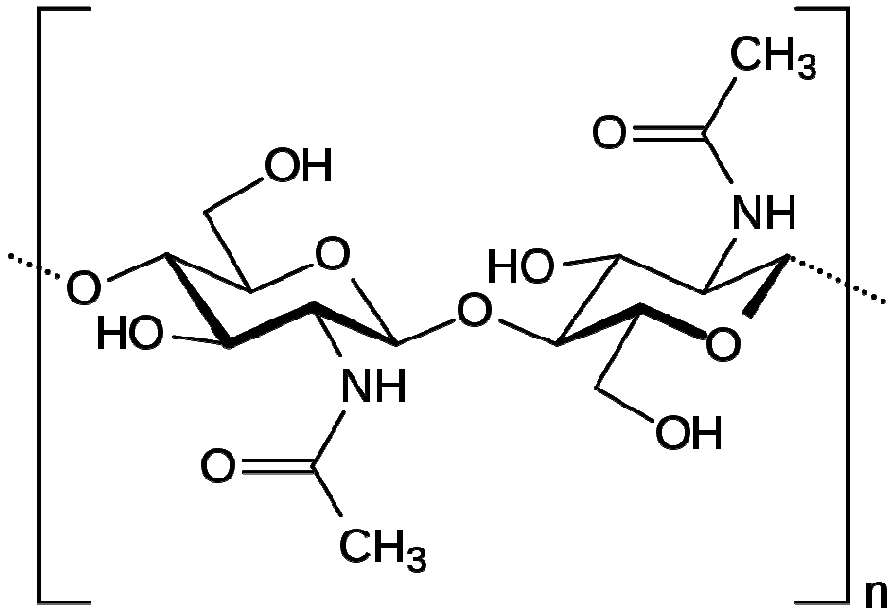


Figure 1.The repeating disaccharide unit in chitin. The picture shows a disaccharide of N-acetylglucosamine (GlcNAc), which is the repeating unit in chitin. The GlcNAc monomers are rotated 180° relative to each other (Aronson, Halloran et al. 2003).

Chitin molecules are not uniform in size, and exist in several lengths and polymorphic forms in nature. After synthesis, chitin molecules are associated with each other via hydrogen bonding between neighboring chains. Chitin exists in three different forms determined by the relative orientation of neighboring chains: α-chitin consisting of anti-parallel chains (Carlstrom 1957; Minke et al. 1978), β-chitin consisting of parallel chains (Dweltz 1961; Gardner et al. 1975) and γ-chitin , which has an arrangement of two parallel chains and one anti-parallel chain (Synowiecki et al. 2003). In nature, the α-form of chitin is the most abundant.

Deacetylation of chitin yields chitosan, a heteropolymer of β(1,4)-linked N-acetylglucosamine (GlcNAc) and D-glucosamine (GlcN). The degree of acetylation of chitin in nature is normally more than 90 % (Kumar 2000). Randomly acetylated chitosan with a degree of acetylation below 65% is water soluble at mildly acidic pH. (Anthonsen 1993).

Chitooligosaccharides (CHOS) are homo-, or heterooligomers of GlcNAc and GlcN. CHOS can be produced from chitin or chitosan by chemical-, or enzymatic conversion. Soluble chitosan variants in both polymeric and oligomeric forms have a variety of applications in fields ranging from agriculture to medicine (Aam et al. 2010).

1.2.1. Applications of chitin, its derivatives and chitin degrading enzymes

Chitin, chitosan and their derivatives have some unique biological properties useful for industrial purposes, and the great abundance of chitin in nature makes it an interesting material for commercial exploitation. There are several advantages of using chitin and chitosan; they are produced from natural resources, and are renewable (Kumar 2000). The polysaccharides are also biologically degradable, non-polluting and non- toxic. Another beneficial feature of chitin and chitosan is their biocompatibility with both plant and animal tissue, which allows application of chitin and chitosan derivatives as pharmaceuticals and medicines.

Chitin is especially important in the fields of agriculture and biotechnology, where it has potential for several applications. It has been shown that incorporation of chitinous mass in soil may protect certain crops against microbial pathogens, probably due to stimulation of natural microbes that work as natural protectors (Tharanathan et al. 2003). As described earlier, chitin is an important constituent of the cell walls of fungi. Hence, the degradation of chitin serves as an important defense mechanism against fungal pathogens. Even in humans, there are genes encoding chitinases; chitotriosidase (CHIT1) is a chitinase (described in section 1.3) extensively expressed by macrophages, and the acidic mammalian chitinase (AMCase), named after its low pH optimum, is found in the gastrointestinal tract and the lung of humans. (Renkema et al. 1995; Boot et al. 2001). Although the functions of these human enzymes are not fully elucidated, there is some speculation that they are involved in pathogen defense. In a clinical study, chitotriosidase activity in the lung was found to be lower than normal in patients suffering from asthma, suggesting the existence of a protective chitinolytic activity in allergic inflammation (Seibold et al. 2008). In agriculture worldwide, fungal diseases cause severe yield losses in crops. Chitinase activity is an important defense strategy initiated by plants upon interaction with fungal pathogens, and these defense mechanisms have been improved in several plants, by using heterologous gene expression (Cohen-Kupiec et al. 1998; Li et al. 2005).

The poor solubility of chitin is the major limiting factor in utilization of the polymer. Chitosan is more soluble than chitin, and with its reactive amine group that allows chemical modification, chitosan is the more applicable of the two polymers. For example, chitosan has been showed to induce a resistance response in wheat seeds, by increasing production of hydrolytic enzymes and anti-fungal compounds (Dutta 2004). Even in the food industry, chitin derivatives have several applications; they are used as preservatives against microbial growth, and also included directly in some foods as fiber and functional ingredients (Tharanathan and Kittur 2003). Chitosan's ability of binding metals has been utilized in wastewater treatment (Onsøyen E. 1990). Although chitosan has a variety of applications, there are some problems due to its low solubility and high viscosity. Therefore, in some settings CHOS seems to be more applicable.

CHOS, being water soluble, are of great interest in several applications of both agricultural and medicinal origin. They have antibacterial activity, antifungal activity, effects on the immune system and have shown promising results in cancer tumor treatment (Jeon 2001; Xu 2007; Aam, Heggset et al. 2010). On the basis of the above, it has been claimed that chitin and its derivatives show a great potential in prevention of diseases, and can make a major contribution to public health (Muzzarelli 1997).

Several fungi and bacteria possess enzymes capable of degrading chitin and chitin derivatives, and research on a variety of species has yielded valuable insight into the machinery of chitin degradation in nature. Understanding how this machinery works will provide knowledge useful for the production of chitin-derived compounds and for utilization of the abundant resource that chitin represents. By understanding the mechanism of chitin degradation, this knowledge can be used for better understanding the process of degradation of other carbohydrates, such as cellulose. Cellulose has a potential in replacing fossil fuels and therefore represents a major resource of human interest.

1.2.2. Degradation of chitin

Bacteria and fungi capable of enzymatic chitin degradation primarily use their chitinolytic machinery to saccharify and consume the substrate as a nutritional source of carbon and nitrogen. Hence, they produce a number of enzymes that convert chitin to soluble products for further degradation through different metabolic pathways. Fungi with chitin containing cell walls also need one or more chitin degrading/ modulating enzymes during cell- expansion, sporulation and division. Enzymatic degradation of chitin can occur via different pathways (Hoell 2009). The major chitinolytic pathway involves hydrolysis of the β(1,4)- glycosidic bond, and the enzymes capable of hydrolyzing these bonds are called chitinases and belong to the glycoside hydrolase (GH) families 18 and 19 (see below, section 1.2.3). An alternative pathway for chitin degradation involves deacetylation of chitin to chitosan. Chitin deacetylases, which belong to family 4 of the carbohydrate esterases (CE), are the enzymes responsible for deacetylation of chitin. Further hydrolysis of the β-(1,4) –glycosidic bond of chitosan is carried out by chitosanases which are found in families 5, 8, 46, 75 or 80 of the GHs.

1.2.3. Glycoside Hydrolases

Because of the huge structural diversity of carbohydrates, there is a need for great diversity among carbohydrate degrading enzymes to accomplish the biological functions of the cell. Glycoside (or glycosyl) hydrolases (GHs) are enzymes hydrolyzing the glycosidic bond in di-, oligo- and polysaccharides, and are found in all living organisms (Davies et al. 1997). The GHs are classified in the CAZy GH database (http://www.cazy.org ; Henrissat 1991; Henrissat et al. 1997), in more than 100 families, based on their amino acid sequence similarities. Enzymes comprised in one family have similar three-dimensional structures and a similar catalytic mechanism, but may show some variety in substrate specificity and in the type of activity (e.g. processivity, endo/exo- activity). For hydrolysis of glycosidic bonds GHs use two acidic amino acid residues, one acting as a general acid and one as a nucleophile or base. This reaction occurs via overall retention or inversion of the anomeric configuration (described in section 1.2.5), depending on the orientation of the catalytic residues and the catalytic mechanism. In some types of retaining enzymes, e.g. those in GH families 18 and 20, the role played by the nucleophilic amino acid residue on the enzyme is played by the acetamido group at carbon 2 of the substrate. This is referred to as substrate- assisted catalysis (see section 1.4.2).

1.2.4. Structure of GHs

The catalytic domain of GHs comprises an active site where the substrate binds and catalysis occurs. The overall active site topology often reflects the activity and substrate specificity of the enzyme, and is divided in three main classes; pocket/crater, cleft/groove and tunnel, as illustrated in Figure 2. GHs often comprise one or more domains in addition to the catalytic domain that usually are carbohydrate binding modules (CBMs) that are thought to increase the binding affinity and specificity of the GH for its substrate.

A.) B.) C.)

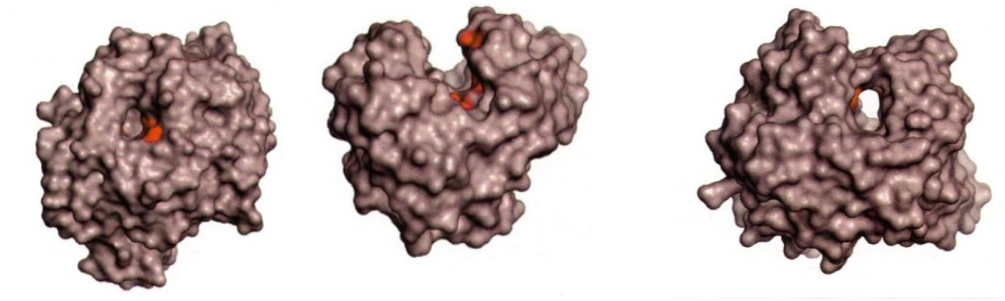


Figure 2. The general classes of active site topologies among Glycoside hydrolases (GHs). The figure shows the three general classes of active site (marked in red) topologies among GHs: A.) Pocket/crater, B.) Cleft/groove and c.) Tunnel. The figure is adapted from (Davies et al. 1995).

In GHs responsible of depolymerization, the catalytic activity is influenced by substrate binding sites distant from the site of bond cleavage (Davies, Wilson et al. 1997). Subsites are sugar binding sites in the active site of the enzyme, and for GHs they are labeled from –n to +n (n is an integer). –n represents subsites where the the non-reducing end of the substrate binds, while +n represents subsites where the reducing end binds. Cleavage of the glycosidic bond occurs between -1 and +1. A schematic overview of sugar-binding subsites in Glycoside hydrolases is shown in Figure 3.

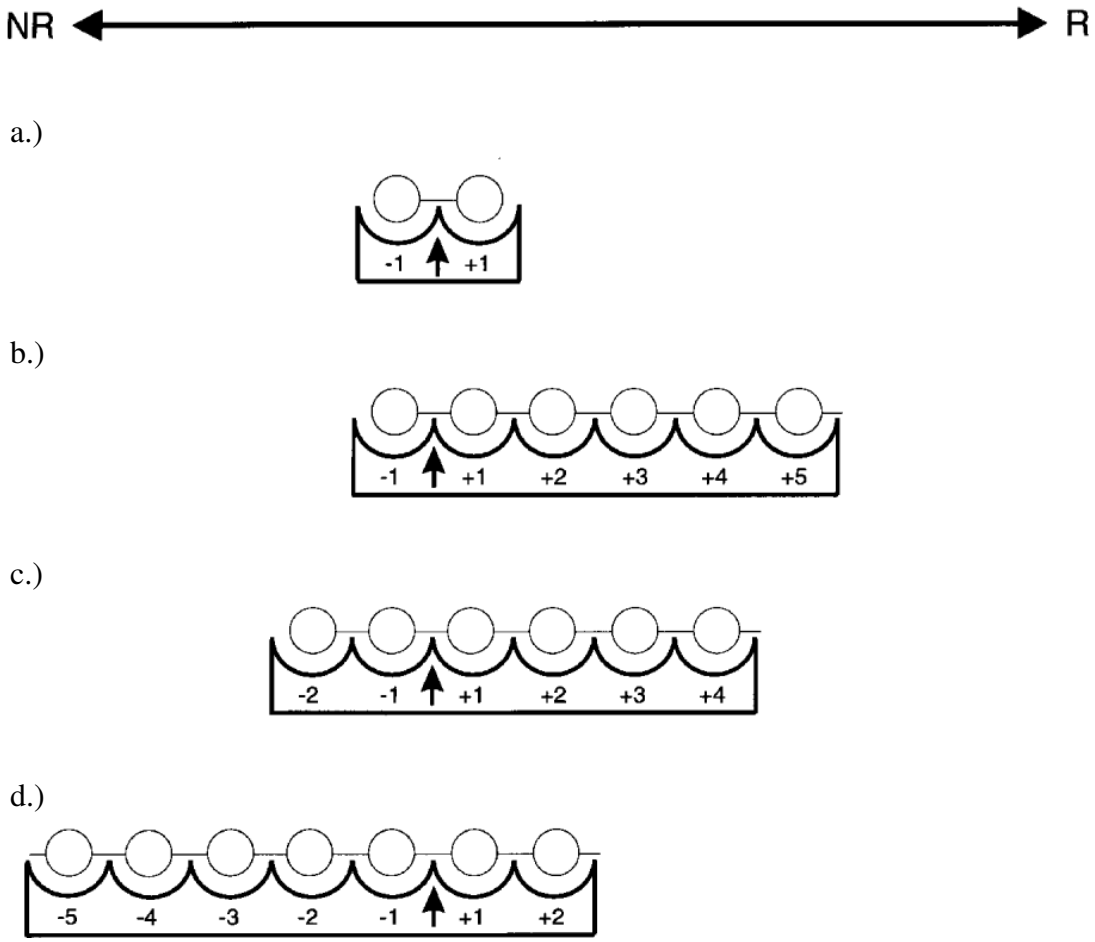


Figure 3. Schematic overview of sugar-binding subsites in Glycoside hydrolases adapted from (Davies, Wilson et al. 1997). The non-reducing end of the substrates is drawn on the left, and the reducing end on the right. The point of cleavage is marked with an arrow. a.) the –n, +n system applied to glycosidases; b.) the -n, +n system applied to enzymes cleaving a monosaccharide from the non-reducing end of the substrate; c.) the –n, +n system applied to enzymes cleaving disaccharide units from the non-reducing end of the substrate, e.g. β-amylase; d.) the –n, +n system applied to enzymes cleaving disaccharide units from the reducing end of the substrate , as proposed for Serratia marcescens Chitinase A.

A similar subsite system is also present among proteinases and nucleases, and the energies and properties of such subsites can be determined experimentally.

1.2.5. Mechanism of GHs

General acid catalysis requires two residues, the catalytic acid, which acts as a proton donor, and a nucleophile (base) (Koshland 1953). Usually among Glycoside hydrolases the proton donor and the nucleophile are an aspartate (D/ Asp) or glutamate (E /Glu) (Davies and Henrissat 1995). As mentioned previously, GHs

primarily employ two catalytic mechanisms, resulting in either retention or inversion of the anomeric configuration. In both mechanisms, the position of the proton donor is identical and within hydrogen bonding distance of the glycosidic oxygen (Davies and Henrissat 1995).

The retaining mechanism is a double displacement mechanism, that proceeds through two steps, illustrated in Figure 4 (Koshland 1953), (McCarter et al. 1994). This mechanism involves a covalent glycosyl-enzyme intermediate, and each step includes an oxocarbenium ion-like intermediate (www.cazypedia.org). The glycosidic oxygen in the scissile bond is protonated by the catalytic acid, and leaving group departure is stimulated by a concomitant nucleophilic attack on the anomeric carbon by the catalytic nucleophile. This proceeds through the formation of an oxocarbenium ion-like transition state and leads to breakage of the glycosidic bond and formation of a covalent bond between the anomeric carbon and the nucleophile. Subsequently, hydrolysis occurs when an activated water molecule attacks the anomeric carbon from a position near the site of the original glycosidic oxygen. Therefore, the anomeric configuration of the sugar is retained (Koshland 1953). The water molecule is activated by the catalytic acid, which is now functioning as a base after donating its proton in the first step of the reaction.

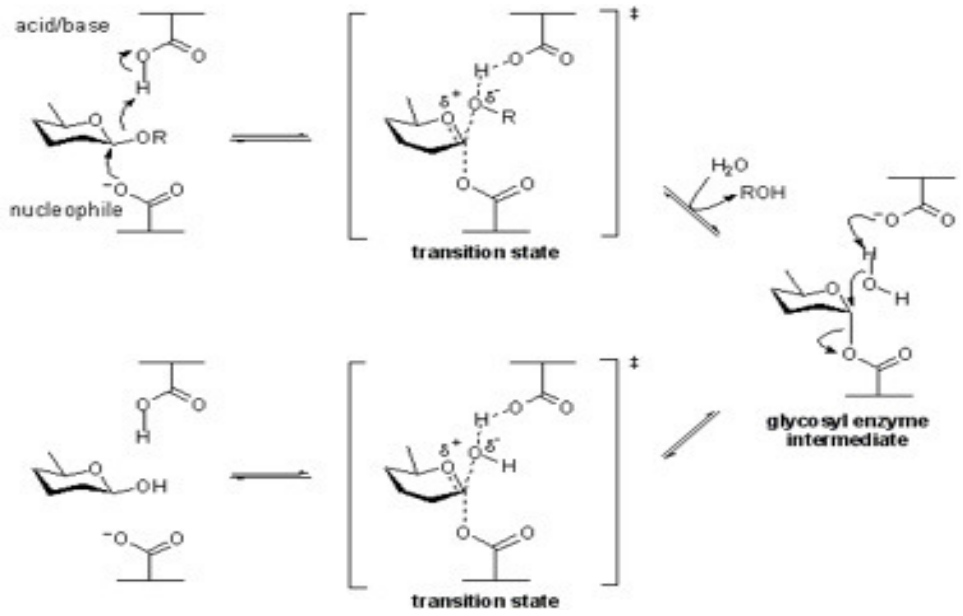


Figure 4. The retaining mechanism of β-glycoside hydrolases. The figure is adapted from (www.cazypedia.org), and shows the mechanism first proposed by Koshland.

The inverting mechanism is a one step mechanism, referred to as a single displacement mechanism, illustrated in Figure 5. The glycosidic oxygen is protonated by the catalytic acid, and leaving group departure is stimulating nucelophilic attack of a water molecule that is activated by the catalytic base. The activated water molecule attacks the anomeric carbon from the opposite site of the substrate. This leads to inversion of the anomeric configuration. This reaction also proceeds through an oxocarbenium ion-like transition state.

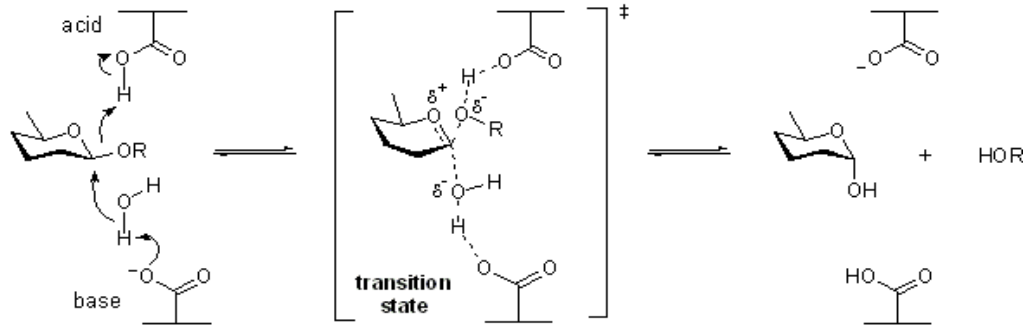


Figure 5. The inverting mechanism of β-glycoside hydrolases. The figure is adapted from (www.cazypedia.org).

Chitinases occur in two families, GH18 and GH19. Family 19 enzymes are inverting and use the standard inverting mechanism. Family 18 chitinases are retaining enzymes, and together with the family 20 enzymes, they employ a variant of

the double displacement mechanism, referred to as the substrate-assisted double displacement mechanism. This substrate-assisted mechanism is further described and illustrated in section 1.4.2.

1.3. The chitinolytic machinery of *Serratia marcescens*

Serratia marcescens is a Gram-negative bacterium belonging to the family of Enterobacteriaceae in the phylum proteobacteria (Hejazi et al. 1997). This soil bacterium is one of the most efficient bacterial degraders of chitin, and when grown in the presence of chitin, it produces chitinolytic and chitin binding proteins (Brurberg 2000; Toratani, Shoji et al. 2008). Three family 18 chitinases, a family 20 Chitobiase and a chitin binding protein, CBP21, have been observed as part of this machinery (Tews, Vincentelli et al. 1996; Brurberg 2000). Recently, the chitin binding protein CBP21, previously thought to be non-catalytic, was shown to have enzymatic properties (Vaaje-Kolstad, Westereng et al. 2010). CBP21 acts on the surface of chitin and introduces chain breaks in the inaccessible polymer, creating oxidized chain ends. It has been shown that this helps speed up the degradation of chitin by chitinases.

The Chitinases convert chitin to oligosaccharides, primarily dimers of GlcNAc. Family 20 GHs are β-N-acetyl hexosaminidases (Chitobiases) that further degrade the oligomers to GlcNAc. The family 20 GHs comprise both eukaryotic and prokaryotic enzymes and are retaining glycoside hydrolases. They catalyze the removal of β-1,4 linked N-acetylhexosamine units from the non-reducing end of chitooligosaccharides and their conjugates.

1.4. Chitobiase from Serratia marcescens

1.4.1. Structure and function

Chitobiase, or *N*-Acetylhexosaminidase, of *S. marcescens* is a large protein of 98.5 kDa consisting of four domains (Tews, Perrakis et al. 1996) (Figure 6 and Figure 7). Chitobiase cleaves off GlcNAc units from the non-reducing end of chitobiose ($GlcNA_2$) and longer oligosaccharides (Drouillard, Armand et al. 1997). The catalytic domain of this large enzyme belongs to a GH family 20, which also comprises the

human hexosaminidases. The human hexoxaminidases catalyze the removal of β (1,4) linked N-acetyl-D-glucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) from the non-reducing end of oligosaccharides and glycoconjugates (Mark et al. 2003). Interest in the human hexosaminidase derives from its association with the lysosomal storage disorders Tay-Sachs disease and Sandhoff disease (discussed in detail in section 1.4.3).

The crystal structure of the *S. marcescens* Chitobiase (Figure 7) represented the first GH family 20 enzyme to have its three-dimensional structure solved. The C-terminal domain protrudes from a compact body formed by the three other domains (Tews, Perrakis et al. 1996). The catalytic site is located in domain III, which has a $(\beta\alpha)_8$-barrel structure with some modifications. Three disulfide bridges stabilize the structure of Chitobiase, and the native enzyme contains no cofactors, metals or other ligands. Domain I may play a role in substrate binding, but this has not yet been addressed experimentally. The roles of domains II and IV are unknown (Figure 6).

Figure 6. The domains of S. marcescens Chitobiase. The scheme which is derived from the Pfam database (http://pfam.sanger.ac.uk)., shows the four domains of S.marcescens Chitobiase. The third domain is the catalytic domain, GH family 20 . The other domains of Chitobiase have unknown functions. The first domain, referred to as CHB_HEX domain, shows similarity with cellulose binding domains. The second and fourth domain have completely unknown functions and have only been observed in connection family GH20 catalytic domains.

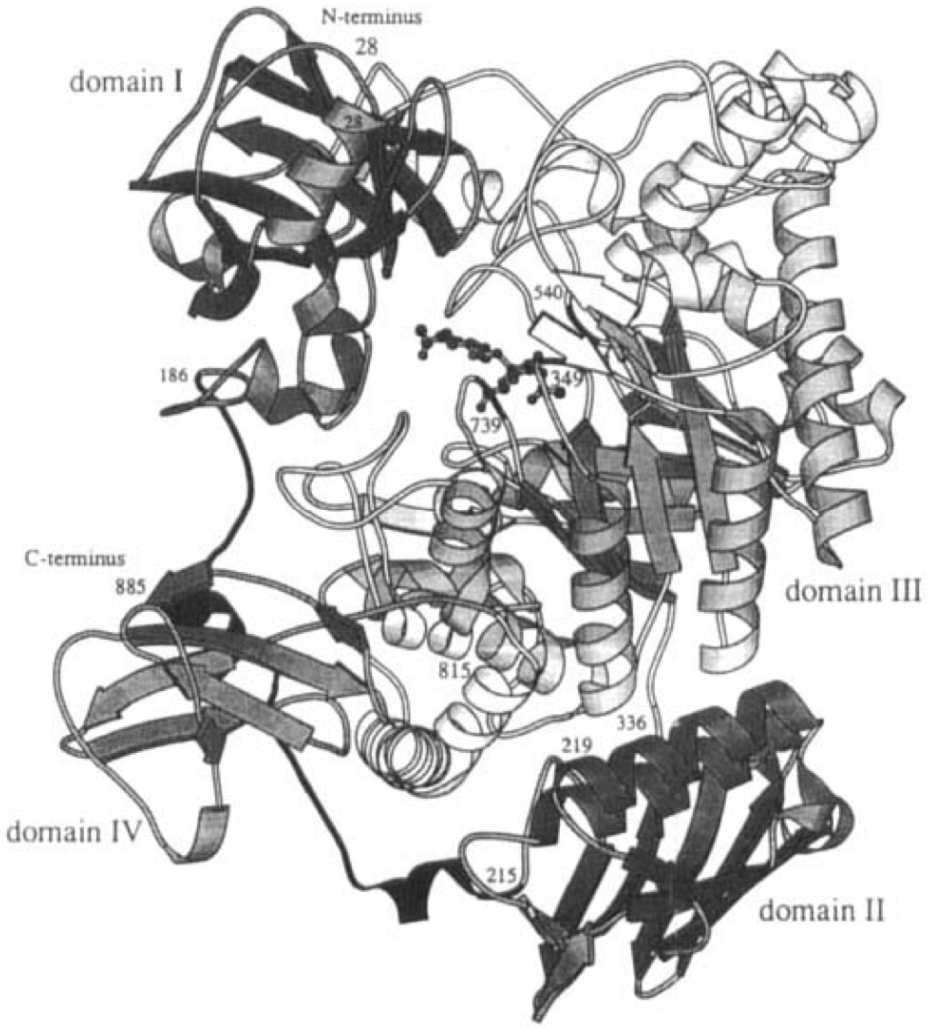


Figure 7. Crystal structure of the four-domain S. marcescens Chitobiase. The figure is taken from (Vorgias et al. 1996), showing the crystal structure of S. marcescens Chitobiase. The catalytic acid Glu540 is shown in ball-stick symbols (see section 1.4.2 for details on catalysis).

1.4.2. Mechanism and active site architecture

Studies on a family 20 human β-hexosaminidase and the Chitobiase from *S. marcescens* have demonstrated that family 20 GHs operate via a substrate-assisted retaining mechanism (Mark et al. 2001) described in section 1.2.5. Like family 18 chitinases, family 20 hexosaminidases and Chitobiases lack the carboxylate group thought to stabilize the oxacarbenium ion transition state in the standard double displacement mechanism (Drouillard, Armand et al. 1997). X-ray structural analysis of Chitobiase from *S. marcescens*, and kinetic studies with inhibitors, as well as crystallographic evidence from a paper on the family 20 β-hexosaminidase from *Streptomyces plicatus* have provided evidence for substrate-assisted catalysis (Mark,

Vocadlo et al. 2001). The acetamido group at carbon 2 of the sugar bound in the -1 subsite replaces the nucleophilic residue of the enzyme, functioning as a nucleophile to generate an enzyme-stabilized oxazolinium ion intermediate. The cyclic intermediate is then hydrolyzed by attack of an activated water at the anomeric carbon. The mechanism is illustrated in Figure 8.

In their original paper, *Tews et al. (1996)* also described the crystal structure of the complex of wild-type Chitobiase and its natural substrate (Tews, Perrakis et al. 1996). The active site is located at the C-terminal end of domain III, in the centre of the curved side of the enzyme. Here, $GlcNAc_2$ binds in pocket. When bound to the enzyme's active site, the sugar is distorted; the two sugar planes are tilted around their glycosidic linkage (Tews, Perrakis et al. 1996). There are several important amino acids involved in binding the substrate, in addition to the catalytic acid. The non-reducing end of the sugar is anchored by hydrogen bonding between the sugar and the amino acid residue Arg349. Glu540 interacts with the glycosidic linkage and is the carboxylate residue acting as the catalytic acid. In family 20 GHs this amino acid is fully conserved, providing further evidence of Glu540 being the catalytic acid. Another conserved residue among family 20 GHs is Asp539 (Prag et al. 2000). This residue is thought to be involved in interaction with the substrate acetamido group of the substrate bound in subsite -1 to promote the attack on the anomeric centre (Hou et al. 2001). Prag et al. investigated the role of the conserved amino acid pair Asp539-Glu540 in *S.marcescens* Chitobiase by site-directed mutagenesis, biochemical characterization and by structural analyses of Chitobiase- substrate complexes. They concluded that both residues are essential for the activity of Chitobiase, with Glu540 acting as the proton donor, and Asp539 being involved in substrate binding as well as in positioning and activating the acetamido group of the substrate in subsite -1.

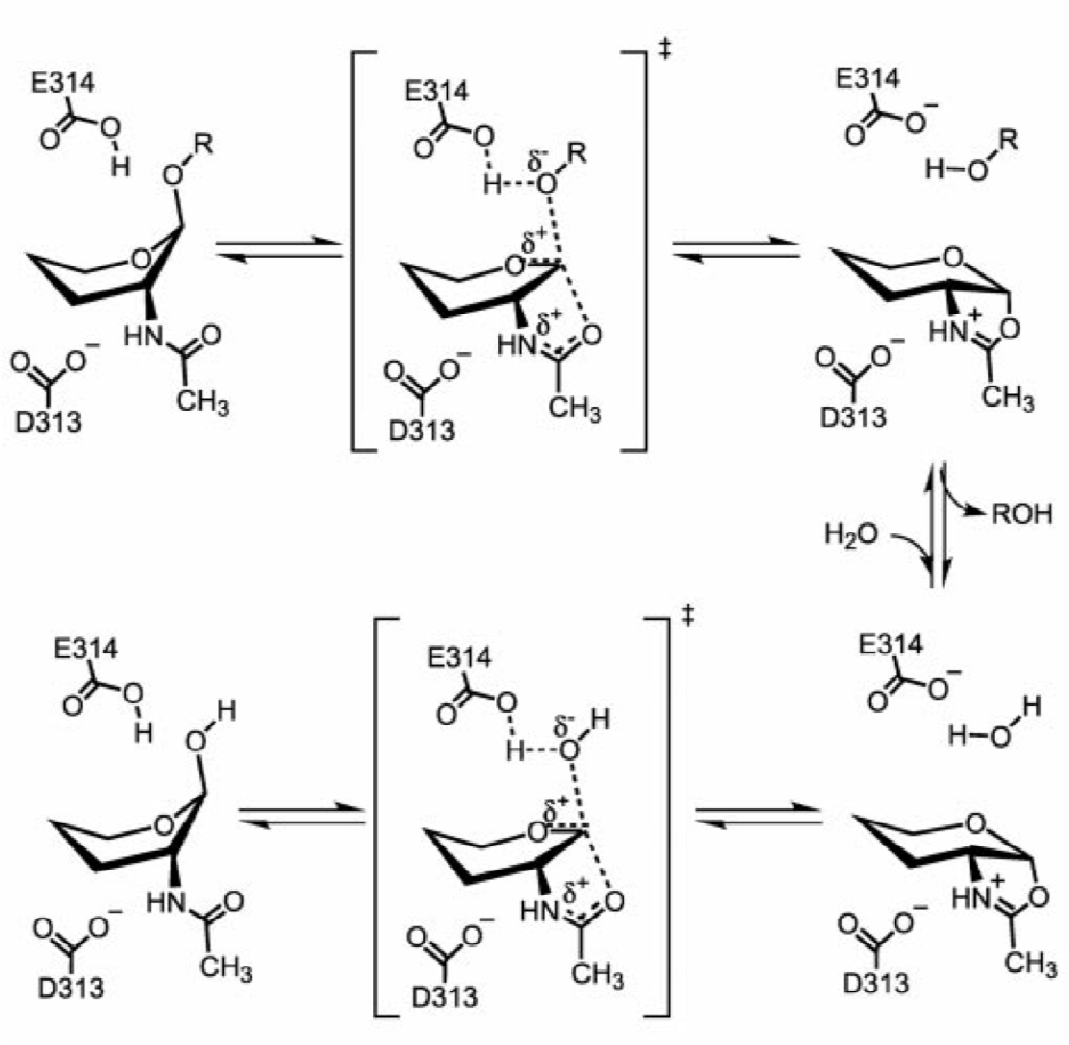


Figure 8. Proposed substrate-assisted catalytic mechanism of family 20 β-hexosaminidases (Mark, Vocadlo et al. 2001). The figure shows the proposed substrate-assisted catalytic mechanism of family 20 β-hexosaminidases. In S. marcescens Chitobiase the corresponding residues are Glu540 (E314 in the figure) and Asp539 (D313 in the figure). The figure is taken from (Mark, Vocadlo et al. 2001).

1.4.3. Involvement of family 20 GHs in Tay-Sachs and Sandhoff disease

In humans, there are two major family 20 β-hexosaminidase isoenzymes, β-Hexosaminidase A (HexA) and β- Hexosaminidase B (HexB) (Hou et al. 2000). The isoenzymes are dimeric enzymes comprising α- and/or β- subunits, encoded by the *HEXA* gene and the *HEXB* gene, respectively. HexA is a heterodimer of subunits α and β, while HexB is a homodimer of the β-subunits. The α- and β-subunits show 60 % sequence identity, and are thought to be structurally and evolutionary related. The

β-Hexosaminidase S (HexS), a dimer of two α-subunits represents a third isoenzyme, but only HexA and HexB can be detected in normal human tissue.

The association of beta-hexosaminidases with inheritable lysosomal storage disorders, such as Tay-Sachs disease and Sandhoff disease, has made these enzymes a target for extensive biochemical and biomedical research (Mark, Mahuran et al. 2003). The lysosomal N-acetyl-β-D-hexosaminidases, catalyzes the removal of β(1,4) linked N-acetyl-D-glucosamine and N-acetyl-D-galactosamine from the non-reducing end of oligosaccharides and glycoconjugates (Mark, Mahuran et al. 2003). However, the hydrolysis of the G_{M2} ganglioside (described below), which contain a charged sialic acid group, is carried out by only the α-subunit of HexA. For hydrolysis, Hex A requires an activator protein, the G_{M2} activator protein, which functions as a lipid transporter that removes G_{M2} from its membrane environment and presents it to HexA. Deficiency of either the α- or β-subunit of HexA, or the G_{M2} activator protein therefore leads to the lysosomal storage disorders. The disorders are characterized by intralysosomal accumulation of an acidic glycolipid G_{M2} ganglioside, primarily in the brain and in peripheral nerve tissue. Gangliosides are molecules composed of a glycosphingolipid (subtype of glycolipids that contain the amino alcohol sphingosine) with one or several sialic acids (e.g. ne-acetylneuraminic acid) linked to the sugar chain (www.wikipedia.org).

Even though the enzymes only show 26 % sequence identity upon alignment between the active site regions of human α-subunit and the monomeric *S.marcescens* Chitobiase, the three-dimensional structure of *S.marcescens* Chitobiase has been used for molecular modeling of human hexosaminidases . The identity between Chitobiase with either subunit of human Hex is restricted to the active site regions of the enzymes. Experiments aimed at investigating some of the conserved active site residues have revealed possible functions of these, and indicate that the catalytic mechanisms and active site architectures of the human enzymes and the bacterial enzyme are highly similar (Hou, Vocadlo et al. 2001), (Hou, Vocadlo et al. 2000). The crystal structures of both human HexA and HexB has been determined, and research on these enzymes provides crucial knowledge for understanding the

mechanism and molecular basis of disorders such as Tay-Sachs disease and Sandhoff disease (Mark, Mahuran et al. 2003), (Lemieux et al. 2006).

1.5. Kinetic characterization of enzymes

Enzymes are biological catalysts; without being altered, they speed up the velocity of biological reactions (Mathews 2000). Most enzymes are proteins. Enzymes do not change the equilibrium between reactants and products, but they change how fast equilibrium is reached by lowering the activation energy. In enzyme catalysis, the substrate(s) is (are) bound to the active site of the enzyme, to form an enzyme-substrate complex, before product(s) is (are) released. The rate-limiting step in a reaction can be substrate binding, the actual conversion of the substrate, or product release.

The velocity of a reaction is measured by monitoring degradation of substrate or formation of products. In steady-state kinetic analysis one employs low concentrations of enzyme and relatively high concentrations of substrate. In that case a condition of steady state will emerge, because the concentrations of unbound substrate and product change slowly and remain relatively high and low, respectively (Figure 10). The most simple enzymatic reactions for which steady-state conditions can be reached may be described by the Michaelis-Menten equation, and the enzyme may be characterized using the parameters, K_M and k_{cat}. For an enzyme showing Michaelis-Menten kinetics, the reaction velocity (V_0) as a function of the substrate concentrations [S] is illustrated in Figure 9. The Michaelis-constant, K_m, describes the substrate concentration, at which the reaction velocity is half of maximum velocity (½ V_{max}). This parameter is often thought to describe the enzyme's affinity for substrate: an enzyme with a high K_M requires a higher substrate concentration for achieving maximum velocity of the reaction, than an enzyme with a low K_M value. k_{cat} measures the rate of the catalytic reaction, that is the number of substrate molecules converted by one enzyme per second. k_{cat} is calculated by dividing an experimentally determined V_{max} by the total concentration of enzyme $[E]_t$. Kinetic characterization of enzymes reveals their most important properties and is also important for elucidating their mechanism. This information can be used in investigation of the structural and mechanistic properties of the enzyme, and provides insight into the interaction

between substrate and enzyme. Further, knowledge of enzyme kinetics can be utilized in manipulation of the enzyme for obtaining e.g. a more stable enzyme (active at different pH's or temperatures), or a more efficient enzyme (by speeding up the rate-limiting step of the reaction). By understanding the features of enzymes, it becomes easier to apply them in or develop them for industrial and medical uses, e.g. in production of insulin, improvement of detergents and degradation of rigid carbohydrates for biofuel production.

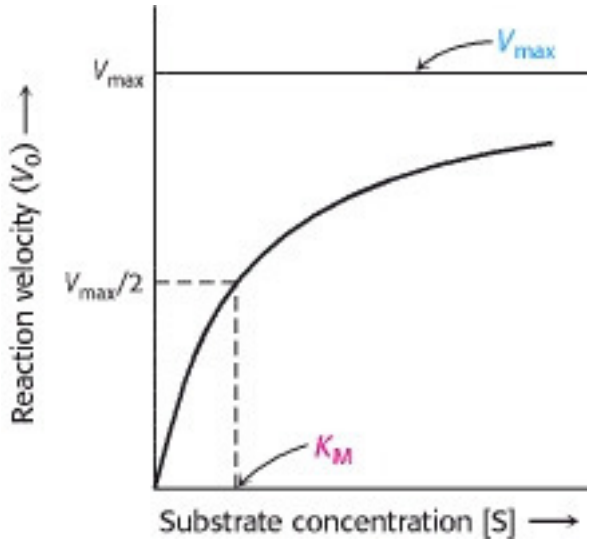


Figure 9. Michaelis-Menten kinetics. A plot of reaction velocity (V0) as a function of the substrate concentrations [S] for an enzyme showing Michaelis-Menten kinetics. The maximum velocity (Vmax) is reached asymptotically, and the KM is the substrate concentration that yields Vmax/2. The figure is taken from Biochemistry 5th edition (Berg 2002).

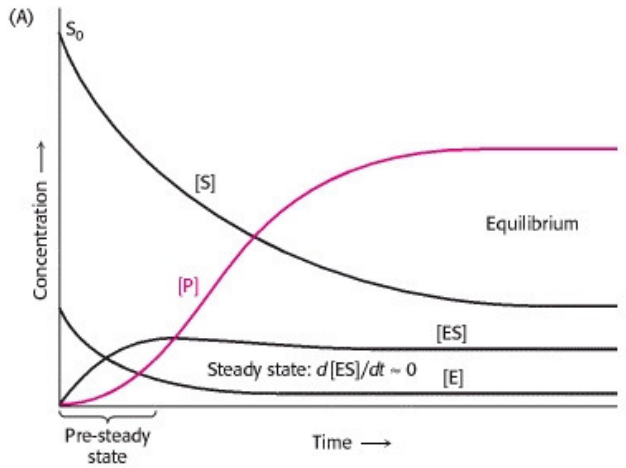


Figure 10. Changes in concentrations of reaction participants in an enzyme-catalyzed reaction during steady state conditions (Berg 2002).

1.6. Enzymatic assays

Artificial substrates such as 4-methylumbelliferyl-$GlcNAc_n$ is often utilized in characterization of chitinases (Krokeide et al. 2007).This substrate produces the fluorescence group 4-MU upon hydrolysis, and represents an easily detectable and rapid method for measuring enzymatic activity. However, there are some disadvantages of using this non-natural substrate, like substrate inhibition, a non-natural leaving group and non-linear kinetics. pNP-$GlcNac_n$ is an alternative artificial substrate that yields the yellow chromophore pNP. Unfortunately, the same disadvantages as for the 4-MU-$GlcNAc_n$ are observed for this substrate. To avoid these problems in kinetic characterization of chitinases, *Krokeide et al.* established a natural substrate assay for chitinases by using high-performance liquid chromatography (Krokeide, Synstad et al. 2007). The experiments showed that using the natural substrate of the enzyme prevented the problems observed for the artificial substrates. Comparing the kinetic parameters from characterization with different substrates may provide information useful in interpretation of the mechanism of the enzyme.

High performance liquid chromatography (HPLC) is a chromatographic method for separation of compounds in solution. The method is widely used in biochemistry and analytical chemistry for identification, purification and quantification of a wide variety compounds. The instrumentation of HPLC includes a pump, an injector, a column for separation (the stationary phase), and a liquid reservoir called mobile phase. The columns are packed with small immobile particles (μm size), and the mobile phase is driven through the column by a high-pressure pump. The compounds loaded to the column interact with the stationary and the mobile phase of the system, and the compounds pass through the column with different retention times because the interactions with the stationary and the mobile phase depend on the nature of the compound. The compounds are eluted from the column, and detected, using one of several possible detection systems including UV absorbace, fluorescence, refractive index (RI) and mass spectrometry (MS).

HPLC is well designed for identification and quantification of chitooligosaccharides: Using columns packed with silica particles

chitooligosaccharides of different sizes show different retention times and separation of the alpha and beta anomers is also possible. Because of the *N*-acetyl groups in chitooligosaccharides, they can be detected by measuring absorbance of UV light, i.e. one can use the simplest and most common detection systems. Hence, based on the intensity of the UV signal and retention times, the concentration and type of chitooligosaccharide can be elucidated.

1.7 Outline and purpose of this study

The overall goal of this study was to gain more insight into the function of the *S. marcescens* Chitobiase. On the one hand we wanted to study enzyme performance towards natural substrates, a topic about which remarkably little information was present in the literature. On the other hand we wanted to investigate possible "unknown" functions and interactions of the Chitobiase. For example, the large size and the presence of three domains additional to the catalytic domain are intriguing and one question asked was whether these domains could play roles in protein-protein interactions.

As part of achieving this overall goal, the work described in this thesis comprised the following elements: cloning of the gene encoding *S.marcescens* Chitobiase, followed by purification, for obtaining pure enzyme for utilization in biochemical experiments. Subsequently, kinetic characterization of *S.marcescens* Chitobiase with both artificial and natural substrate was performed, to verify the activity of the recombinant enzyme, as well as investigating the kinetics of the enzyme with its natural substrate, previously not described in literature. Degradation of crystalline β-chitin with Chitobiase in combination with other chitin degrading enzymes from *S.marcescens* (ChiB, ChiC and CBP21) was observed to reveal possible synergistic effects among the enzymes, giving knowledge about how the chitinolytic machinery of *S.marcescens* works in nature.
Some pulldown experiments were performed for investigation of possible interaction partners of Chitobiase, with the overall goal of understanding the functions of the three domains additional to the catalytic domain.

2. Materials

2.1. Laboratory equipment and instruments

Laboratory equipment and instruments utilized for the experimental work described in this thesis are listed in Table 1.

Table 1. Laboratory equipment and the instrumentation utilized in this thesis.

Equipment/ instrument	**Supplier**
Finnpipettes	Labsystems
LC621P and 1602 weights	Sartorius
RCT classic magnetic stirrer	IKA
Eppendorf tubes	Axygen
HPLC vials	
Micro freeze tubes 2 ml	Sarstedt
Nunc tubes, 15 and 50 ml	BIO101, Inc
Falcon 2059 Polypropylene Round-Bottom tube	Sarstedt
Sterile filters, 0.22µm	Micro Filtration Systems
AmiconUltra-0.5 and -15	Millipore
Centrifuge 5415 R	Eppendorf
Centrifuge 5430 R	Eppendorf
Avanti JM-25 centrifuge	Coulter Beckman
Biophotometer	Eppendorf
Mastercycler gradient, pcr cycler	Eppendorf
CO8000 cell density meter	WPA
Hoefer DyNa Quant 200 Fluorimeter	Hoefer
Sterile bench	TELSTAR
pH meter 827	Metrohm
Incubator cabinet	Termaks

Wather bath	Julabo
Äkta purifier	GE healthcare
Frac920	GE Healthcare
LabTips	Thermo scientific
Cuvettes	Brand
Bio Rad gel system	Bio Rad
Bio Rad Powerpac 300	Bio Rad
WWR Nitrile disposable gloves	WWR
HPLC Ultimate 3000	Dionex
Agilent 1290 Infinity UHPLC	Agilent technologies
MALDI-TOF MS: Ultra flex TOF/TOF	Bruker Daltonics
LC-MS: Ion trap	Bruker Daltonics
LC-MS: HPLC	Agilent

2.2. Chemicals and reagents

The chemical and reagents used in this thesis is shown in Table 2.

Table 2. Chemicals, reagents and their suppliers .

Chemical	**Supplier**
2-β-mercaptoethanol	Sigma-Aldrich
Acetic acid	Merck
Aceton	WWR International
Acetonitril	Fulltime chemical
Bacto Agar	Saveen Werner AB
Agarose, seakem LE Agarose	MedProbe Cambrex
Ammonium acetate	Merck
Ammonium Bicarbonate (AmBic)	Fluka analytical
Ammonium sulphate	Merck
Bacto Yest Extract	Becton Dickinson
Bacto Tryptone	Becton Dickinson
Bio-Rad Protein Assay, Dye Reagent	Bio-Rad

Concentrate	
Citric acid	Sigma-Aldrich
Coomassie Brilliant Blue R250	Bio-Rad
Disodium carbonate	AppliChem
Disodiumhydrogenphosphate dihydrate, Na_2HPO_4 x 2 H_2O	Merck
Dithiothreitol, DTT	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethanol (EtOH)	AS Vinmonopolet
Ethidium bromide, EtBr	Sigma-Aldrich
Formic acid	Sigma-Aldrich
Glucose	WWR International
Glycerol	Merck
Glycine	Merck
4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)	Sigma-Aldrich
Hydrochloric acid (HCl)	Merck
Iodoacetamide (IAA)	Merck
Imidazole	Sigma-Aldrich
Isopropanol	AS Vinmonopolet
Isopropyl β-D-1- thiogalactopyranoside, (IPTG)	Sigma-Aldrich
Kanamycin sulphate	Sigma-Aldrich
Magnesium sulphate	Merck
Methanol	Merck
NEB Buffer 4	Nex England Biolabs
NuPAGE® LDS Sample Buffer 4X	Invitrogen
NuPAGE® Sample Reducing Agent 10X	Invitrogen
Potassium dihydrogen phosphate	Merck
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich
Reduced glutathione	Sigma-Aldrich
Sodium acetate	Merck

Sodium chloride, NaCl	Merck
Sodium dodecyl sulfate (SDS), $C_{12}H_{25}O_4SNa$	AppliChem
Sodiumdihydrogenphosphate monohydrate, NaH_2PO_4 x H_2O	Merck
Sodium hydroxide (NaOH)	Merck
Sodium phosphate, dibasic	Merck
Sodium phosphate, monobasic	Merck
Trifluoroacetic acid (TFA)	Fluka analytical
Trizma base, Tris (hydroxymethyl) $C_4H_{11}NO_3$	Sigma-Aldrich
Tris-HCl, $C_4H_{11}NO_3$ x HCl	Sigma-Aldrich

2.3. Growth media for Bacteria

2.3.1. Luria-Bertani (LB)

Recipe:

10 g Bacto Trypton

5 g Bacto Yeast extract

10 g NaCl

After mixing the medium components, dH_2O was added up to a total volume of 1 L. The medium was autoclaved for 15minutes at 115°C before use.

Agar plates:

For agar plates, 1.5 % (w/v) agar was added prior to autoclaving. Appropriate antibiotics were supplemented after cooling the autoclaved medium to temperatures below 55°C. The medium was then transferred to petri dishes.

2.3.2. Terrific Broth Media

Recipe:

12 g Bacto Trypton

24 g Bacto Yeast extract

4 ml Glycerol

The components were solved in 900 ml, and autoclaved at 115°C for 15minutes. After cooling to room temperature, 100 ml of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 , sterilized by filtration, was added to the medium.

2.3.3. Basal Salt Buffer

0.375 g Na_2CO_3
0.375 g KH_2PO_4
0.325 g $(NH_4)_2SO_4$
0.250 g NaCl
0.125 g $MgSO_4$
33.35 g HEPES
Carbon sources were 1 w/v % chitin or 6.2 ml/l glycerol
dH_2O was added to an end volume of 1000 ml.

2.4. Bacterial strains

Three bacterial strains were used in this study, two *E. coli* strains for cloning and gene expression and a *Serratia* strain as source of chitinolytic enzymes. The strains:
E. coli NovaBlue GigaSingles™ Competent Cells
E. coli BL21 (DE3) Competent Cells
Serratia marcescens BJL200

2.5. Proteins/enzymes

The proteins and enzymes utilized in this thesis are listed in Table 3.

Table 3. Proteins and enzymes used in the experiments described in this thesis.

Bovine serum albumin (BSA)	New Englang Biolabs
Lysozyme	Sigma-Aldrich
Benchmark Protein Ladder	Invitrogen
Phusion DNA polymerase	Finnzymes
Restriction enzymes; XhoI and AatII	New England Biolabs
Chitobiase (*S. marcescens BJL200*)	Cloned and expressed in *E.coli* (this work)
Chitinase B (*S. marcescens BJL 200*)	(Brurberg et al. 1995)
Chitinase C (*S.marcescens BJL 200*)	(Synstad et al. 2008)
CBP21 (*S. marcescens BJL 200*)	(Vaaje-Kolstad et al. 2005)

2.6. Enzyme Substrates

The enzyme substrates utilized in this thesis are listed in Table 4.

Table 4. Substrates utilized in the experiments described in this thesis.

Substrate	Supplier
4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide, 4-MU-GlcNAc	Sigma-Aldrich
N-acetylglucosamin, GlcNAc	Sigma-Aldrich
Di-N-acetylchitobiose, $GlcNAc_2$	Sigma-Aldrich
Tri-N-actylchitotriose, $GlcNAc_3$	Sigma-Aldrich
Tetra-N-acetylchitotetraose, $GlcNAc_4$	Sigma-Aldrich
Penta-N-acetylchitopentaose, $GlcNAc_5$	Sigma-Aldrich
Hexa-N-acetylchitohexaose, $GlcNAc_6$	Sigma-Aldrich
Hexamer of chitosan, partly deacetylated,	Berit Bjugan Aam

2.7. Kits

The kits utilized in this thesis are listed in Table 5.

Table 5. This table lists the kits utilized in this thesis.

Kit	Supplier	Application
E.Z.N.A Bacterial DNA kit	Omega Bio-tek	Purification of bacterial chromosomal DNA
Nucleospin Plasmid kit	Macherey-Nagel	Purification of plasmid DNA
Nucleospin Extract kit	Macherey-Nagel	Purification of DNA from agarose gels
pET-30 Xa/LIC Vector Kit	Novagen	Ligation Independent Cloning (LIC)
BigDye Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems	Sequencing
Pierce Silver Stain for Mass Spectrometry	Thermo Scientific	Staining method (SDS-PAGE)

2.8. Primers

The primers designed and used for cloning and sequencing of the Chitobiase encoding gene from *S. marcescens* are shown in Table 6. The primers were designed using the Gentle software and synthesized by Eurofins. The pET-30 Xa/LIC vector was used for cloning. This vector is designed for ligation independent cloning (described in section 3.1.8), and the primers were specially designed for LIC-cloning (for further details, section 3.1.5 and 3.1.8).

Table 6. The primers utilized for cloning and sequencing of the Chitobiase encoding gene from Serratia marcescens.

Name	Sequence (5'-> 3')
Chb LIC FP	GGTATTGAGGGTCGCGATCAACAGCTGGT
Chb LIC RP	AGAGGAGAGTTAGAGCCCTAGACCTTCTCGGC
Chb seq1 FP	CCGTTCACCGGCGACCAGTGGAAG
Chb seq2 FP	CGCGGCATTTTCCTCGACGTGGCG
Chb seq3 FP	TCCTCCCAGCGCTTTGTCGACAAG
Chb seq4 FP	GACTTCCCTTACGAGGTGAATCCG
Chb seq1 RP	GCTTTTGGCGTTGAAGTGGTTGCCGTC
Chb seq2 RP	CAGGCGGATGTTTTTCGCCTCATC
Chb seq 3 RP	ATTGAGCTTGTAAGCCGCCATCTG
Chb seq4 RP	CAGCGTCTGCAGATCGGCATTGCT

2.9. Software

Gentle Software	http://gentle.magnusmanske.de/	Primer design
BioEdit	www.mbio.ncsu.edu/BioEdit/bioedit.html	DNA sequence analysis
GraphPad Prism	graphpad.com/	Non-linear regression

2.10. Web services

CAZy	www.cazy.org

3. Methods

3.1. Cloning, sequencing and expression of the Chitobiase encoding gene from *Serratia marcescens*

3.1.1. Cultivation and storage of Bacteria

E. coli strains were grown using either LB medium or Terrific Broth medium at 37°C. 100 µg/ml Kanamycin was added for selection of bacteria containing the plasmid of interest. When using liquid media, incubation was performed in a shaking incubator, while agar plates where incubated in a heating cabinet.

For long time storage of bacteria, glycerol stocks of the bacteria was prepared and stored at -80°C. Using sterile techniques, a single colony was selected from an agar plate and grown in liquid medium over night. The glycerol stock was prepared by gently mixing 300 µl of 87% (v/v) glycerol with 1 ml of overnight bacterial culture. The mixtures were frozen immediately at -80 °C.

3.1.2. Recombinant DNA technology

Biotechnology, defined as "the use of living cells to produce materials useful to people" (Savada 2008) has been utilized for several thousands of years. Use of bacterial cultures in production of cheese and yogurt, and the use of yeast in brewing beer and wine are techniques representing the earliest examples of biotechnology, even though one at the time was not aware of the science behind these processes. Today, biotechnology has become an important science, both industrially and academic. Development of recombinant DNA technology, has revolutionized biotechnology, by allowing creation of new species utilized for protein production. In 1973, *Cohen et al.* were the first to demonstrate this artificial manipulation of the DNA molecule, by creation of plasmids with DNA fragments from several sources and further transformation into *Escherichia coli* (Cohen 1973*).* In this master's thesis, recombinant DNA technology has been utilized for cloning, sequencing and expression of the *Serratia marcescens* Chitobiase- encoding gene.

3.1.3. Purification of bacterial chromosomal DNA

Purification of *S. marcescens* chromosomal DNA was carried out using the E.Z.N.A Bacterial DNA kit (Omega bio-tek). The protocol was executed in accordance to the user manual provided by the manufacturer, with only minor modifications.

Materials:

- E.Z.N.A™ Bacterial DNA Kit (Omega bio-tek),
    - TE Buffer/Elution buffer: Aqueous solution of Tris (hydroxymethyl) amino methane/Hydrochloric acid.
    - BTL Buffer: Aqueous solution of Tris (Hydroxymethyl) Aminomethane/hydrochloric acid, Sodium Chloride and detergent
    - BDL Buffer: Aqueous solution of isopropanol and guanidine hydrochloride
    - DNA Wash Buffer : Aqueous solution of Tris (hydroxymethyl) amino methane/Hydrochloric acid and sodium chloride + absolute (96-100%) ethanol
    - RNaseA
    - ProteinaseK
    - Lysozyme
    - HiBind™ DNA spin-column
    - 2 ml collection tube

Procedure:

1. An overnight bacterial culture (3 ml) was centrifuged (4000 x g, 10 minutes) and the supernatant discarded. The bacterial cells were resuspended in 100 µl TE buffer.
2. Lysozyme (18 µl 50 mg/ml) was added to promote lysis of the cells, followed by incubation for 75 minutes at 30°C.
3. The lysed cells were pelleted by centrifugation (5000 x g, 5 minutes), and resuspended in 200 µl Buffer BTL. 25 µl Proteinase K (20 mg/ml) was added for removal of proteins, followed by brief vortexing and incubation at 55°C in

a water bath for one hour. The cells were vortexed briefly every 15 minutes during the incubation.

4. 5 µl RNase A was added to the samples, and the tube was inverted several times, before incubation at room temperature for 2 minutes. Subsequently, 220 µl of Buffer BDL was added to the tubes, which were then shaken briefly and incubated at 65°C for ten minutes.
5. After the incubation, 220 µl ethanol was added, and the samples were mixed by vortexing. The HiBind™ DNA spin-column was assembled in a 2 ml collection tube, and the entire sample was loaded onto the column. The tube was centrifuged for 1 minute at 8000 x g, and the flow-through was discarded. To remove trace salts and protein contaminants, two wash steps with a DNA wash buffer containing ethanol were included. The flow troughs were discarded, and the spin-column was finally "dried" by centrifugation at maximum speed for 2 minutes.
6. Finally, the column was placed in a sterile 1.5 microcentrifuge tube and 50 µl preheated (65°C) elution buffer was added, followed by incubation at room temperature for five minutes. Finally, the DNA-containing buffer solution was eluted by centrifugation at 8000 x g for 1 minute.

3.1.4. Agarose gel electrophoresis

Agarose gel electrophoresis is a separation technique often used for visualization and identification of DNA molecules of different size (Watson 2008). In an electric field, ionized chemical species wander towards a negative (cathode) or positive (anode) electrode, depending on their charge. DNA molecules are negatively charged, and will migrate to the anode. The larger molecules have more difficulty of passing through the pores of the gel, and will therefore travel slower through the gel than smaller molecules. The UV-fluorescence chemical ethidium bromide, which intercalates between the DNA bases, was added to the gel making visualization of the DNA fragments possible by UV light. In this study, 1 % agarose gels were utilized.

Materials:

Agarose

TAE-buffer (Tris-acetate), 50 x

- 242 g Tris-base
- 57.1 ml acetic acid
- 100 ml 0.5 M EDTA, pH 8.0
- dH_2O to 1 L

Ethidium bromide, 10 mg/ml (aqueous)

Loading Dye 10 x

DNA standards

Procedure:

1. Agarose gel was prepared by mixing 0.5 g agarose and 50 ml 1 x TAE buffer followed by heating in a microwave oven for dissolving the agarose.

2. After cooling the solution to approximately 60 °C, ethidium bromide was added to a final concentration of 0.5 μg/ml, and the solution was poured into a tray for solidification (20-30 minutes). A well comb was placed in the tray.

3. The solidified gel was transferred to an electrophoresis chamber, and covered with 1 x TAE buffer.

4. The DNA samples was mixed with 10 x loading dye in the ratio 0.1 : 1and applied to the wells in the gel. A DNA standard, with DNA fragments of known sizes was included on the gel to determine the size of the DNA molecules in the unknown samples.

5. Normally, the gel was run with a voltage of 90 V for 30-60 minutes, before visualization of the DNA by UV-light.

3.1.5. Primers

Primers (oligonucleotides) are short strands of nucleic acids, and represent the starting point for DNA synthesis (Watson 2008). The enzymes that catalyze DNA synthesis require these short strands of DNA, because they can only add new nucleotides to an existing strand of DNA. In PCR, the oligonucleotides define the region of the template DNA to be amplified. Two primers, one forward and one reverse primer, are required for the specific area. In this thesis, primers were designed for amplifying the Chitobiase gene from *S. marcescens*, and also for sequencing of the gene. The cloning primers were specially designed for LIC-cloning (see section 3.1.8), by using the Gentle software.

3.1.6. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a powerful tool in molecular biology, which allows exponential amplification of DNA molecules (Watson 2008). In a living cell, DNA polymerase is the enzyme responsible for duplication of the DNA during mitosis or meiosis. In PCR, the properties of DNA polymerase are utilized for amplification of DNA molecules in vitro. For amplification of a certain DNA fragment, the template DNA is mixed with a pair of specific oligonucleotides (primers), nucleotides (dNTPs) and a thermostable DNA polymerase. After denaturation of DNA by high temperature, the decrease in temperature allows binding of specific oligonucleotides to the template DNA, thereby initiating DNA synthesis by the DNA polymerase. The DNA polymerase utilized for PCR is isolated from a thermophile organism and withstands the high temperatures in the reaction. In this study PCR was utilized for amplification of the Chitobiase-encoding gene for cloning in the pET-30Xa/LIC vector.

The following reagents were added to a 50 µl PCR tube:

27.5 µl dH_2O

10 µl HF Phusion buffer

1 µl 10 mM dNTP mix

5 µl forward primer (5µM)

5 µl reverse primer (5µM)

1 µl template DNA (chromosomal DNA isolated from *Serratia marcescens*)

0.5 µl Phusion DNA polymerase

The parameters for PCR- amplification of the Chitobiase encoding gene are shown in Table 7.

Table 7. Conditions for PCR optimized for the Phusion DNA polymerase.

Function	Temperature	Time	Cycles
Initial denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	35
Annealing	72°C	20 seconds	35
Extension	72°C	40 seconds	35
Final extension	72°C	5 minutes	1
Storage	4°C	hold	

3.1.7. Purification of DNA from Agarose gels

The size of the PCR product was verified by Agarose gel electrophoresis, and the DNA excised from the gel and purified by using the Nucleospin® extract II kit (Macherey-Nagel). The procedure was executed as described in the manual provided with the kit.

Materials:

Nucleospin® extract II kit

- Buffer NT (binding)
- Buffer NT3 (wash, concentrate added ethanol)
- Buffer NE/elution (5 mM Tris-HCl pH 8.5)
- Nucleospin® extract II columns
- Collection tubes

Procedure:

1. The desired fragment of DNA was excised from the gel using a sterile scalpel, weighed, and solubilized with Buffer NT. 200 µl buffer was added for each 100 mg of gel. The mixture was incubated at 50°C until the gel was completely dissolved (about 5-10 minutes).

2. The sample was loaded onto a microspin column for binding DNA, and centrifuged at 11000 x g for 1 minute. The flow-through was discarded.
3. To wash the column, 700 µl Buffer NT3 was added, followed by centrifugation for 1 minute at 11000 x g. The flow-through was discarded, and the empty column was centrifuged for 2 minutes for drying the silica membrane.
4. The column was placed in a sterile 1.5 ml eppendorf tube, and after adding 50 µl elution buffer followed by incubation in room temperature for 1 minute the DNA was eluted by centrifugation at 11000 x g for 1 minute.

3.1.8. Ligation Independent Cloning (LIC)

Ligation independent cloning is a method that allows direct cloning of PCR products without the use of restriction enzymes, ligase or alkaline phosphatase (Hoell 2009). Compared to conventional cloning, which includes creation of phosphodiester bonds between vector DNA and a DNA fragment by DNA ligase, LIC has a number of advantages. LIC vectors are designed for directional cloning without the need of certain restriction enzyme sites, and LIC ensures that the DNA fragment is cloned in the correct reading frame. A schematic overview of the procedure is shown in Figure 11.

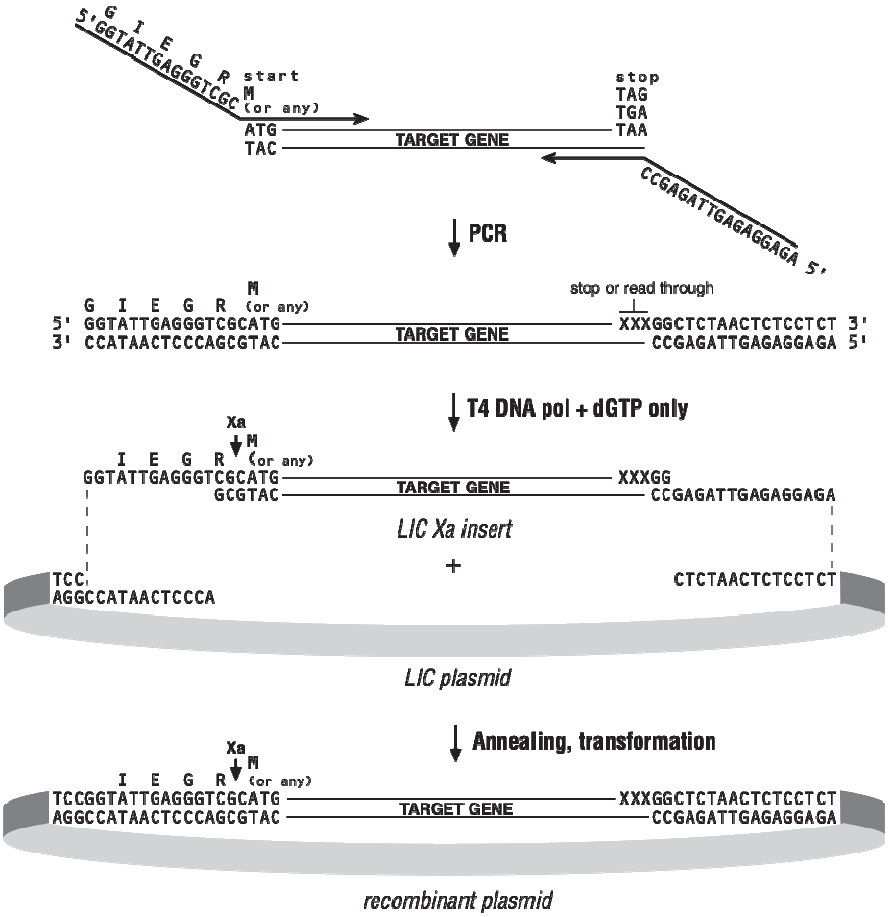


Figure 11. Schematic overview of the LIC-procedure. The figure is taken from the manual provided by the manufacturer (Novagen).

Materials:

Xa/LIC Cloning kit (Novagen):

- pET-30Xa/LIC Vector
- T4 DNA Polymerase (LIC-qualified)
- 10x T4 DNA polymerase buffer
- 100 mM DTT
- 25 mM dGTP
- 25 mM EDTA
- nuclease free water
- NovaBlue GigaSingles™ Competent cells
- BL21 (DE3) Competent cells
- SOC medium

Procedure:

1. Preparation of insert:

Primers complementary to the vector overhangs where designed (section 2.8, Table 6) containing the following specific sequences at their ends:
Sense primer: 5' GGT ATT GAG GGT CGC – insert-specific sequence 3'.
Antisense primer: 5' AGA GGA GAG TTA GAGCC – insert-specific sequence 3'.
The PCR products were verified by agarose gel electrophoresis, and subsequently extracted from the gel before T4 DNA Polymerase treatment.

2. T4 DNA Polymerase treatment of target insert:

For generation of the specific vector compatible overhangs, the inserts were treated with T4 DNA Polymerase in the presence of dGTPs.
The following reagents (reaction volume 20 µl) were mixed and kept on ice:

- 1.5 µl purified PCR product in up to 14.6 µl TlowE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0).
- 2 µl 10x T4 DNA Polymerase Buffer
- 2 µl 25 mM dGTP
- 1 µl 100 mM DTT
- 13.1 µl Nuclease-free Water
- 0.4 µl 2.5 U/ µl T4 DNA Polymerase (LIC-qualified; 0.5 unit per 0.1 pmol PCR product)

The reaction was started by the addition of T4 DNA Polymerase, and incubated at 22°C, After 30 minutes, the enzyme was heat inactivated at 75°C for 20 minutes.

3. Annealing the Vector and Xa/LIC Insert:

The following reagents were mixed and incubated at 22°C.

- 1 µl Xa/LIC Vector
- 2 µl T4 DNA Polymerase treated Xa/LIC insert from the previous reaction (0.02 pmol)
- After 5 minutes, 1 µl 25 mM EDTA was added making the total volume of 4 µl. The reaction mix was stirred with a pipette and incubated at 22°C for another 5 minutes.

4. Transformation of pET-30Xa/LIC vector comprising the Chitobiase encoding gene into competent cells:

The pET-30Xa/LIC vector comprising the Chitobiase encoding gene was transformed into NovaBlue Gigasingles™ competent cells, provided in the Xa/LIC cloning kit, as described below. The cells containing the plasmid, was selected by using the antibiotic Kanamycin, and a glycerol stock was created from the transformants.

3.1.9. Transformation

20µl Nova Giga Singles cells and 1µl pET-30Xa/LIC vector comprising the Chitobiase encoding gene were mixed, and left on ice for 20 minutes (5-30minutes). The tube was incubated at 42°C in a water bath for exactly 30 seconds without shaking, and thereafter put on ice for 2 minutes. This was followed by addition of 250 µl SOC medium to the cells, and incubation at 37°C in a shaking incubator for 3 hours. The cells were plated out on preheated LB agar plates with 100 µg/ml Kanamycin, and incubated overnight at 37°C.

3.1.10. Plasmid isolation from Escherichia coli

The Nucleospin® Plasmid kit (Macherey-Nagel) was used for isolation of the pET-30Xa/LIC Vector with the Chitobiase gene insert. The protocol was executed in accordance to the manual provided by the manufacturer.

Materials:

Nucleospin® Plasmid kit

- Buffer A1 (resuspension, added RNaseA)
- Buffer A2 (lysis)
- Buffer A3 (neutralization)
- Buffer AW (wash)
- Buffer A4 (wash, concentrate added ethanol)
- Buffer AE (elution: 5 mM Tris-HCl pH 8.5)
- Nucleospin® Plasmid Columns
- Collection tubes 2 ml

Procedure:

1. An overnight culture with the appropriate antibiotic, Kanamycin, was prepared.
2. 1.5 ml of bacterial culture was transferred to a sterile 1.5 eppendorf tube, and centrifuged for 30 seconds at 11000 x g. The supernatant was discarded.
3. The cell pellet was resuspended in 250 µl buffer A1.
4. Subsequently 250 µl buffer A2 was added. The suspension was mixed by gently inverting the tube 6-8 times (NB! Do not vortex), and incubated at room temperature for 5 minutes.
5. 300 µl buffer A3 was added, followed by gently mixing by inverting the tube.
6. For clarification of the lysate, the suspension was centrifuged for 5 minutes at 11000 x g.
7. For binding of DNA, the supernatant from the previous step was loaded to a microspin column placed in a collection tube, and centrifuged at 11000 x g for 1 minute. The flow-through was discarded.
8. For washing the Silica membrane, 500 µl preheated (50°C) AW buffer was added, followed by centrifugation at 11000 x g for 1 minute. The flow-through was discarded.
9. Another washing step was executed by adding 600 µl buffer A4, followed by centrifugation at 11000 x g for 1 minute. The flow- through was discarded.
10. The column was "dried" by centrifugation at 11000 x g for 2 minutes.
11. Finally, the column was placed in a sterile 1.5 ml micro centrifuge tube, and 50 µl elution buffer was added, and then incubated at room temperature for 1 minute. DNA was eluted by centrifugation at 11000 x g for 1 minute.

3.1.11. Restriction endonuclease digestion

Restriction digestion of DNA by enzymes is a mechanism of microbial defense, which has a wide range of scientific applications (Savada 2008). Restriction enzymes cut foreign double stranded DNA of invading pathogens, e.g. phages. These enzymes cleave only at specific DNA sequences, and cleavage can occur at the same position in both strands (blunt end), or at different positions (sticky ends/overhangs). Plasmid vectors for expression often comprise multiple cloning sites to enable

insertion of genes by utilizing restriction endonucleases. Restriction enzymes have different optimal conditions for digestion, and when preparing an experiment pH, temperature and type of buffer has to be taken into consideration.
In this study, restriction endonucleases were utilized to cleave plasmid constructs to verify that the gene was inserted into the LIC-vector. The vector was digested with an enzyme with a restriction site only present in the vector (XhoI), and an enzyme with restriction sites only present in the inserted gene (AatII).

Materials:
Restriction enzyme(s)
10x NEB buffer 4
BSA

Procedure:
DNA and sterile water was mixed with BSA and buffer recommended by NEB for the enzyme(s) chosen. The reaction was initiated by addition of restriction enzyme(s), followed by briefly mixing and incubation at the temperature recommended by NEB (37°C) for 3 hours. The resulting DNA fragments were separated on agarose gels and visualized by UV light.

3.1.12. DNA sequencing

Sequencing of the plasmid construct was performed to confirm the correct sequence of the Chitobiase encoding gene, and to ensure that mutations had not occurred. For the sequencing, an ABI prism BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems) was used.
The following reagents were mixed, and a PCR reaction was executed as described in Table 8.
Reagents:

- 4 µl 5x sequencing buffer (Applied Biosystems)
- 2 µl Premix (Applied Biosystems)
- 1 µl Primer
- 3 µl Template DNA
- 10 µl dH_2O

Table 8. Reaction conditions optimized for sequencing PCR.

Temperature	Time	Cycles
96°C	1 minute	1
95°C	10 seconds	25
50°C	5 seconds	25
60°C	4 minutes	25
4°C		Hold

The PCR products were precipitated with EDTA and ethanol. The following amounts were added to the PCR reaction mixture (20 µl) and the reaction mixture was incubated at room temperature for 15 minutes, and centrifuged (maximum speed) at 4°C for 30 minutes.

- 2 µl 125 mM EDTA
- 62.5 µl 96% ethanol

The supernatant was immediately removed, and the pellets were air dried on ice for 30 minutes and then kept at 4°C until sequencing.

3.1.13. Transformation to E. coli BL21

After verification of the inserted Chitobiase encoding gene by sequencing, the resulting plasmid was transformed into the BL21(DE3) competent cells for protein expression. The transformation was executed as described in section 3.1.9, with BL21 cells replacing the Nova Giga Single cells.

3.1.14. Over-expression of the Chitobiase encoding gene

An (2.5 ml) overnight culture of *E.coli* BL21 (DE3) containing the Chitobiase encoding recombinant plasmid was diluted in 250ml culture medium, with 100 µg/ml kanamycin, and incubated at 37°C. At OD 0.5, the cultures were induced for protein production by addition of IPTG (end concentration 0.1 mM), and incubated at 30°C for 3 hours.

3.1.15. Cell harvesting and cell lysis

The cells were centrifuged at 8000 rpm in the JA14 rotor of the Avanti JM-25 centrifuge (Coulter Beckman) for 10 minutes. The supernatant was discarded, and the cells were resuspended in Tris-HCl pH 8 buffer with 1 mg/ml lysozyme, followed by incubation for 20 minutes in room temperature. A cell extract was prepared by sonication (10 minutes, 5 seconds on/off), and after centrifugation at 11000 rpm in the JA 25.50 rotor of the Avanti JM-25 centrifuge (Coulter Beckman), the extracts were sterile-filtrated and added PMSF (2 µl/ml). The extracts were stored in the refrigerator.

3.2. Purification of His-tagged Chitobiase

3.2.1. Purification of His-tagged Chitobiase

The recombinant Chitobiase was designed carrying a polyhistidine-tag (His-tag) that binds strongly to divalent metal ions such as nickel (Ni). Thus, for purification of His-tagged Chitobiase, immobilized metal affinity chromatography (IMAC) was used. Ni- ions are bound to chelating absorbents such as nitriloacetic acid (NTA), which is a tetradendate chelating absorbent that occupies four out of six ligand binding sites of the Ni-ion. This leaves two ligand binding sites free for interaction with the his-tag of the protein. Binding of the His-tagged protein to the Ni-NTA column is followed by a washing step, to remove unspecifically bound proteins. The protein of interest is eluted by adding imidazole, which competes with the His-tag for binding to the metal ions. Another option is to decrease pH, which will decrease the affinity for the tag to interact with the Ni-NTA.

In this thesis, recombinant His-tagged Chitobiase was bound to the Ni-NTA column and eluted by using an imidazole concentration of 1 M. Pure protein was obtained by two-step purification on the 5 ml HisTrap tandem column. The first step included the superloop (50 ml) and purification of the prepared bacterial cell extract. Impure fractions from this purification were pooled, concentrated (with change of buffer), followed by another round of purification. After purification, the buffer was changed to 100 mM Tris HCl pH 8, and the fractions were analyzed by SDS-PAGE (described in section 3.2.2.).

Materials:

- Äkta purifier (GE Healthcare)
- 5 ml HisTrap™ column
- Superloop 50 ml
- Loop 500 µl
- Plastic syringes
- Needle
- Fraction collector
- Eppendorf tubes
- Washing buffer: 100 mM TrisHCl pH 8/ 20 mM Imidazole

- Elution buffer: 100 mM TrisHCl pH 8/ 1 M Imidazole

Procedure:

1. The system was washed with 20 % ethanol, before running washing buffer through the system.
2. When the UV absorbance base line and the pressure were stabilized, 50 ml of the bacterial extract was slowly loaded onto the column using a superloop and a flow rate of 0.5 ml/min. For the second time purification, the impure protein was loaded on the column by a smaller (500 µl) loop.
3. Unbound and non-specifically bound proteins were washed through the column by applying the washing buffer, using a flow rate of 2.5 ml/min. When the UV baseline was stable, the mobile phase was altered to 100 % elution buffer, and the fractions containing protein were collected. The purity of collected protein fractions was analyzed by SDS-PAGE (described in section 3.2.2).

3.2.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins have a variety of charges and structures. By treating proteins by the strong ionic detergent sodium dodecyl sulfate (SDS) and a reducing agent, such as β-mercaptoethanol, the proteins denature and are provided with a global negative charge. This allows the proteins to be separated by gel electrophoresis based on their size. The NuPAGE® Novex® Bis-Tris Mini Gels from Invitrogen were utilized in this thesis. After electrophoresis, the proteins can be visualized by different staining methods, such as Coomassie brilliant blue or silver staining. Both staining methods were used in this thesis.

Reagents:

10 µl sample

5 µl NuPAGE® LDS sample buffer (4X)

2 µl NuPAGE® Reducing agent (10X)

3 µl H_2O

Procedure:

The samples were prepared by mixing the reagents described above, and heating of the mixtures at 70°C for 10 minutes. 1 X SDS Running buffer was prepared by adding 50 ml 20 X NuPAGE® MOPS SDS Running Buffer to 950 ml dH_2O. The gel (10% NuPAGE® Novex® Bis-Tris Mini Gel) was placed in the electrophoresis chamber, and both the inner and outer chambers were filled with 1X SDS Running buffer. The samples were loaded, and the gel ran for 50-55 minutes at 200 V. A protein standard, with proteins of known size, Benchmark Ladder, was included to verify protein size.

Staining of the SDS-PAGE gel

SDS-PAGE coloring solution:

- 0.1% Coomassie Brilliant Blue R250
- 50 % methanol
- 10 % acetic acid

SDS-PAGE destaining solution:

- 10% methanol
- 10% acetic acid

Procedure:

1. The gel was gently placed in a gel box, staining solution was added, and the gel was incubated at room temperature with agitation.
2. After 30 minutes, the staining solution was replaced by destaining solution and the gel was incubated with agitation for at least one hour.
3. The gel was photographed using a CANON photo scanner.

Silver staining:

Silver staining is a more sensitive staining method for protein gels. This method detects protein amounts in the nanogram range. The Silver® STAIN for Mass Spectrometry kit from Pierce was utilized. The protocol was executed in accordance to the manual included in the kit.

Materials:

- Silver® STAIN for Mass Spectrometry kit
    - Sensitizer working solution (1 part Silver Stain Sensitizer (provided in the kit)+ 500 parts ultrapure water).
    - Enhancer working solution (1 part Silver Stain Enhancer+ 100 parts Silver stain, both provided in the kit)
    - Developer working solution (1 part Silver Stain Enhancer+ 100 parts Silver Stain Developer)

- Fixation solution:
    - 30 % (v/v) ethanol
    - 10 % (v/v) acetic acid
- Ethanol wash solution
    - 10 % (v/v) ethanol
- Stop solution
    - 5 % (v/v) acetic acid

Procedure:

1. The gel was gently placed in a gel box, and washed twice in dH_2O for 5 minutes.
2. Water was discarded, and the gel was incubated in 30 ml fixation solution for 15 minutes. This step was repeated once.
3. The fixing solution was replaced by the ethanol wash solution. The gel was washed twice for 5 minutes, followed by two 5 minutes washes in dH_2O.
4. The gel was added sensitizer working solution and incubated for 5 minutes, and subsequently washed twice in dH_2O (1 minute pr wash).

5. Enhancer working solution was added, followed by incubation for 5 minutes, before two times 20 seconds wash in deionized water.
6. Next, the gel was incubated in developer working solution until protein bands developed with the desired intensity.
7. Developer working solution was replaced with 5 % acetic acid (stop solution), and the gel was incubated for 10 minutes. The gel was photographed by using the CANON photo scanner.

3.2.3. Concentrating purified protein

Materials:

Amicon® Ultra-0.5 and -15 Centrifugal Filter Devices, 10000 MWCO

Procedure:

The pure protein fractions were added to the Amicon® Ultra-0.5 or -15 Centrifugal Filter Devices, and centrifuged rat 14000 x g or 4500 x g, respectively, at 4°C using a fixed angle rotor (eppendorf centrifuge). The time of centrifugation depended on sample volume and the desired protein concentration. A pipette was used for collecting the concentrated protein when using an Amicon® Ultra-15 device. When using the Amicon® Ultra-0.5 device, the protein was collected by inverting the filter device in the collection tube, and centrifugation for 2 minutes at 1000 x g.

3.2.4. Determination of protein concentration (Bradford assay)

Protein concentrations were determined by using the Bio-Rad Protein Assay, which is based on the method developed by Bradford (Bradford 1976). This method involves the binding of the Coomassie Brilliant blue G250 dye to proteins, primarily via aromatic and basic amino acids. The utilized dye solution was the red acidic color solution (Protein assay dye reagent) with absorption maximum at 465 nm (www.bio-rad.com). When the dye binds to proteins, it turns blue and the absorbance maximum is altered to 595 nm. The samples were measured at 595 nm using a spectrophotometer, and the protein concentrations were determined by using a standard curve of bovine serum albumin (BSA) ranging from 1.2 µg/ml to 10 µg/ml.

Solutions:

- Bio-Rad Protein Assay Dye Reagent Concentrate
- Purified BSA (Promega)
- Sample buffer

Procedure:

1. The samples were diluted to 800 µl in the sample buffer, and added 200 µl protein assay dye reagent concentrate. The blank sample was prepared by mixing 800 µl sample buffer with 200 µl protein assay dye reagent concentrate.
2. The samples were incubated in room temperature for 10 minutes.
3. Absorbance at 595 nm was measured using a spectrophotometer (Biophotometer).
4. Protein concentrations were calculated using the BSA standard curve.

3.3. Kinetic analysis

3.3.1. pH optimum determination

The enzymatic activity of Chitobiase was measured in buffers with pH varying from 3 to 10. Reaction mixtures (500 µl) contained the purified enzyme (0.03 nM), 0.1 mg/ml BSA and 100 µM 4-MU-GlcNAc in various buffers (0.1 M) listed below. Reactions were started by adding the enzyme and after 6, 12 and 18 minutes the reactions were quenched by adding 100 µl of the reaction volum to 1.9 ml Na_2CO_3. Three parallel reactions were used for estimating the reaction velocity for each pH.

Buffers:

pH	Buffer
3	Citrate-Phosphate
4	Citrate-Phosphate
5	Citrate-Phosphate
6	Sodium Phosphate
7	Sodium Phosphate
8	Sodium Phosphate
9	Tris-HCl
10	Glycine-NaOH

3.3.2. Standard 4-MU-GlcNAc assay

A working solution of Chitobiase (1 nM) was prepared with 0.1 mg/ml BSA in 20 mM Tris-HCl. Solutions containing the substrate, 4-MU-GlcNAc, at various concentrations from 10-640 µM in 0.1 M pH 6 sodium-phosphate buffer containing 0.1 mg/ml BSA were pre-incubated at 37°C for about 2 minutes using glass assay tubes. The enzyme reactions were initiated by adding enzyme to an end concentration of 0.03 nM (reaction volume 500 µl). Several enzyme concentrations were tested for obtaining conditions yielding substrate hydrolysis compatible with the calibration of the Hoefer DyNa Quant 200 Fluorimeter. Consumption of substrate was also taken into consideration to avoid consumption of too much substrate in the low-substrate concentration samples. When studying enzyme kinetics, it is generally accepted that the consumption of substrate should be kept at less than 10 % of the total substrate in the reaction (Wu et al. 2003).

Three parallel reactions were performed for every substrate concentration. After 6, 12 and 18 minutes, the reactions were stopped by adding 100 µl of the reaction volume to 1.9 ml Na_2CO_3, and stored dark until 4MU fluorescence was measured by using a fluorometer.

For calibration of the instrument, 100µl 1µM 4-MU was added to 1.9 ml of Na_2CO_3, and mixed thoroughly. This 50 nM 4-MU solution was set to equal 500 on the fluorometer display. This means that the value 500 equals 100 nmol cleaved 4-

MU. Before calibration, the instrument was set to zero by measuring the fluorescence of 1.9 ml of Na_2CO_3 (blank sample).

3.3.3. $GlcNAc_2$ assay

Reaction mixtures containing the substrate, $GlcNAc_2$, at various concentrations (20-800 µM) in 0.1 M ammonium acetate buffer, pH 6.0, containing 0.1 mg/ml BSA were pre-incubated at 37°C for about 2 minutes in glass assay tubes. The enzyme reactions were started by adding enzyme to an end concentration of 0.2 nM (reaction volume 500 µl). Several enzyme concentrations were tested for obtaining conditions yielding product formation compatible with the sensitivity of the HPLC. Consumption of substrate was also taken into consideration to avoid consumption of too much substrate in the low-substrate concentration samples. Three parallel reactions were performed for every substrate concentration. After 6, 12 and 18 minutes, the reactions were stopped by mixing 90 µl sample from the reaction volume with 10 µl 10 % acetic acid in an eppendorf tube, which decreased pH to 2 and completely inactivated the enzyme. The samples were transferred to HPLC vials and analyzed analyzed by High performance Liquid Cromatography (HPLC) using a TSK Amide-80 column for separation of GlcNAc (product) and $GlcNAc_2$ (substrate) by length and anomeric configuration.

Materials:
Mobil phase 70 % Acetonitrile (ACN), 0.1 ml/minute
Dionex Ultimate 3000 HPLC system
Chromeleon Chromatography data system software v 6.8.

Procedure:
The UV detector was configured to detect signal at 195 nm, and the samples were analysed (2 µl injection volume) after stabilization of the system pressure and base line. Standard samples (100 µM) of GlcNAc and $GlcNAc_2$ were included at regular intervals.

The concentrations of produced GlcNAc were used in the calculations of the kinetic parameters of Chitobiase.

3.4. Degradation of chitooligosaccharides by recombinant Chitobiase

To further investigate the activity of Chitobiase, the enzyme was incubated with $GlcNAc_3$ and $GlcNAc_4$, to observe a possible activity against these higher oligosaccharides. The reaction conditions and enzyme concentrations were the same as used in the $GlcNAc_2$ assay. The samples were analyzed by HPLC as described in section 3.3.3.

The ability of Chitobiase to degrade partially acetylated chitooligosaccharides was analyzed using a hexameric oligomer as substrate produced by Berit Bjugan Aam by degrading acetylated chitosan with ChiC from *S. marcescens* followed by size exclusion chromatography to purify hexamers (Berit Bjugan Aam, personal com.). This hexamer mixture was incubated with Chitobiase and degradation products were analysed by MALDI-TOF MS.

Materials:

- Hexamer (ending at –DAA; see Horn et al. 2006)
- Purified recombinant Chitobiase
- 20 mM Ammonium acetate buffer pH 6
- 2,5-dihydroxybenzoic acid (DHB)
- Water bath
- MALDI plate with stage
- MALDI-TOF MS: Ultra flex TOF/TOF (Bruker Daltonics)

Procedure:

The hexamer was incubated with chitobiase at 37°C over night: 1 mg/ml hexamer (end concentration) was incubated with 50 nM Chitobiase in 20 mM Ammonium acetate buffer pH 6. 1 µl sample was mixed with 2 µl DHB (matrix), and spottet on the MALDI-plate. The degradation products were analyzed by MALDI-TOF MS, and compared with the control sample containing the hexamer before degradation with Chitobiase.

3.5. Binding of chitooligosaccharides to α- and β-chitin

Materials:

- Crystalline β-chitin from squid pen, France Chitin
- Crystalline α-chitin, Hov-Bio
- Chitooligosaccharides; $GlcNAc_{2-4}$
- 0.1M Ammonium acetate buffer pH 6

Procedure:

1. 10 mg/ml α- chitin or β-chitin were incubated with 100 µM $GlcNAc_2$/ $GlcNAc_3$/ $GlcNAc_4$ in 0.1 M ammonium acetat buffer pH 6 (reaction volume 300 µl) at room temperature with vertical rotation at 20 rpm.
2. Samples (50 µl) were taken from the reaction after 1 hour, 2 hours and 6 hours, transferred to eppendorf tubes and centrifuged at maximum speed for 10 minutes, before they were transferred to HPLC vials and stored at -20°C until analysis by UHPLC.
3. A sample taken out before addition of chitin, as well as standard samples (100 µM of $GlcNAc_2$/ $GlcNAc_3$/ $GlcNAc_4$), were compared with the other samples to investigate if any chitooligosaccharides had bound to the chitin.

3.6. Degradation of β-chitin

Enzymatic degradation experiments on β-chitin were done in triplicates with crystalline β-chitin and another β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad). Enzymatic degradation of β-chitin was analyzed by using UHPLC and HPLC for product detection. β-chitin was degraded by addition of pure chitinolytic recombinant enzymes to reaction mixtures containing BSA, buffer, (and in presence of CBP21 a reducing agent such as reduced glutathione). By combining Chitobiase with Chitinase B (ChiB), Chitinase C (ChiC) and CBP21, respectively, the degradation products of β-chitin were analyzed and quantified.

Materials (UHPLC analysis)

- Acetonitril, 100 %
- dH_2O
- Column: ACQUITY UPLC BEH Amide, 2.1 x 50 mm, 1.7 µm
- Pre-column: ACQUITY BEH HILIC VanGuard Pre-column, 2.1 x 5 mm, 1.7 µm
- 20 mM Ammonium acetat pH 6
- 0.1 mg/ml BSA (end concentration)
- Recombinant, pure enzyme(s): Chitobiase, CBP21, ChiB, ChiC
- β-chitin from squid pen, France Chitin
- GlcNAc, $GlcNAc_2$
- Reduction agents: Ascorbic acid (end concentration 1 mM)
- Shaking incubator 37°C

Materials (HPLC analysis)

- 5 mM H_2SO_4 mobile phase, (10 mM were used for stopping the reactions by inactivation of proteins by lowering pH)
- Column: Rezex RFQ-Fast Fruit H+, 7.8 x 100 mm
- Pre-column: Carbo-H, 4 x 3.0 mm
- Flow 1.0 ml/min
- Detection UV absorbance 195
- Column oven 85°C
- Injection volume 6 µl
- 20 mM Ammonium acetat pH 6
- 0.1 mg/ml BSA (end concentration)
- Recombinant, pure enzyme(s): Chitobiase, CBP21, ChiB, ChiC
- β-chitin from squid pen, Gustav Vaaje-Kolstad
- GlcNAc, $GlcNAc_2$
- Reduction agents: reduced glutathione (end concentration 1 mM)
- Shaking incubator 37°C

Procedure:

1 mg/ml of the β-chitin substrates, 0.1 mg /ml BSA and 20 mM ammonium acetate buffer were mixed before addition of enzyme(s). Reducing agents (end concentration 1 mM) were added to samples containing CBP21. ChiB, ChiC and CBP21 were added to a final concentration of 0.5 μM, while the final concentration of Chitobiase was 50 nM. Samples were taken at 0.5, 1, 2, 4, 6, 8 and 24 hours: 50 μl of the reaction mixture was mixed with 50 μl of 100 % ACN / 20 mM H_2SO_4 followed by centrifuged at maximum speed for 10 minutes, transfer of supernatants to HPLC vials, and storage at -20°C until product analysis by UHPLC / HPLC. Products were detected using the UV signal at 195 nm.

3.7. Pulldown experiment of possible interaction partners

An attempt to detect possible interaction partners of Chitobiase was done by attaching the recombinant his-tagged Chitobiase to a nickel column, and running the supernatant or a cell extract of *Serratia marcecens* cultivated on chitin through the column.

Procedure: (Preparation of extracts):

The bacteria were cultivated in Basal Salt Buffer (BSB) containing 1 w/v % chitin. An overnight culture of *S. marcescens* in LB- medium were centrifuged and the medium discarded, and the cells were resuspended in BSB and diluted in 100 or 250 ml cultures with BSB containing chitin. The bacteria were grown for about 5-6 days (until minimal amounts of chitin was observed in the culture). The culture were harvested by centrifugation; 8000 rpm in the JA14 rotor of the Avanti JM-25 centrifuge (Coulter Beckman) for 10 minutes. The supernatant was sterile filtrated (0.2 um) and added PMSF and stored in the refridgerator. The cell pellet were resuspended in 0.1 M Tris-HCl pH 8 buffer with 1 mg/ml lysozyme, followed by incubation for 20 minutes in room temperature. A cell extract was prepared by sonication (10 minutes, 5 seconds on/off), and after centrifugation at 11000 rpm in the JA 25.50 rotor of the Avanti JM-25 centrifuge (Coulter Beckman), the extracts were sterile-filtrated (0.2 μm) and added PMSF (2 μl/ml). The extracts were stored in the refrigerator.

Materials (pulldown experiment)

- Äkta purifier (GE Healthcare)
- 5 ml HisTrap™ column
- Superloop 50 ml
- Loop 500 µl
- Plastic syringes
- Needle
- Fraction collector
- Eppendorf tubes
- Washing buffer: 100 mM TrisHCl pH 8/ 20 mM Imidazole
- Elution buffer: 100 mM TrisHCl pH 8/ 1 M Imidazole
- Recombinant His-tag Chitobiase
- *Serratia marcescens* supernatant
- *Serratia marcescens* cell extract prepared by sonication
- Washing buffer 100 mMTris-HCl pH 8 / 20 mM Imidazole
- Elution buffer 100 mM Tris-HCl pH 8 / 1 M Imidazole
- 1.5 ml sterile eppendorf tubes

Procedure:

4. The Äkta purifier system was washed with 20 % ethanol; thereafter washing buffer was run through the system.
5. After the UV absorbance reached base line level and the pressure had stabilized, the pure recombinant His-tag Chitobiase (0.1 mg/ml) was loaded onto the column, by a 1 ml syringe.
6. Unbound and non-specifically bound proteins were washed through the column by the washing buffer.
7. When the UV absorbance base line and the pressure were stabilized, the bacterial extract/supernatant from the super loop was slowly loaded through the column.
8. When the UV baseline was stabilized, the mobile phase was altered to 100 % elution buffer, and the fractions containing protein with its possible interaction partners were collected. The collected protein fractions were analyzed by SDS-PAGE (described in section **3.2.2**).

9. Matrix-assisted-laser-desorption/ionization (MALDI-TOF) Mass Spectrometry (MS) was used for analysis of the protein bands of interest. The pre-treatment of samples before MALDI-TOF MS analysis is described in sections 3.7.1 and 3.7.2.

3.7.1. Reduction, alkylation, in-gel trypsin digestion and peptide extraction

Materials:

- NuPAGE® Novex® Bis-Tris Mini Gels
- Reduction, alkylation, in-gel trypsin digestion and peptide extraction:
    - Scalpel
    - 100 % ACN
    - 100 mM Ammonium Bicarbonate (AmBic)
    - 50 % ACN / 50 mM (AmBic)
    - 10 mM DTT in 100mM AmBic
    - 55 mM Iodo acetamide (IAA) in 100mM AmBic
    - Trypsin buffer: 10mM AmBic / 10 % ACN
    - Trypsin solution: 1 vial of trypsine
    - Extraction solution: 5 % formic acid mixed 1:2 with 100 % ACN

Procedure:

1. Protein bands of interest were cut out of the gel, and transferred to eppendorf tubes. The gel pieces were added 100 µl 50 % ACN / 50 mM Ambic, and incubated in room temperature for 15 minutes.
2. The solution was removed, and discarded, before addition of 200 µl 100 % ACN, and further incubation at room temperature for 15 minutes. In this step the gel pieces are dehydrated.
3. All liquid was removed, and the tubes left with the lids open in a hood to dry the gel pieces for about 15 minutes. The gel pieces should be completely dry before proceeding.
4. 50 µl DTT solution was added to the gel pieces, and the tubes incubated for 30 minutes in a waterbath of 56°C.
5. The samples were cooled to room temperature, and all liquid was removed.

6. Subsequently, 50 µl IAA solution was added, and the samples incubated for 30 minutes (room temperature) in the dark.
7. All liquid was removed, and 200 µl 100 % ACN added before incubation for 15 minutes (room temperature).
8. The liquid was discarded, and the tubes left with the lids open in a hood to dry for about 15 minutes.
9. The samples were put on ice. A trypsin solution was added to cover the gel pieces and the samples were then incubated on ice for 30 minutes to one hour. This made the gel pieces swelling. If necessary, more trypsin solution was added, before proceeding with incubation for at least one hour.
10. Excess trypsin solution was removed, and replaced with 10% ACN / 10 mM AmBic. The samples incubated in a shaking incubator at 37°C over night.
11. The samples were briefly centrifuged, then added 50 µl extraction solution, and incubated at 37°C with shaking for 15 minutes.
12. To ensure extraction of all peptides, the samples were sonicated for 5 minutes, followed by centrifugation at 16000 x g for 5 minutes. The supernatants, which contained the peptides, were transferred to clean eppendorf tubes.
13. An additional 50 µl ACN was added to the gel pieces, which were then incubated for 15 minutes at room temperature with shaking, before centrifugation. Finally, the supernatants were pooled with the supernatants from the previous step.
14. The extracts were dried in a SpeedVac for 20-30 minutes, and stored at -20°C until purification and concentration of peptides, described in section 3.7.2.
15. Before purification and concentration of peptides, addition of 15 µl 0.1 % TFA to the dried extracts were followed by vortexing, and sonication for 30 seconds.

3.7.2. Purification and concentration of peptides by modified STAGE micro columns for MALDI-TOF MS analysis

Materials:

- 20 µl GELoader tips (eppendorf)
- C_{18} Empore Discs (3M)
- Fused silica
- Hollow tool for preparing the columns
- Plastic syringe
- Methanol
- 0.1 % TFA
- 70 % ACN / 0.03 % TFA

Procedure:

1. The column was prepared by corking out the mesh from the C_{18} Empore Discs, by using a hollow blunt end needle, and the piece of C_{18} was pressed down in the GELoader tip.
2. A plastic syringe was utilized for pressing the liquids through the columns.
3. The column material was activated by 5 µl methanol.
4. Subsequently, the column was equilibrated twice with 10 µl 0.1 % TFA.
5. 10 µl sample was run through the column, and the column was washed twice with 0.1 % TFA.
6. The peptides were eluted with 1 µl 70 % ACN / 0.03 % TFA.

3.7.3. MALDI-TOF MS analysis of extracted peptides

Prior to the MALDI analysis, the desalted peptides were mixed 1:1 with α-cyano-4-hydroxicinnamin acid (HCCA) matrix in a total of 1.5 µl, applied to a spot on the MALDI-target plate, and air dried. The MALDI was operated in a range of 450-4000 *m/z,* and the laser intensity was adjusted manually for each run. The MS peptide mass fingerprints (PMFs) and MS/MS peak lists created by analyzing the peptides on the MALDI-TOF was searched against databases

located on the in-house local MASCOT-server for identification of the proteins from which the peptides originate.

The following parameters were used searching MASCOT:

- Species: *Serratia marcescens*
- Allowed number of missed cleavages: 1
- Enzyme: Trypsin
- Variable post-translational modifications: methionine oxidation
- Fixed modifications: carbamidomethyl oxidation of cysteines (resulting from alkylation with iodoacetamide).
- Peptide mass tolerance: 20-50 ppm
- Peptide charge: 1

3.8. Secreted proteins of *Serratia marcescens* induced by cultivation on different carbon sources

Supernatants of *S marcescens* cultures grown on two different carbon sources (6.2 ml /L Glycerole or 1 w/v % chitin) were analyzed by LC-MS to detect proteins induced by the presence of chitin. These proteins might possibly contribute to the degradation of chitin.

Proteins in the supernatants were precipitated with acetone (described below) and analyzed by SDS-PAGE. The areas on the gel that revealed differences in expression of proteins were excised from the gel (that is, also the corresponding area on the gel not showing expression was analyzed). The proteins were reduced, alkylated and trypsinated (in gel), and the peptides were extracted, as described in 3.7.1. The peptides were concentrated and purified as described in 3.7.2. The samples were diluted by 10 µl 0.1 % TFA, transferred to HPLC-vials, then analyzed by an Agilent 1100 series nano-flow HPLC system coupled to an esquire HCT ion trap from Bruker Daltonics. In short, samples were loaded on a trap column (C18 Hotsep Tracy 300 µm i.d x 5 mm) for final clean-up before passing the samples trough the analytical column (C18 Hotsep kromasil 100 µm i.d x 15 cm). The separation was performed using a mobile phase system consisting of buffer A (water with 0.1 % (v/v) trifluoroacetic

acid (TFA) and 2 % acetonitrile (ACN)) and buffer B (ACN with 0.1 % (v/v) TFA) for a 70 min gradient from 2-60 % buffer B, followed by a washing step up to 90 % buffer B.

Procedure (Precipitation of proteins with acetone):

- The bacterial culture supernatant was diluted by 4 volumes ice-cold acetone in a total volume of 2 ml (that is 400 µl supernatant+ 1.6 ml acetone).
- The acetone with the supernatant were incubated at -20°C for at least one hour
- Following incubation, the samples were centrifuged at 4°C at maximum speed for ten minutes.
- The liquid was discarded after centrifugation, and the tubes left in a hood to air-dry for about 20 minutes.
- The samples were boiled in SDS-PAGE loading buffer before it was analyzed by SDS-PAGE gel electroforesis.

4. Results

4.1. Cloning, sequencing and expression of the Chitobiase encoding gene from *Serratia marcescens*

Primers for amplification of the *S. marcescens* Chitobiase encoding gene without its signal peptide were designed for cloning in the pET-30 Xa/LIC vector (Table 6).

As described in section 3.1, PCR amplification of the Chitobiase encoding gene was performed on chromosomal DNA isolated from *Serratia marcescens* using E.Z.N.A bacterial DNA kit, and the Phusion DNA polymerase with HF Phusion buffer was utilized in the PCR reaction. The PCR fragment was inserted in the pET 30 Xa/LIC vector, which was subsequently transformed into NovaBlue GigaSingles™ Competent cells. The plasmid was isolated and digested by restriction enzymes to verify correct insertion of the gene in the vector. The verification was positive, and sequencing of the inserted gene did not show any mutations. The pET- 30 Xa/LIC vector containing the Chitobiase encoding gene was transformed into *E. coli* BL21 (DE3) competent cells optimized for protein production.

Cells from Chitobiase producing *E.coli* BL21 (DE3) transformants were harvested by centrifugation and lysed by sonication (section 3.1.15). After centrifugation of the lysate, both supernatant and pellet fractions were analyzed by SDS-PAGE. Figure 12 shows that the protein was expressed and that most protein appeared in soluble form (i.e. in the supernatant of the cell lysate), but there was also seen insoluble protein as inclusion bodies (aggregated protein) in the cell pellet after cell lysis and centrifugation (Figure 13). To find the conditions yielding most protein the cells were induced with different concentrations of IPTG and incubated at different temperatures after induction and this analysis led to the following final protocol for Chitobiase production: cultivation of Chitobiase producing *E.coli* BL21 (DE3) cells until OD=0.5, induction by 0.1 mM (end concentration) of IPTG followed by incubation at 30 °C for 3 hours. Thereby preparation of a cell extract as described in section 3.1.15.

Protein activity was confirmed by assaying activity towards 4-MU-GlcNAc, by incubating 50 µl cell extract with 50 µl 100 µM 4-MU-GlcNAc in Sodium phosphate buffer pH 6 for 10 minutes before addition of 1.9 ml Na_2CO_3 and analysis by Hoefer DyNa Quant 200 Fluorimeter. Control sample (incubated substrate+ buffer) did not give any fluorescence.

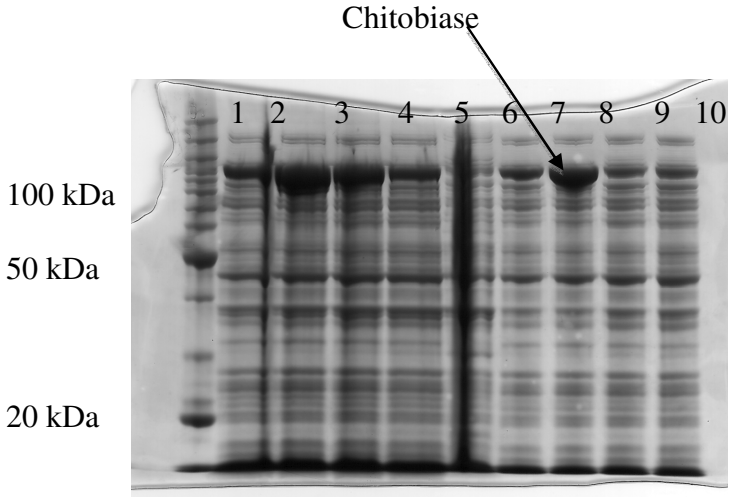


Figure 12. SDS-PAGE analysis of cells induced for Chitobiase production. Lane 1, Benchmark protein ladder; Lanes 2-5 show boiled cells 3 hours after induction (i.e. soluble and insoluble proteins). Lane 7-10 show cell extracts prepared by sonication 3 hours after induction (i.e. soluble proteins). Lanes 2 and 7, cells induced by 0.250 mM IPTG and incubated at 30°C after induction; lanes 3 and 8, cells induced by 0.1 mM IPTG and incubated at 30°C after induction; lane 4 and 9, cells induced by 0.250 mM IPTG and incubated at 37°C after induction; lanes 5 and 10, cells induced by 0.1 mM IPTGand incubated at37°C after induction. Lane 6 shows boiled cells from cells containing the vector only, induced with 0.1 mM IPTG.

The insoluble fractions of protein from the cell extracts showed in Figure 12 were analyzed by SDS-PAGE, and are shown in Figure 13.

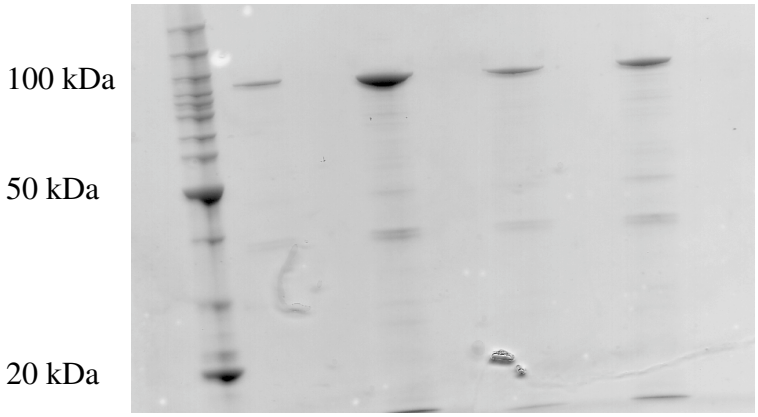


Figure 13. SDS-PAGE analysis of insoluble fractions of Chitobiase. After cell lysis by sonication, the cell extracts were centrifugated, and the pellet resuspended and analyzed by SDS-PAGE. Lane 1= Benchmark ladder. Lane 2, 4, 6 and 8 shows the pellets after cell lysis, representing the insoluble fractions of Chitobiase. Lane 2 is the insoluble fraction of protein from cells induced by 0.250 mM IPTG and incubated at 30°C after induction. Lane 4 is the insoluble fraction of protein from cells induced by 0.1 mM IPTG and incubated at 30°C after induction. Lane 6 is the insoluble fraction of protein from cells induced by 0.250 mM IPTG and incubated at 37°C after induction. Lane 8 is the insoluble fraction of protein from cells induced by 0.1 mM IPTG and incubated at 37°C after induction.

4.2. Purification of His-tagged Chitobiase

The Chitobiase was purified by immobilized metal affinity chromatography (IMAC), as described in section 3.2. In a typical experiment 50 ml of cell extract was applied to the 5 ml nickel column and Chitobiase-containing fractions were collected, concentrated (with change of buffer) and subjected to a second round of purification described in section 3.2.1. A typical chromatogram from such a second purification round is shown in Figure 14. The purity of protein fractions was analyzed by SDS-PAGE (Figure 15). Fractions containing pure Chitobiase were pooled and concentrated by Amicon® Ultra -15 Centrifugal Filter Devices, 10000 MWCO, before protein concentration determination by Bradford's assay. The protein thus purified was used for the kinetic analyses described below.

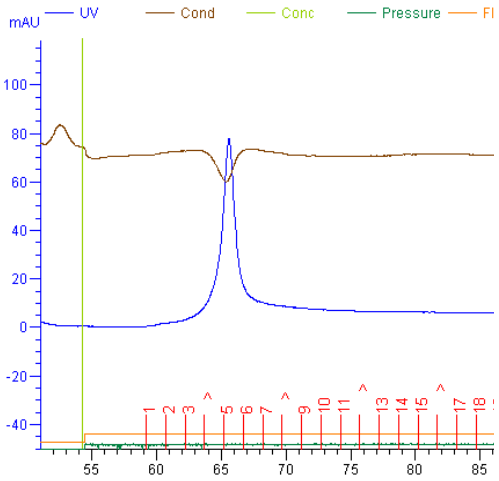


Figure 14. Protein purification by nickel column chromatography. The chromatogram shows the result of the second chromatography run. The peak in the UV-trace (blue) contains chitobiase activity. An SDS-PAGE analysis of these fractions is depicted in Figure 14. The vertical red lines indicate the fractions collected. The vertical green line indicates 100 % elution buffer.

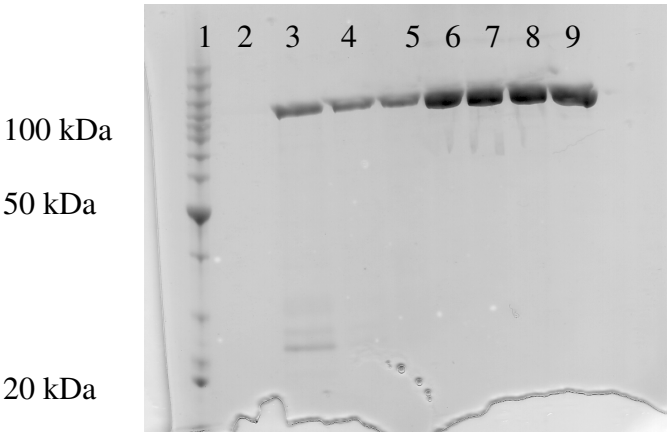


Figure 15. SDS-PAGE analysis of collected fractions from of the second round of protein purification, as depicted in Figure 13. Lane 1= Benchmark protein ladder. Lane 2= flow through. Lane 3-9= fractions from elution. The gel shows some impurities in they “early” fractions (lane 3 and 4); such fractions were not used in further work. The fractions containing pure material (in this case lanes 5 - 9) were pooled and concentrated, and used for further biochemical experiments.

4.3. Kinetic analysis

Figure 16 shows a pH-activity curve for Chitobiase. The curve shows that Chitobiase has high relative activity over a broad range of pH, with a maximum occurring at pH 6. The experiment was performed with only one substrate concentration (100 µM), and the pH activity profile is thus not complete in the sense that the profile may give mixed K_m and k_{cat} effects. Still, for the rest of this study, it was assumed that the curve of Figure 16 gives a good estimate of the pH optimum of the enzyme.

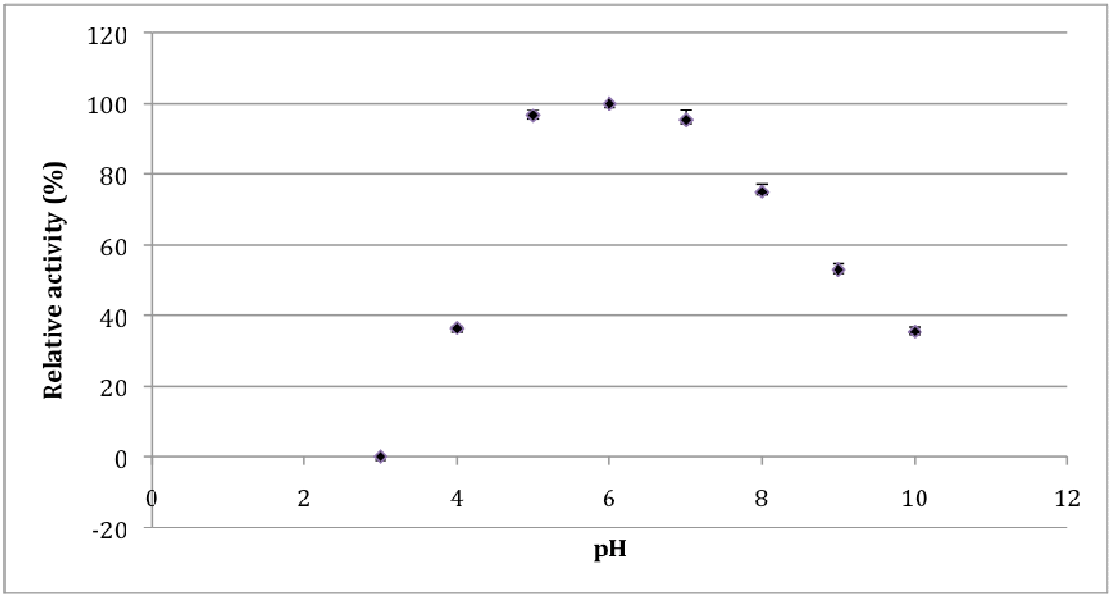


Figure 16. pH activity profile for Chitobiase on 4-MU-GlcNAc. Activities were determined as initial rates with 100 □M 4-MU-GlcNAc as substrate. The assay time was 18 minutes, and product formation was linear over time at all pH values tested, except pH 3, where the enzyme did not show any activity; no product formation was observed. The plot shows the average values of three parallels, and the error bars shows the standard deviations.

The Michaelis-Menten curve for hydrolysis of the 4-MU-GlcNAc substrate at pH 6.0 was obtained by non-linear regression of the experimental data (initial rate measurements) by using the GraphPad Prism software (Figure 17). The kinetic parameters were determined as follows: K_M = 52 +/- 3.5 µM and k_{cat} =129 +/- 3 s^{-1}. The experiment was performed in triplicates, that is, for every substrate concentration, three reaction mixtures were incubated and for each of these samples, and product formation after 6, 12 and 18 minutes were analyzed.

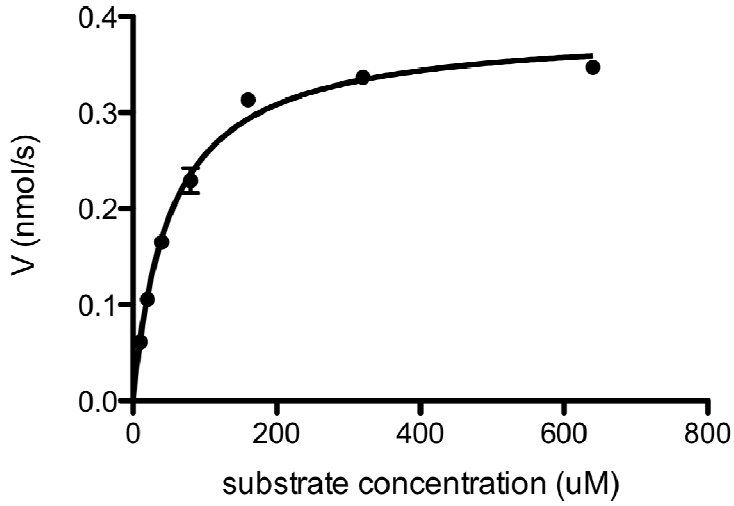


Figure 17. Michaelis-Menten curve for hydrolysis of 4-MU-GlcNAc by chitobiase. The velocities were calculated from initial linear rates of formation of 4-MU. The experiment was performed in triplicates. The filled circles show the experimental data whereas the curve results from non-linear regression using the Graphpad Prism software. The calculated kinetic parameters are KM = 52 +/- 3.5 µM and Kcat =129 +/- 3 s-1.

The Michaelis-Menten curve of the natural substrate was obtained by non-linear regression, and the kinetic parameters K_M = 55 +/- 9.17 µM and K_{cat} = 135 +/- 5.975 s^{-1}, for Chitobiase, were calculated by using the GraphPad Prism software (Figure 18). The experiment was performed in triplicates, that is, for every substrate concentration, three reaction mixtures were incubated and for each of these samples, and product formation after 6, 12 and 18 minutes were analyzed.

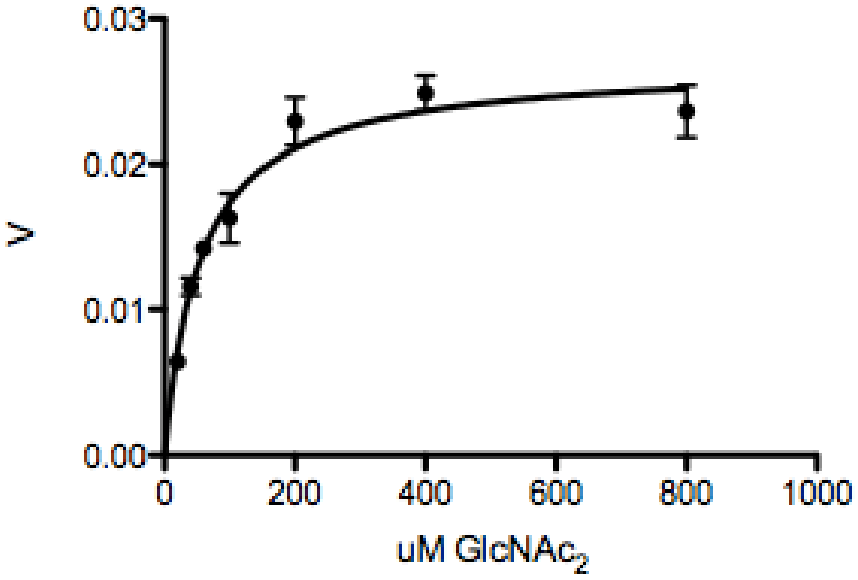


Figure 18. Michaelis-Menten curve from hydrolysis of GlcNAc2. The velocities (nmol/s) were calculated from initial linear rates of product formation, GlcNAc, analyzed by HPLC. The experiment was performed in triplicates. The filled circles show the experimental data whereas the curve results from non-linear regression using the Graphpad Prism software. The kinetic parameters were calculated by using the Graphpad Prism software: KM = 55 +/- 9.17 µM and Kcat =135 +/- 5.975 s^{-1}.

4.4. Degradation of chitooligosaccharides by recombinant Chitobiase

Samples containing 100 µM $GlcNAc_3$(A3) and 100 µM $GlcNAc_4$ (A4), respectively, were incubated with 0.2 nM Chitobiase at pH 6 and 37°C HPLC analysis of the samples showed that chitobiase digests these oligosaccharides. In degradation of trimer (A3), products of both monomer (A1) and minor amounts of dimer (A2) was observed. In degradation of tetramer (A4), the products formed and observed were monomer (A1) and trimer (A3).

An experiment with a mixture of randomly acetylated hexamers showed that Chitobiase also has activity against this substrate. The sequence of this substrate had not been determined, but the substrate was prepared by digestion of chitosan with ChiC. Since chitosan hydrolysis by ChiC only is productive when two acetylated sugars are present in the +1 and +2 subsites (Horn, Sorbotten et al. 2006), it was

assumed that all hexamers had at least two acetylated residues (AA) at their reducing end. The results are shown in Figure 19 and Table 9.

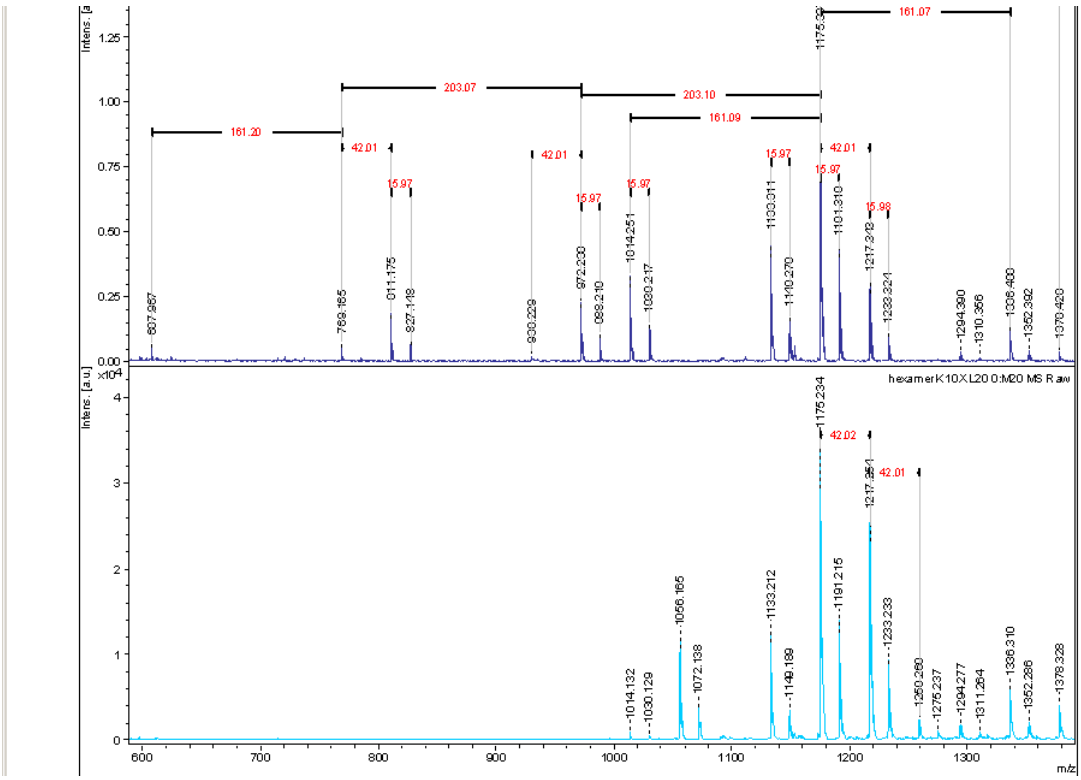


Figure 19. MALDI-TOF MS analysis of the degradation of a mixture of an acetylated hexamers. The upper panel shows degradation products from incubation with Chitobiase. The lower panel is a control sample taken before addition of Chitobiase. The identified chitooligosaccharides are listed in Table 9.

Table 9. Chitooligosaccharides identified using the MALDI-TOF MS spectra shown in Figure 18. The table lists m/z, composition (based on mass) and proposed sequence. X equals D or A. The chitooligosachharides only seen after incubation with Chitobiase are marked with an asterix.

M/Z	Chitooligosaccharide	Proposed chitooligosaccharide sequence
1378	D2A5 (+Na)	DXXXXAA
1217	DA5 (+Na)	DAAAAA
1175	D2A4 (+Na)	DXXXAA
1133	D3A3 (+Na)	DXXXAA
1014	DA4 (+Na)	DAAAA
972*	D2A3 (+Na)	DXXAA
930*	D3A2 (+Na)	DDDAA
811*	DA3 (+Na)	DAAA
769*	D2A2 (+Na)	DDAA
607*	DA2 (+Na)	DAA

The chitooligosaccharides were identified by their molecular masses. The proposed chitooligosaccharide sequences are discussed in section 5.2.

4.5. Binding of chitooligosaccharides to α- and β-chitin

The hypothesis behind this experiment was that chitooligosaccharides attach to the chitin polymer and thus make it more inaccessible for degradation by Chitinases. Thus, the presence of Chitobiase, which has shown activity against the dimer, trimer and tetramer of chitin, may increase the rate of chitinases action because it removes these attached chitooligosaccharides. An attempt was made to check for such oligosaccharide binding.

The experiments were set up by mixing 10 mg/ml crystalline β-chitin (from squid pen, France Chitin) or crystalline α-chitin (Hov-Bio) with 100 μM of $GlcNAc_{2-4}$ in 0.1 M Ammonium acetate buffer, pH 6 and by following the concentration of $GlcNAc_{2-4}$ during incubation at room temperature. The results for A2, A3 and A4 are shown in Figure 20-Figure 25. The experiment was also performed with $GlcNAc_5$ and $GlcNAc_6$, but the analysis of these samples with UHPLC was interrupted by technical problems with the UHPLC. A new experiment was set up for these chitooligosaccharides, but enduring technical issues with the instrumentation prevented analysis.

The data depicted in Figure 20-Figure 25 clearly show that no significant binding was observed in the experiments on incubation with α- and β-chitin with chitooligosaccharides, at least not for the shorter oligomers A2, A3 and A4 (during the conditions in these experiments).

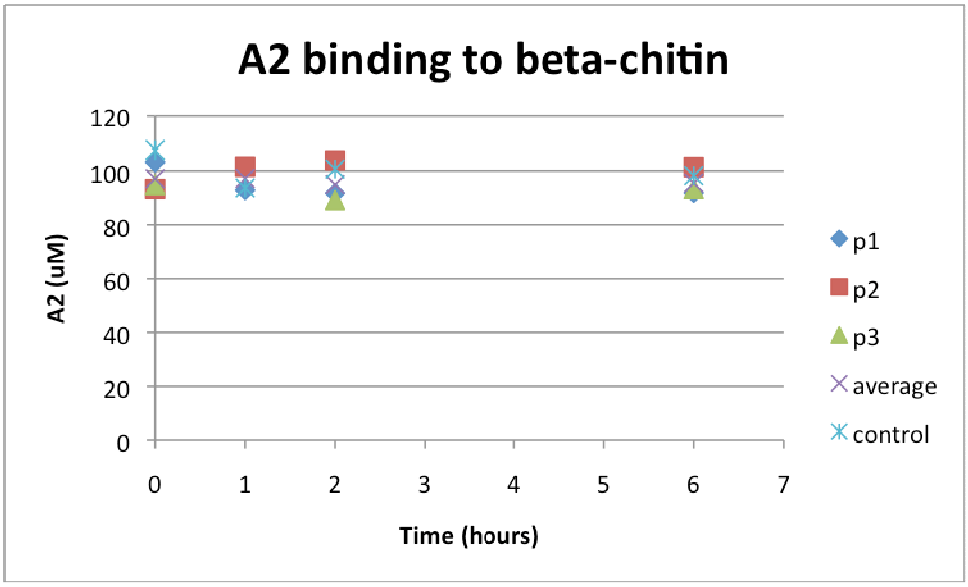


Figure 20. Plot showing the GlcNAc2 (A2) concentration (µM) vs. incubation time with 10 mg/ml crystalline β-chitin. The experiment was performed at pH 6 in 0.1 M ammonium acetate buffer. There is no significant decrease in the A2 concentration (100 µM), meaning that significant binding to the crystalline substrate does not occur. Control samples: 100 µM A2 in 0.1 M Ammonium acetate buffer, pH 6, without addition of chitin.

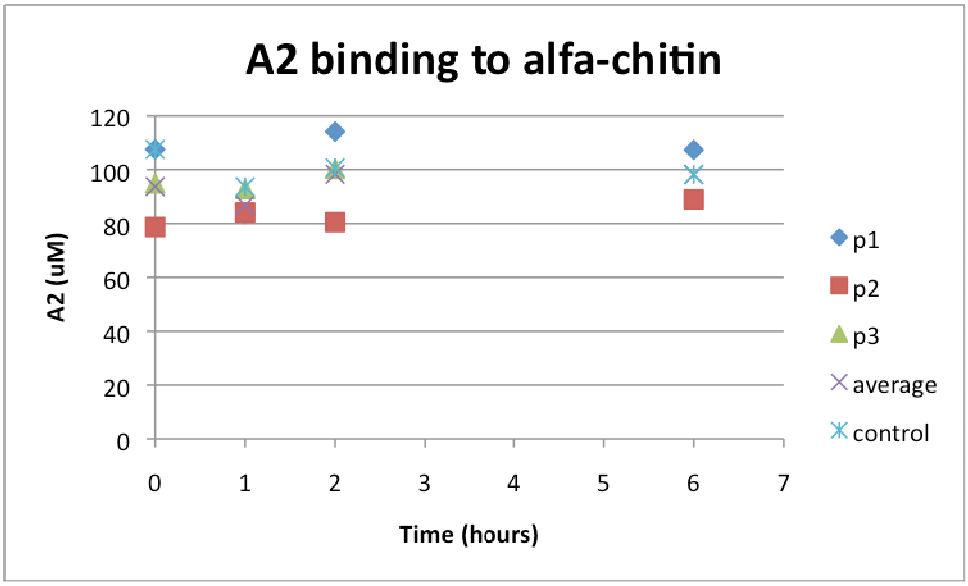


Figure 21. Plot showing the GlcNAc2 (A2) concentration (µM) vs. incubation time with 10 mg/ml crystalline α-chitin. The experiment was performed at pH 6 in 0.1 M ammonium acetate buffer. There is no significant decrease in the A2 concentration (100 µM), meaning that significant binding to the crystalline substrate does not occur. Control samples: 100 µM A2 in 0.1 M Ammonium acetate buffer, pH 6, without addition of chitin.

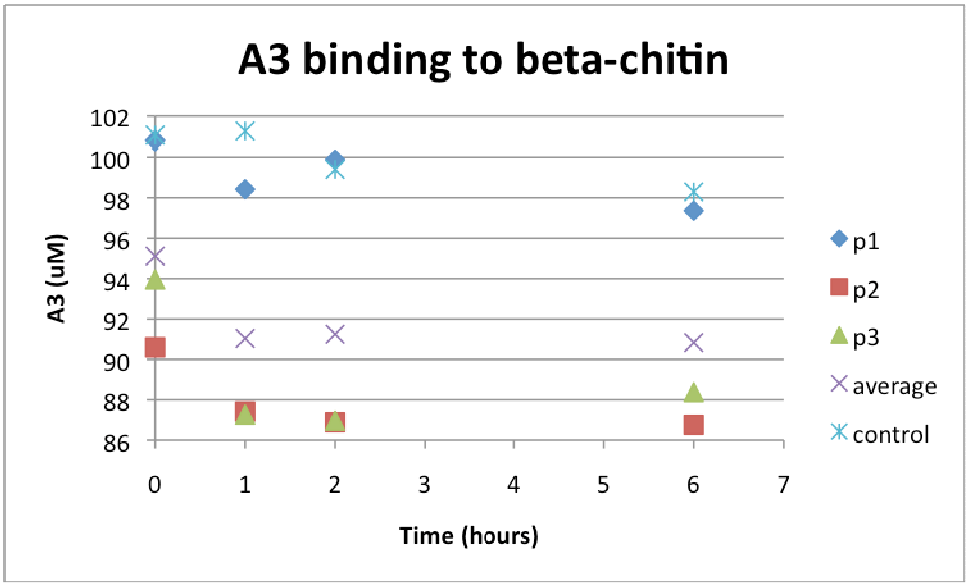


Figure 22. Plot showing the GlcNAc3 (A3) concentration (µM) vs. incubation time with 10 mg/ml crystalline β-chitin. The experiment was performed at pH 6 in 0.1 M ammonium acetate buffer. There is no significant decrease in the A3 concentration (100 µM), meaning that significant binding to the crystalline substrate does not occur. Control samples: 100 µM A3 in 0.1 M Ammonium acetate buffer, pH 6, without addition of chitin.

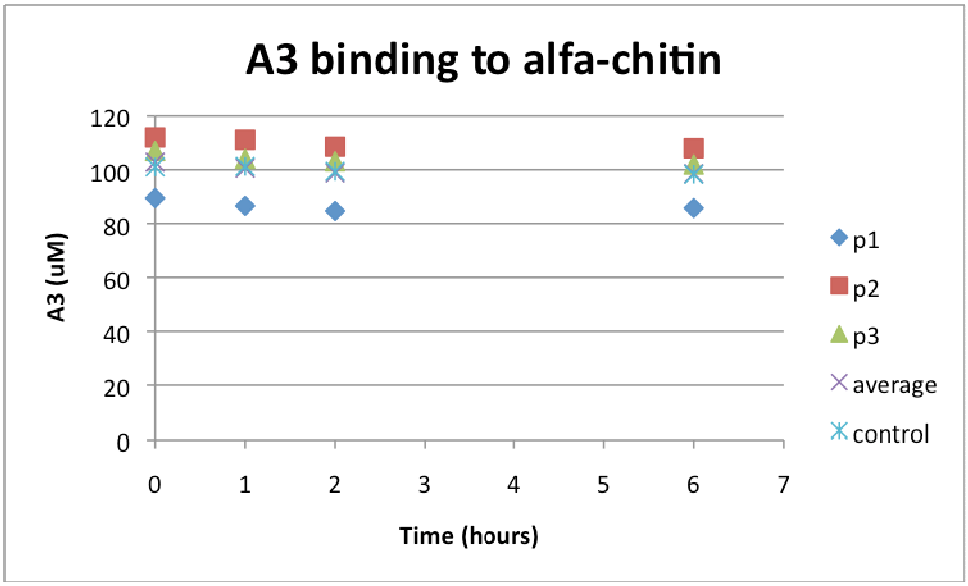


Figure 23. Plot showing the GlcNAc3 (A3) concentration (µM) vs. incubation time with 10 mg/ml crystalline α-chitin. The experiment was performed at pH 6 in 0.1 M ammonium acetate buffer. There is no significant decrease in the A3 concentration(100 µM), meaning that significant binding to the crystalline substrate does not occur. Control samples: 100 µM A3 in 0.1 M Ammonium acetate buffer, pH 6, without addition of chitin.

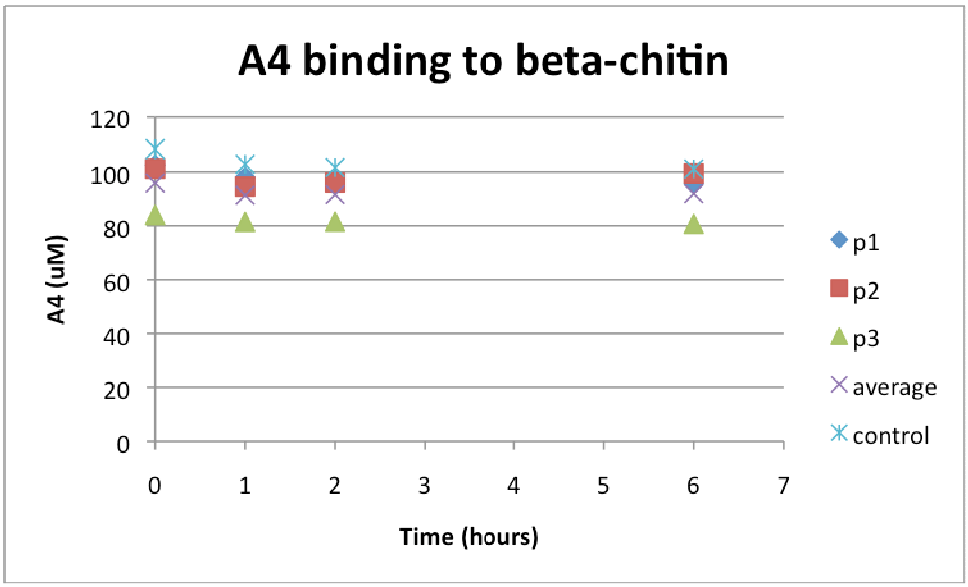


Figure 24. Plot showing the GlcNAc4 (A4) concentration (µM) vs. incubation time with 10 mg/ml crystalline β-chitin. The experiment was performed at pH 6 in 0.1 M ammonium acetate buffer. There is no significant decrease in the A4 concentration (100 µM), meaning that significant binding to the crystalline substrate does not occur. Control samples: 100 µM A4 in 0.1 M Ammonium acetate buffer, pH 6, without addition of chitin.

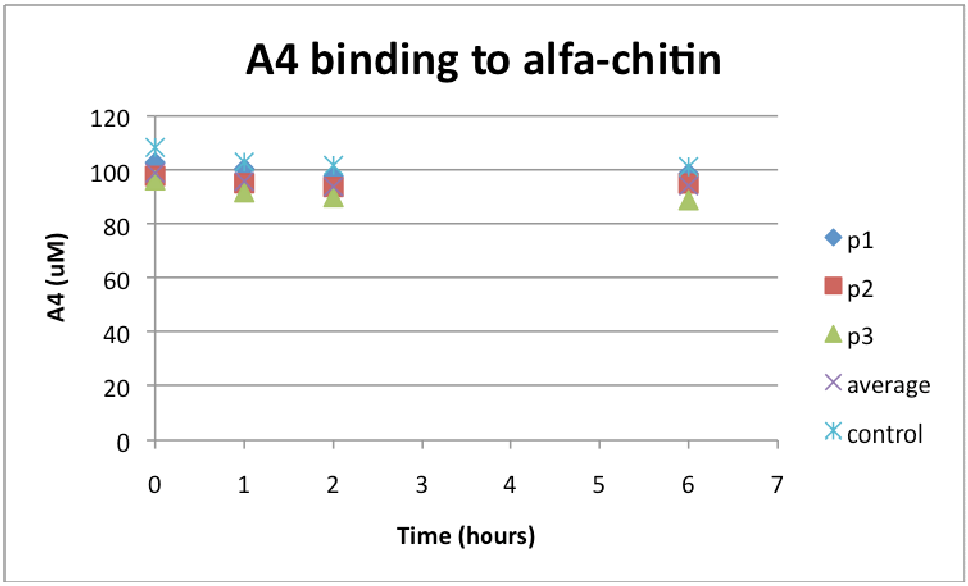


Figure 25. Plot showing the GlcNAc4 (A4) concentration (µM) vs. incubation time with 10 mg/ml crystalline α-chitin. The experiment was performed at pH 6 in 0.1 M ammonium acetate buffer. There is no significant decrease in the A4 concentration (100 µM), meaning that significant binding to the crystalline substrate does not occur. Control samples: 100 µM A4 in 0.1 M Ammonium acetate buffer, pH 6, without addition of chitin.

4.6. Degradation of β-chitin

To learn more about Chitobiase's role in the degradation of chitin, it was interesting to see if there was an effect on the rate of degradation when comparing degradation of chitin with chitinases alone, and with both a chitinase and Chitobiase, as well as degradation of β-chitin with Chitobiase +/- CBP21. Degradation experiments (described in section 3.6) were done in triplicates with crystalline β-chitin and another β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad. The results showed in this section are results from enzymatic degradation of a β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad). The degradation products were analyzed by HPLC (Ultimate 3000), with the Rezex RFQ-Fast Fruit H+, 7.8 x 100mm column and the Carbo-H, 4 x 3.0mm pre-column and 5 mM H_2SO_4 as mobile phase (isocratic chromatography).

4.6.1. Quantitative calibration and detection of background peaks

Before HPLC analysis of product formation were carried out, several control experiments were carried out for quantitative calibration (Figure 26) and for detecting background peaks (Figure 27 and Figure 28).

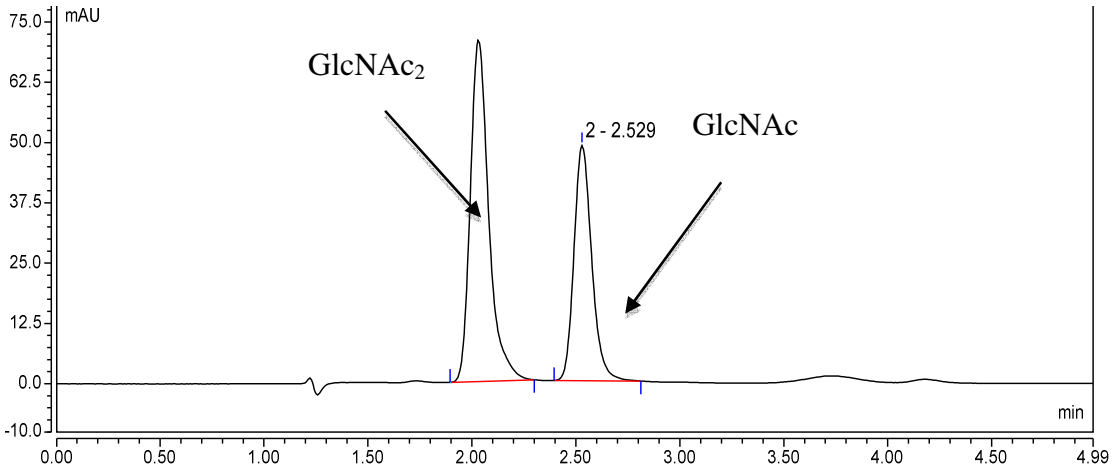


Figure 26. HPLC analysis (Ultimate 3000- Isocratic, 5 mM H2SO4) of a standard mixture comprising 100 µM GlcNAc (A1) and 100 µM GlcNAc2 (A2). Several control experiments with standard mixtures of 100 µM GlcNAc (A1) and 100 µM GlcNAc2 (A2) were performed, and the areas of the peaks identified as A1 and A2 were used in quantification of product formation.

Control samples without addition of enzymes were analyzed to define background peaks from the reaction mixtures comprising only buffer and the β-chitin substrate (Figure 27), as well as reaction mixtures containing reduced glutathione, buffer and the β-chitin substrate (Figure 28). The background peaks were observed to be identical over time, and are neglected in the analysis of the samples.

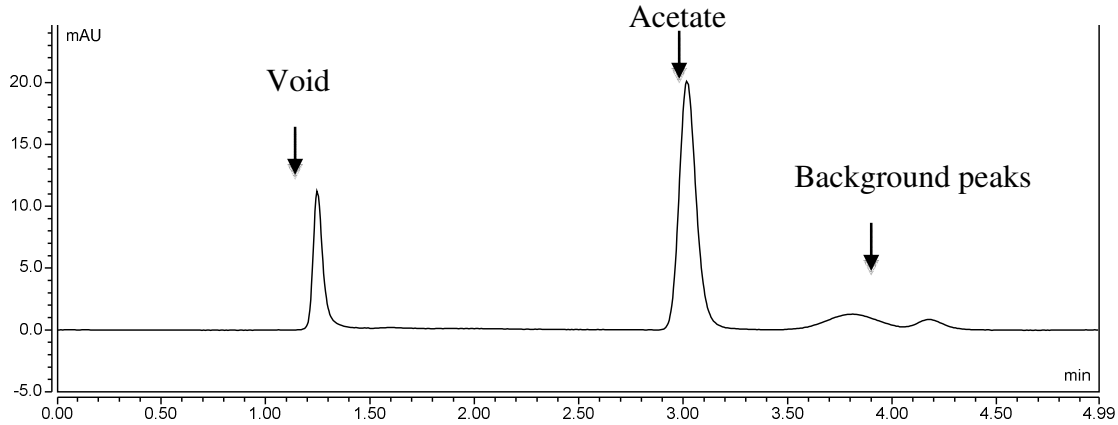


Figure 27. HPLC (Ultimate 3000- Isocratic, 5 mM H2SO4) analysis of a control sample comprising buffer and β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad).The chromatogram shows background peaks whose size did not change over time during incubations with enzymes; these peaks are therefore neglected in the analyses described below.

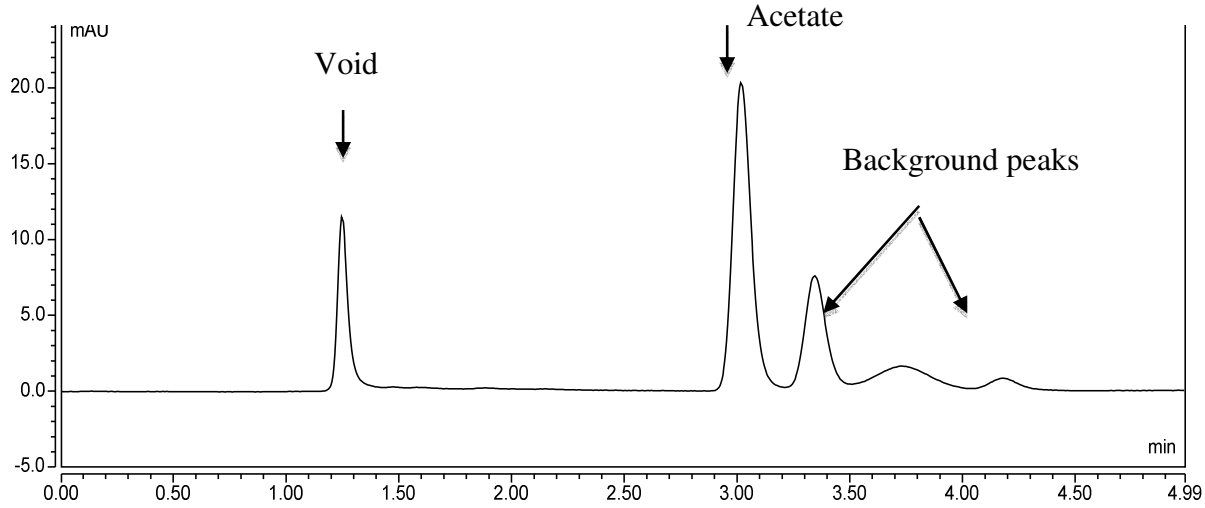


Figure 28. HPLC (Ultimate 3000- Isocratic, 5 mM H2SO4) analysis of a control sample comprising buffer, reduced glutathion and β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad).The chromatogram shows background peaks whose size did not change over time during incubations with enzymes; these peaks are therefore neglected in the analyses described below.

4.6.2. Degradation of β-chitin with ChiB vs. ChiB+Chitobiase

The degradation products from enzymatic degradation of a β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) by using the recombinant, pure chitinolytic enzymes ChiB and Chitobiase were analyzed by HPLC. Degradation products from incubation of ChiB alone, as well as the degradation products from combining ChiB and Chitobiase were analyzed. The samples were collected in a time-scale from 0.5 hour-24 hours, as described in 3.6), prior to analysis by HPLC (Ultimate 3000).

The chromatogram below represents HPLC analysis of a sample from the degradation experiment collected after 24 hours of incubation with ChiB (Figure 29). As seen from the figures, the primary product from hydrolysis with ChiB is $GlcNAc_2$.

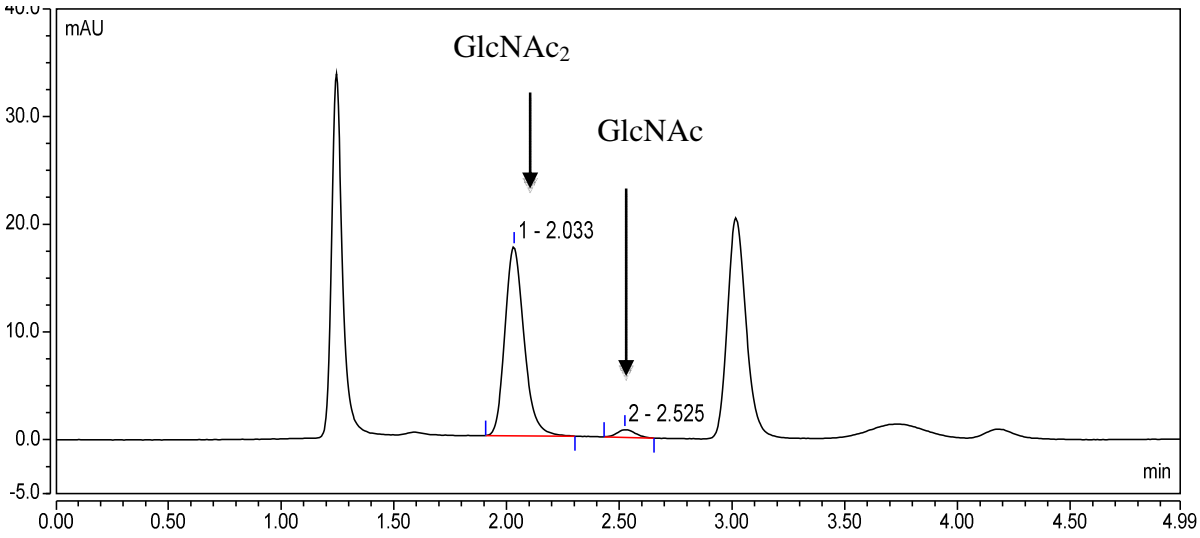


Figure 29. Chromatogram from HPLC analysis (Ultimate 3000- Isocratic, 5 mM H2SO4) of products from hydrolysis of a β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) incubated with Chitinase B for 24 hours. Primarily, hydrolysis of β-chitin with Chitinase B yields GlcNAc2, but also a minor amount of GlcNAc. All other peaks are background peaks, as shown in Figure 27.

This second (below) chromatogram represents HPLC analysis of a sample from the degradation experiment collected after 24 hours of incubation with ChiB and Chitobiase (Figure 30). As seen from the figure, the incubation with ChiB and Chitobiase together, results in the β-chitin polymer being fully the degraded to the monomer of chitin, GlcNAc.

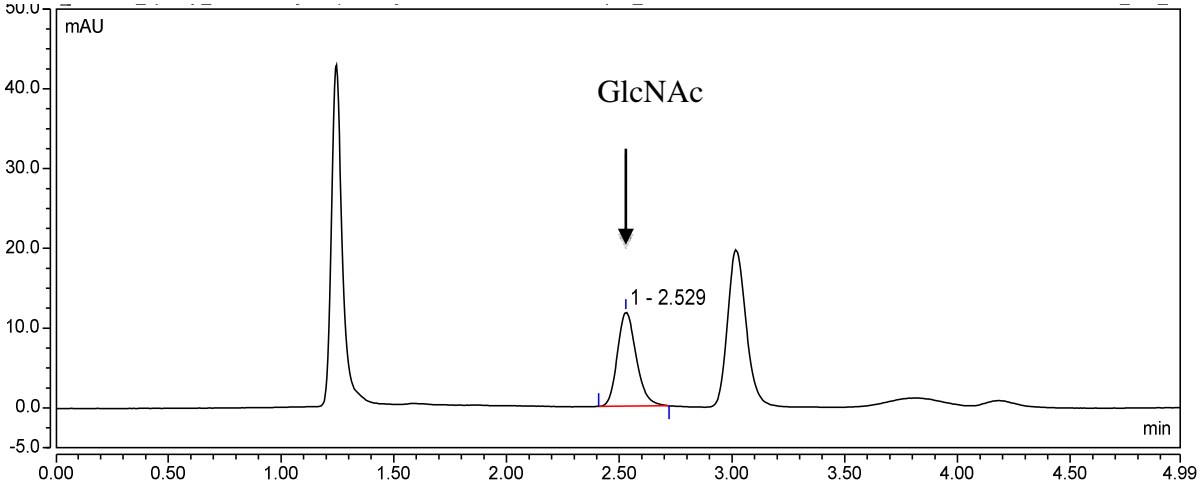


Figure 30: Chromatogram from HPLC analysis (Ultimate 3000- Isocratic, 5 mM H2SO4) of products from hydrolysis of a β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) incubated with Chitinase B and Chitobiase for 24 hours. When Chitobiase is present, the major product formed is GlcNAc. All other peaks are background peaks, as shown in Figure 27.

Figure 31 shows a time curve of the average product formation (sugar concentrations in mg/ml) from the degradation experiments on β- chitin by ChiB alone vs. ChiB+Chitobiase. The sugar concentrations were calculated from peak areas of produced GlcNAc/$GlcNAc_2$ observed upon incubation with the enzymes. Three parallel reactions of β-chitin degradation with ChiB, and three parallel reactions of β-chitin degradation with ChiB and Chitobiase were set up. Samples collected from these reactions at different time points provided data used for determining the average sugar concentration at each time point. As seen from the figure, comparison of the degradation of β-chitin by ChiB vs. ChiB+Chitobiase did not reveal any enhancing effects on the rate of degradation by the presence of Chitobiase.

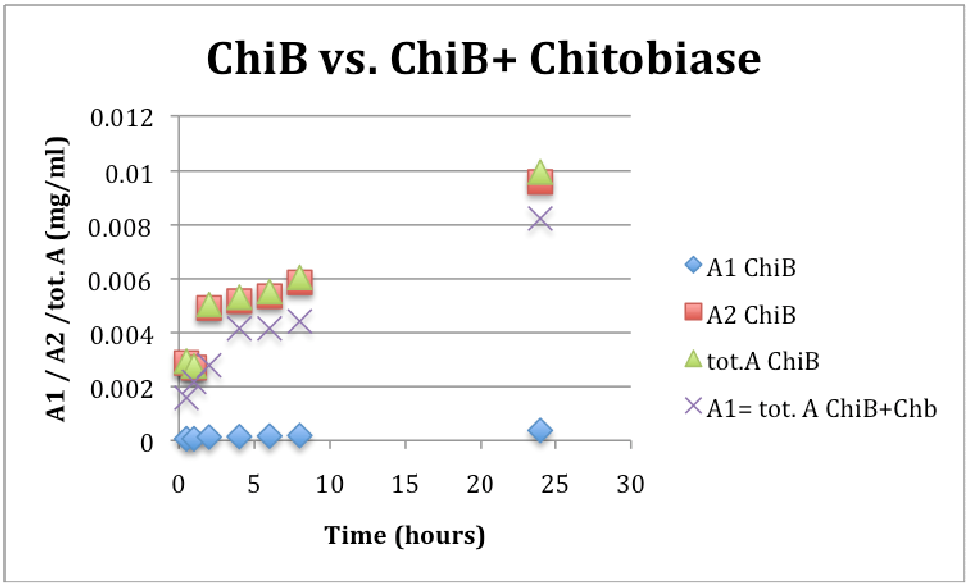


Figure 31. Product formation during degradation of a β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad)with ChiB and Chitobiase (Chb). A1= GlcNAc, A2= GlcNAc2, and tot.A = total sugar (GlcNAc+GlcNAc2). The reaction conditions were: pH 6, 37°C, [Chitobiase]= 50 nM, [ChiB]=0.5 µM. Each point represents the average of 3 independent measurments. The standard deviations were quite high, in the range of 0.002-0.003 mg/ml. A1 ChiB+Chb equals tot. A ChiB+ Chb . When Chitobiase is present, the major product formed is GlcNAc; all A2 are degraded (therefore, no GlcNAc2 (A2) are seen upon incubation with Chitobiase)

4.6.3. Degradation of β-chitin with ChiC vs ChiC+Chitobiase

The degradation products from enzymatic degradation of a β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) by using the recombinant, pure chitinolytic enzymes ChiC and Chitobiase were analyzed by HPLC. Degradation products from incubation of ChiC alone, as well as the degradation products from combining ChiC and Chitobiase were analyzed. The samples were collected in a time-scale from 0.5 hour-24 hours, as described in 3.6), prior to analysis by HPLC (Ultimate 3000).

The chromatogram below represents HPLC analysis of a sample from the degradation experiment collected after 24 hours of incubation with ChiC (Figure 32). As seen from the figures, the primary product from hydrolysis with ChiC is $GlcNAc_2$.

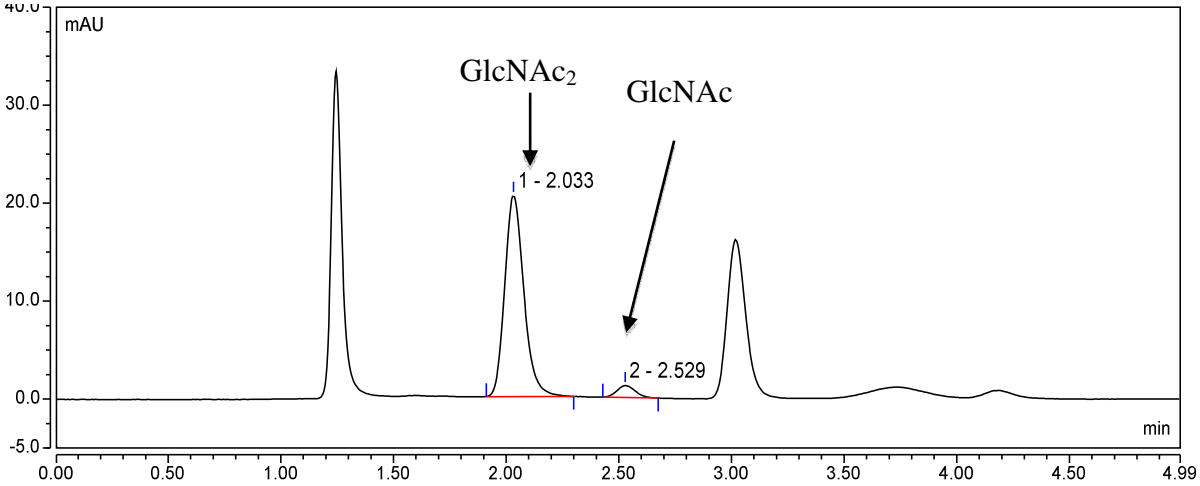


Figure 32: Chromatogram from HPLC analysis (Ultimate 3000- Isocratic, 5 mM H2SO4) of products from hydrolysis of a β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) incubated with 0.5 µM Chitinase C for 24 hours. Primarily, hydrolysis of β-chitin with Chitinase C yields GlcNAc2, but also a minor amount of GlcNAc. All other peaks are background peaks, as shown in Figure 27.

This second (below) chromatogram represents HPLC analysis of a sample from the degradation experiment collected after 24 hours of incubation with ChiC and Chitobiase (Figure 30). As seen from the figure, the incubation with ChiC and Chitobiase together, results in the β-chitin polymer being fully the degraded to the monomer of chitin, GlcNAc.

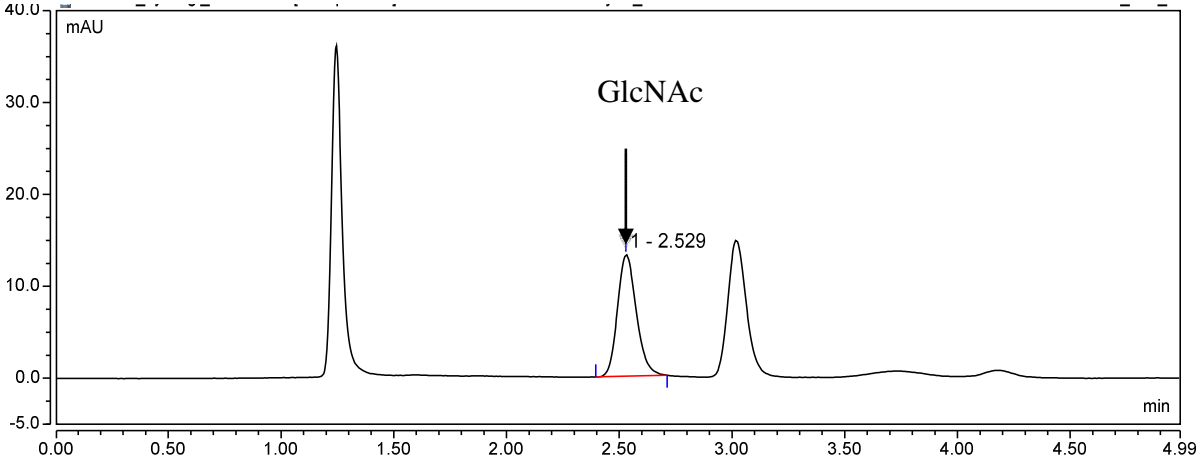


Figure 33: Chromatogram from HPLC analysis (Ultimate 3000- Isocratic, 5 mM H2SO4) of products from hydrolysis of β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) incubated with 0.5 µM Chitinase C and 50 nM Chitobiase for 24 hours. When Chitobiase is present, the major product formed is GlcNAc. All other peaks are background peaks, as shown in Figure 27.

Figure 34 shows a time curve of the average product formation (sugar concentrations in mg/ml) from the degradation experiments on β- chitin by ChiB alone vs. ChiC+ Chitobiase. The sugar concentrations were calculated from peak areas of produced GlcNAc/$GlcNAc_2$ observed upon incubation with the enzymes. Three parallel reactions of β-chitin degradation with ChiC, and three parallel reactions of β-chitin degradation with ChiC and Chitobiase were set up. Samples collected from these reactions at different time points provided data used for determining the average sugar concentration at each time point.
As seen from the figure, comparison of the degradation of β-chitin by ChiC vs. ChiC+ Chitobiase did not reveal any enhancing effects on the rate of degradation.

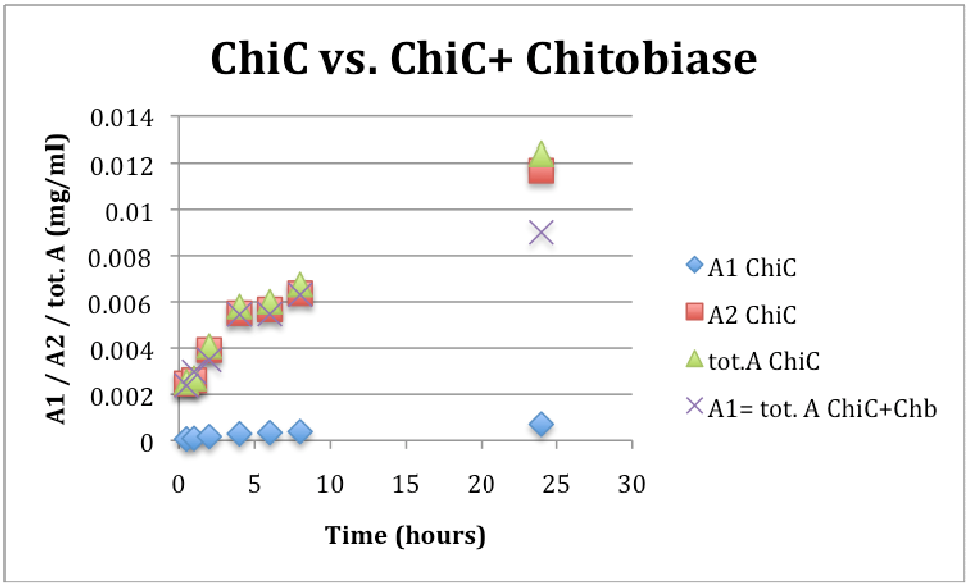


Figure 34. Plot illustrating product formation from degradation of β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) with only Chitinase C vs. Chitinase C in company with Chitobiase. The product formation is observed from 0.5 hours to 24 hours. A1= GlcNAc, A2= GlcNAc2, and tot.A = total sugar (GlcNAc+GlcNAc2). The reaction conditions were: pH 6, 37°C, [Chitobiase]= 50 nM, [ChiB]=0.5 µM. Each point represents the average of 3 independent measurements. The standard deviations were in the range of 0.003-0.004 mg/ml. A1 ChiC+Chb equals tot. A ChiC+ Chb . When Chitobiase is present, the major product formed is GlcNAc; all A2 are degraded (therefore, no GlcNAc2 (A2) are seen upon incubation with Chitobiase)

4.6.4. Degradation of β-chitin with Chitobiase and CBP21 (in presence of reduced glutathione)

The degradation products from enzymatic degradation of a β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) by using the recombinant, pure chitinolytic enzymes CBP21 and Chitobiase (in presence of reduced glutathione) were analyzed by HPLC. Degradation products from incubation of Chitobiase+ reduced glutathione, as well as the degradation products from combining CBP21, reduced glutathione and Chitobiase were analyzed. The samples were collected in a time-scale from 0.5 hour-24 hours, as described in 3.6), prior to analysis by HPLC (Ultimate 3000).

The chromatogram below represents HPLC analysis of a sample from the degradation experiment collected after 24 hours of incubation with Chitobiase+ reduced glutathione (Figure 35). As seen from the figures, the primary product from hydrolysis with Chitobiase+ reduced glutathione is GlcNAc. The product formation from β-chitin degradation by Chitobiase in presence of reduced glutathione was only detectable after 24 hours of incubation. At earlier time-points only minor amounts of produced GlcNAc were observed, and the areas of the peaks in the chromatograms could not be integrated because they were below the detection limit.

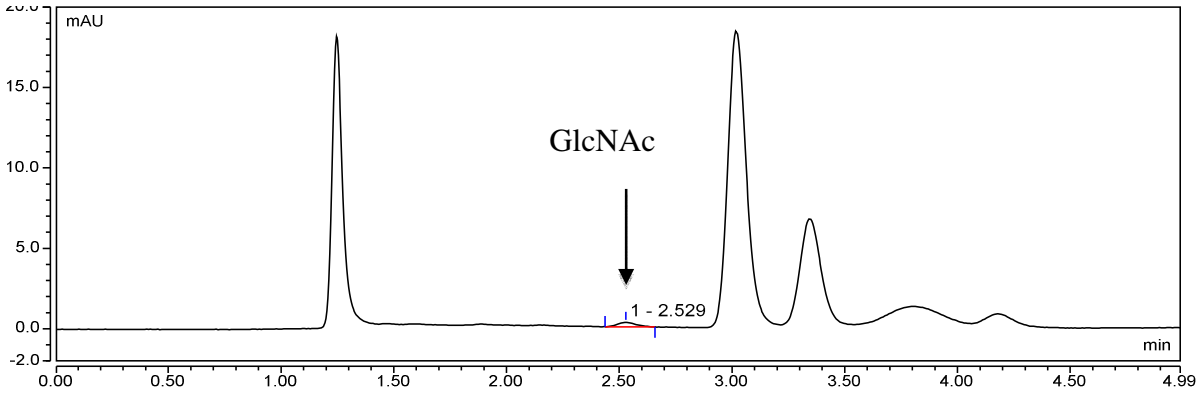


Figure 35: Chromatogram from HPLC analysis (Ultimate 3000- Isocratic, 5 mM H2SO4) of products from hydrolysis of a β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) incubated with 50 nM Chitobiase (in presence of 1 mM reduced glutathione) for 24 hours. The major product formed is GlcNAc, only present in minor amounts. In fact, the product formation from β-chitin degradation by Chitobiase in presence of reduced glutathione was only detectable after 24 hours of incubation, and non-detectable by this method at earlier time-points. All other peaks are background peaks, as shown in Figure 28.

In presence of CBP21 (and reducing agent) the production of GlcNAc is increased compared to GlcNAc production by only Chitobiase and reducing agent. The chromatogram below represents HPLC analysis of a sample from the degradation experiment collected after 24 hours of incubation with CBP21+Chitobiase+ reduced glutathione (Figure 36). Additionally an unidentified product, which matches the retention time of $GlcNAc_3$, is observed upon β-chitin degradation with Chitobiase+CBP21 and reducing agent. Unfortunately, the attempts to identify this product with MALDI-TOF MS failed.

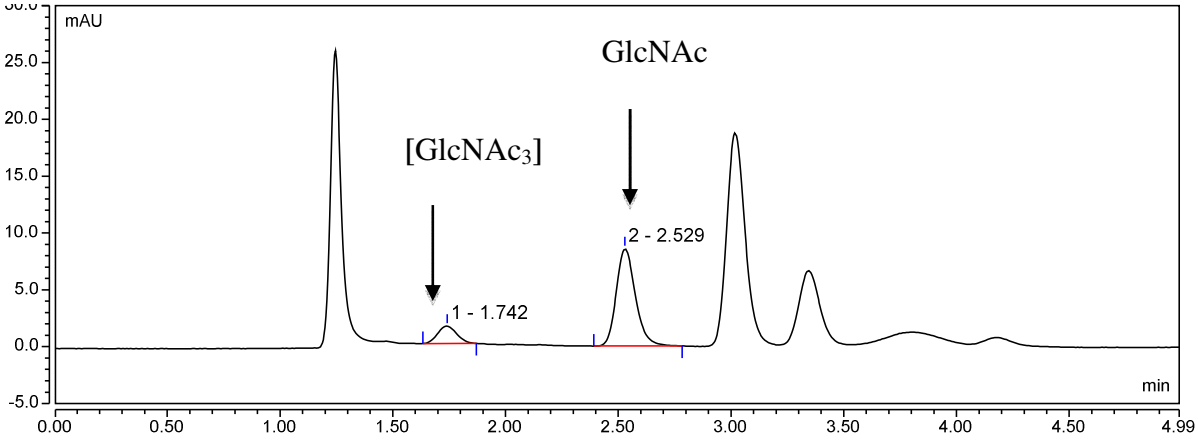


Figure 36: Chromatogram from HPLC analysis (Ultimate 3000- Isocratic, 5 mM H2SO4) of products from hydrolysis of β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) incubated with 0.5 µM CBP21, 1 mM reduced glutathion and 50 nM Chitobiase for 24 hours. The major product formed is GlcNAc, but there is also observed a product that matches the retention time of GlcNAc3, marked in brackets. All other peaks are background peaks, as shown in Figure 28.

Figure 37 shows a time curve of the average product formation (sugar concentrations in mg/ml) from the degradation experiments on β- chitin by CBP21 and Chitobiase in presence of reduced glutathione. The sugar concentrations were calculated from peak areas of produced GlcNAc/GlcNAc observed upon incubation with the enzymes. Three parallel reactions of β-chitin degradation with Chitobiase+ reduced glutathione, and three parallel reactions of β-chitin degradation with CBP21+ Chitobiase+ reduced glutathione were set up. Samples collected from the reactions with with CBP21+ Chitobiase+ reduced glutathione at different time points provided data used for determining the average sugar concentration at each time point. As mentioned, the product formation from β-chitin degradation by Chitobiase in presence

of reduced glutathione was only detectable after 24 hours of incubation, therefore, no time curve could be made from these reactions. Thus, it seems that the production formation from β-chitin degradation by Chitobiase is enhanced by presence of CBP21.

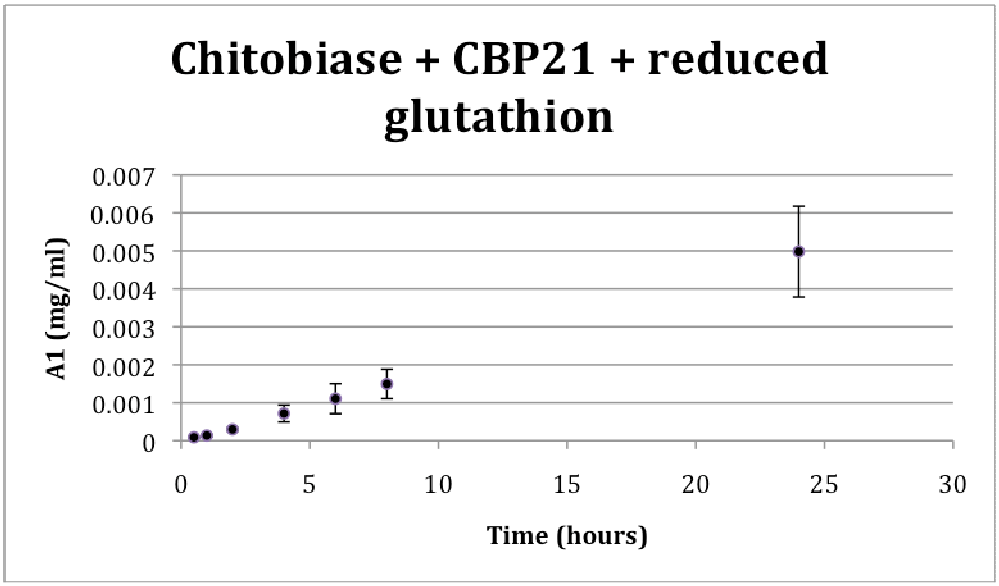


Figure 37. Plot illustrating formation of GlcNAc (A1) from degradation of β-chitin substrate extracted from squid pen (provided by Gustav Vaaje-Kolstad) with 0.5 μM CBP21 and 50 nM Chitobiase, in the presence of reduced glutathione. Note that product formation is linear over time. The average of three parallel reactions and the standard deviations of these are illustrated in the plot. The reaction conditions were: pH 6, 37°C, [Chitobiase]= 50 nM, [ChiB]=0.5 μM. As seen from the plot, standard deviations were in the range of 0.001-0.002 mg/ml).

4.7. Pulldown experiment to detect possible interaction partners

The main goal of this experiment was to find interaction partners of Chitobiase. Finding such interaction partners could perhaps reveal new elements of the chitinolytic machinery of *Serratia marcescens* or could shed light on the function of the three domains of unknown function in this large enzyme. The pulldown experiment was executed with the supernatant from a *Serratia marcescens* culture that had been cultivated on chitin for about five days (minimal chitin left) (described in section 3.7), and with a cell extract (soluble proteins) of such a culture. The total cell extract was prepared by sonication, as described in section 3.7. The fractions obtained from the pulldown experiment (see section 3.7 for experimental details) were analyzed by SDS-PAGE, and bands of interest (were cut out of the gel for protein identification as described in section 3.7 (reduction, alkylation and trypsination, followed by MALDI-TOF MS analysis of extracted peptides). Bands of interest means bands different from Chitobiase only present after elution in pulldown experiments with recombinant Chitobiase attached to the column. Running the extracts through the column without attached recombinant Chitobiase, followed by elution, was performed as a control experiment. Both Coomassie staining and Silver Staining were utilized for visualization of proteins. Silver staining is a more sensitive staining method, and was chosen to possibly reveal proteins not detected by Coomassie staining.

The SDS-PAGE gel (Figure 38) shows the pure recombinant Chitobiase, the cell extract and supernatant utilized in the pulldown experiment, as well as flow through and fractions from the experiment. The bands that were seen in addition to Chitobiase in the fractions from elution were selected and analyzed by MALDI-TOF. However, the further analysis showed that all selected bands were proteolytic cleavage products of Chitobiase.

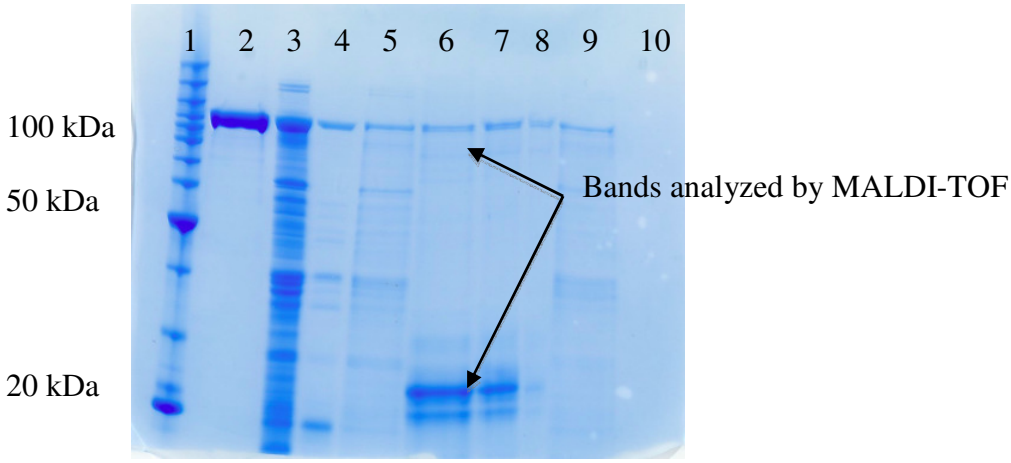


Figure 38: SDS-PAGE analysis of extracts utilized in the experiment, and fractions obtained by elution after running the full cell extract from Serratia marcescens through the column with attached recombinant Chitobiase. Coomassie blue staining was utilized for staining this gel. Lane 1= Benchmark Ladder. Lane 2: pure recombinant Chitobiase attached to the His-trap column. Lane 3:Cell extract of Serratia marcescens prepared by sonication. Lane 4: Supernatant from Serratia marcescens cultivated on chitin. Lane 5: flow through when running the extract slowly through the column. Lane 6, 7 and 8: Eluted proteins. Lane 9 and 10: Flow through and eluted proteins without Chitobiase attached to the column. The bands excised and analyzed by MALDI-TOF are marked with arrows.

The same result was observed with the supernatant, the fractions from this experiment were analysed by SDS-PAGE and stained by silver staining (a more sensitive staining method)(Figure 39), but only protein bands of proteolytic cleavage products of Chitobiase were observed.

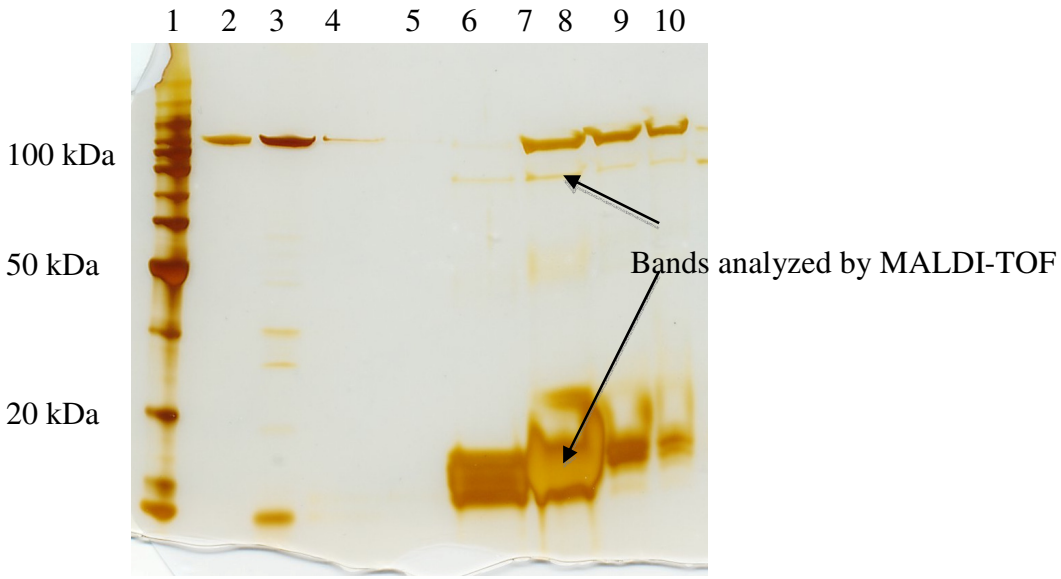


Figure 39. Lane1= Benchmark Ladder; Lane 2= pure, recombinant Chitobiase,Lane 3: Supernatant Serratia marcescens cultivated on chitin. Lane 4 and 5 = Eluted proteins without Chitobiase attached to the column. Lane 5-10= Eluted proteins. The bands excised and analyzed by MALDI-TOF are marked with arrows.

4.8. Secreted proteins of *Serratia marcescens* induced by cultivation on different carbon sources

Proteins precipitated from supernatants from *Serratia marcescens* cultivated in the presence of two different carbon sources were analyzed by LC-MS/MS to detect proteins induced by the presence of chitin, as described in section 3.8. The goal of this experiment was to detect proteins previously unknown to contribute to the degradation of chitin. The proteins were separated by SDS-PAGE prior to LC-MS to reveal differences between the cultures and to select proteins (i.e. gel pieces) for analyses. In this preliminary experiment we only focused on proteins/bands that seemed to be produced in the presence of chitin or in the presence of glycerol only. The experiment was thus not "complete".

Table 10: Serratia marcescens proteins expressed during cultivation on two different carbon sources. Several proteins were only expressed when cultivated on chitin. The proteins where analyzed by SDS-PAGE prior to LC-MS to reveal the differences. Only protein bands not found expressed on both carbon sources where analysed. Proteins in red were only analyzed in the gel band from the cells grown with chitin as carbon source, while the proteins with green text

Full= hemolysin *	165095	Chitin
SSP-h1 *	107817	Chitin
Surface layer protein	101124	Both
putative phosphoenolpyruvate-utilizing enzyme *	99699	Chitin
Aspartokinase I-homoserine dehydrogenase I	89124	Chitin
PTS system enzyme I *	63410	Both
Chitinase	61336	Chitin
Chain A, Crystal Structure Of Chitinase A From S. Marcescens At 1.55 Angstroms	58774	Chitin
60 kDa chaperonin	56751	Both
HAS ABC exporter outer membrane component	54405	Chitin
metalloprotease	52073	Both
Chitinase C	51791	Chitin
N,N'-diacetylchitobiose utilization, enzyme IIC of PTS transport system	48629	Chitin
Porin	41414	Chitin
NlpBsm	38079	Chitin
putative O-methyl transferase	37690	Chitin
Full=Fe(3+)-binding periplasmic protein	36135	Both
Glyceraldehyde-3-phosphate dehydrogenase	31527	Chitin
dihydrodipicolinate synthetas	31285	Chitin
elongation factor Tu A	28661	Chitin
F1hd *	13505	Chitin
HTH-type transcriptional regulator merD; *	13174	Both
Major outer membrane lipoprotein *	8291	Both

Table 10 shows 23 proteins identified when analyzing the gel bands showing differences in expression between the two cultures. 16 proteins (marked in red) were only found in samples from the chitin culture, whereas 7 proteins (marked in green) were only found in the cells cultivated on both substrates. The protein hits reported from the MASCOT search are given with the predicted molecular mass of the protein. Since the bands are excised from a SDS-PAGE band, the approximate size of the band should match the theoretical size of the protein. This is true for most of the hits, but some discrepancies were observed, and these proteins are marked with an asterix (*) in the table above.

5. Discussion

5.1. Production and purification of the Chitobiase

The Chitobiase enzyme characterized in this study originates from *S. marcescens*, has been shown to be present both inside and outside the cells (Toratani, Shoji et al. 2008). Chitobiase has a leader peptide in its precursor form, and when cultivated in *E.coli*, the protein is secreted to the periplasm (Tews, Vincentelli et al. 1996). Therefore, intracellular Chitobiase from *S.marcescens* is thought to be localized in the periplasm (Toratani, Shoji et al. 2008). In this study only the gene sequence encoding the mature protein (without leader peptide) was cloned. The construct was made such that the protein was expressed intracellularly with an N-terminal His_6-tag. Thus, recombinant protein had to be purified from cytoplasmic cell extracts.

The pET-30 Xa/LIC vector is designed to fuse a His_6-tag to the protein of interest for easily purification of the resulting His-tagged protein.The His-tagged Chitobiase bound very tightly to the column, and, therefore an elution buffer with an exceptionally high concentration of imidazole (1 M) had to be used. Standard elution buffers for hexa-histidine tagged proteins are in the range of 100-200 mM imidazol. The presence of several histidine residues on the surface of the protein may cause this tight binding. The protein fractions obtained from the first round of purification were not pure enough for kinetic experiments. Therefore, a second round of purification had to be run in order to obtain satisfactory purity. Before the second round of purification could be performed, the collected fractions had to be concentrated, followed by change of buffer, which gave some protein loss and thus a lower final protein yield. At least two runs were necessary and used routinely. In some cases, additional runs were required to reach the desired level of purity.

These complications made protein purification time-consuming, and since protein is inevitably lost during the purification procedure, the final yield could be as low as 1 mg per L of culture. It would thus be worthwhile to look for optimized purification procedures that are faster and give better yields. For example a

combination of different methods for purification could be utilized, alternatively, one might invest more time in optimizing the nickel column-based purification procedure, e.g. by varying conditions such as pH. Because of the large size of the enzyme, e.g. gel filtration could be included as a step in the protocol. Another option for purification of the Chitobiase from *S. marcescens* is to cultivate *S. marcescens* in Basal Salt Buffer with chitin as the only carbon source. This induces production of Chitobiase, and the enzyme could be purified from the extract by, e.g. size exclusion chromatography or by methods based on chitin-affinity chromatography, which have also been used by other researchers to purify the enzyme (Watanabe et al. 1997).

5.2. Kinetic analysis and degradation of chitooligosaccharides by recombinant Chitobiase

The pH profiling of Chitobiase revealed maximum activity of the enzyme at pH 6, but, as observed from the bell-shaped pH activity curve (Figure 16), the enzyme had a relatively high activity over a broad pH range. This experiment was performed with only one substrate concentration. Since K_M may vary at different pHs pH-activity profiling with only one substrate concentration may be partly invalid. However, this experiment was performed to get a rough estimate of the pH activity profile in a realistic setting and not to elucidate the pH optimum of k_{cat}. The substrate concentration (100 μM) was quite high compared to the K_M determined later (52 +/- 3.5 μM) so it would seem that the pH-activity profile presented in Figure 16 provides a rather realistic picture.

The kinetic experiments were performed at pH 6, and the reaction velocities were determined by determining released 4 MU from the 4-MU-GlcNAc substrate (Figure 40). The Graphpad Prism software, using a built in algorithm that fits the data to the Michaelis-Menten equation, was used for calculation the kinetic parameters. The K_M for this artificial substrate was calculated to be 52 +/- 3.5 μM, and the enzymatic turnover rate (k_{cat}) was calculated to be 129 +/- 3 s^{-1}. In comparison, kinetic analysis of the *S. marcescens* Chitobiase with the artificial substrate pNP-GlcNAc (Figure 40) by Drouillard et al. gave the following values; K_M= 56.7 +/- 4.3 μM and K_{cat}=111.0 +/- 1.7 s^{-1} (Drouillard, Armand et al. 1997). Thus, it seems that

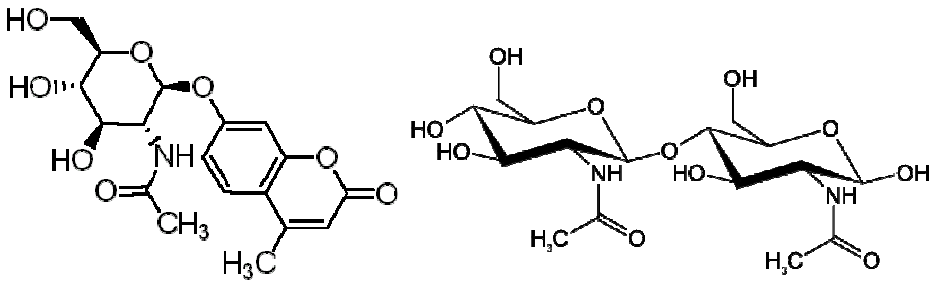
there is no significant difference in the kinetic parameters of Chitobiase for these two artificial substrates.

Kinetic analysis on a natural substrate of Chitobiase, $GlcNAc_2$, was performed at pH 6, and product formation was analysed by HPLC. The kinetic parameters were calculated using the GraphpadPrism Software and gave K_M = 55 +/- 9.2 µM and k_{cat} = 135 +/- 6 s^{-1}. The kinetic parameters from this study, as well as the parameters calculated from kinetic analyses with the pNP-GlcNAc substrate (Drouillard, Armand et al. 1997) are summarized in Table 11, whereas Figure 40 shows all the three substrates.

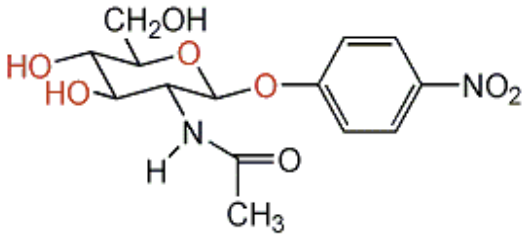
Table 11. Kinetic parameters calculated for three different substrates1).

Substrate	**K_M**	**K_{cat}**
4-MU-GlcNAc	52 +/- 3.545 µM	129 +/- 2.517s^{-1}
pNP-GlcNAc	56.7 +/- 4.3 µM	111 +/- 1.7 s^{-1}
$GlcNAc_2$	55 +/- 9.17 µM	135 +/- 5.975 s^{-1}

[1)] Values for 4-MU-GlcNAc and $GlcNAc_2$ are from this study; values for pNP-GlcNAc are from (Drouillard, Armand et al. 1997).



a) b.)



c.)

Figure 40. The figure shows the substrates utilized for kinetic analysis in this thesis; a.) 4 MU GlcNAc, artificial substrate, where the leaving group is 4-Methylumbelliferone. b.) di-N-acetyl glucosamine, a natural substrate of Chitobiase, where the leaving group is GlcNAc c.) p-nitrophenyl N-acetyl-β-D-glucosaminide (pNP-GlcNAc), where the leaving group is pNP.

The leaving groups of the three substrates depicted in Figure 40 are rather different both in size and chemical composition. The difference in the chemical properties of the leaving groups would be expected to affect the catalytic efficiency of the enzyme, due to the ability of the substrate to interact with the substrate binding site of the enzyme. Nevertheless the kinetic parameters for the three substrates are remarkably similar. This sheds some light on the rate-limiting step in the reaction mechanism of Chitobiase and on the roles of the -1 and +1 subsites in substrate binding.

Since the character of the leaving group seems to have little effect on kinetics, it may seem that substrate-binding, formation of the oxazolinium ion intermediate and product release from the +1 subsite are not rate-limiting. It is more likely that hydrolysis of the oxazolinium ion and/or subsequent release of the product (i.e. a monosugar) from the -1 subsite are rate-limiting. Very strong binding in the – 1 subsite is known to be necessary in enzymes with this type of catalytic mechanism due to the necessary distortion of the -1 sugar (Brameld 1998). It is surprising that, apparently, binding of a sugar in this subsite alone gives enough binding energy to

compensate for the distortion. This is supported by the active site architecture. As seen from Figure 41 and Figure 42, the -1 subsite is more buried in the binding pocket, whereas the +1 subsite seemes remarkably open, and it is conceivable that the sugar residue in subsite +1 is more freely bound than the sugar residue bound in -1, and that the binding in subsite -1 is strong. This is supported by Figure 43 which illustrates several active site residues interacting with the sugar residue bound in subsite -1. As mentioned, hydrolysis of the oxazolinium ion and/or subsequent release of the product from subsite -1 thus may be the rate-limiting factor in this catalysis.

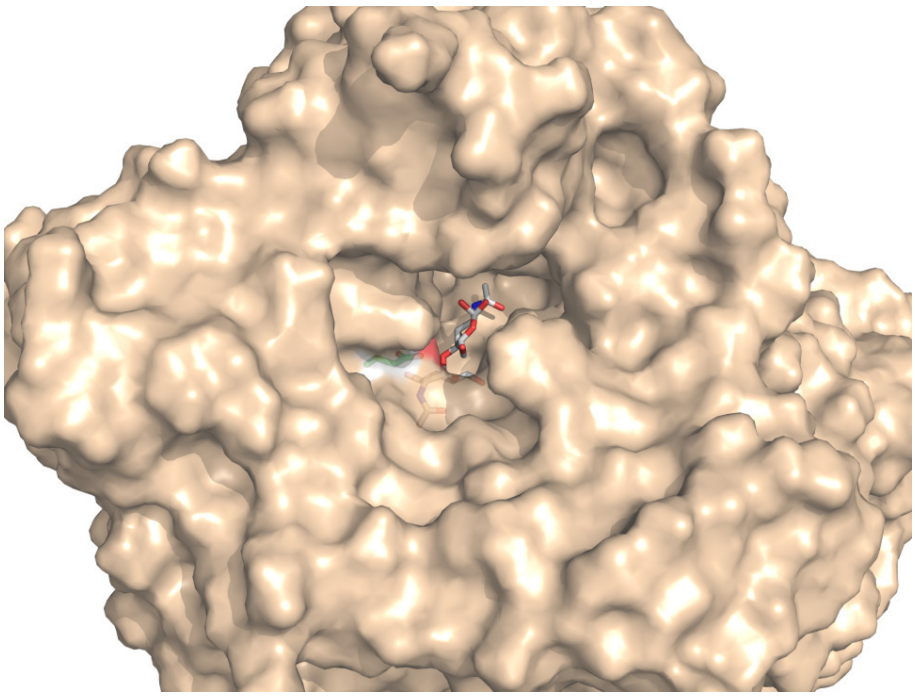


Figure 41. Chitobiase active site with the natural substrate of the enzyme, GlcNAc2 (Bacterial Chitobiase complexed with chitobiose (1QBB) (modified in pyMOL by Gustav Vaaje-Kolstad). The non-reducing sugar residue is buried in the pocket (-1 subsite), while the sugar bound in subsite +1 protrudes from the pocket.

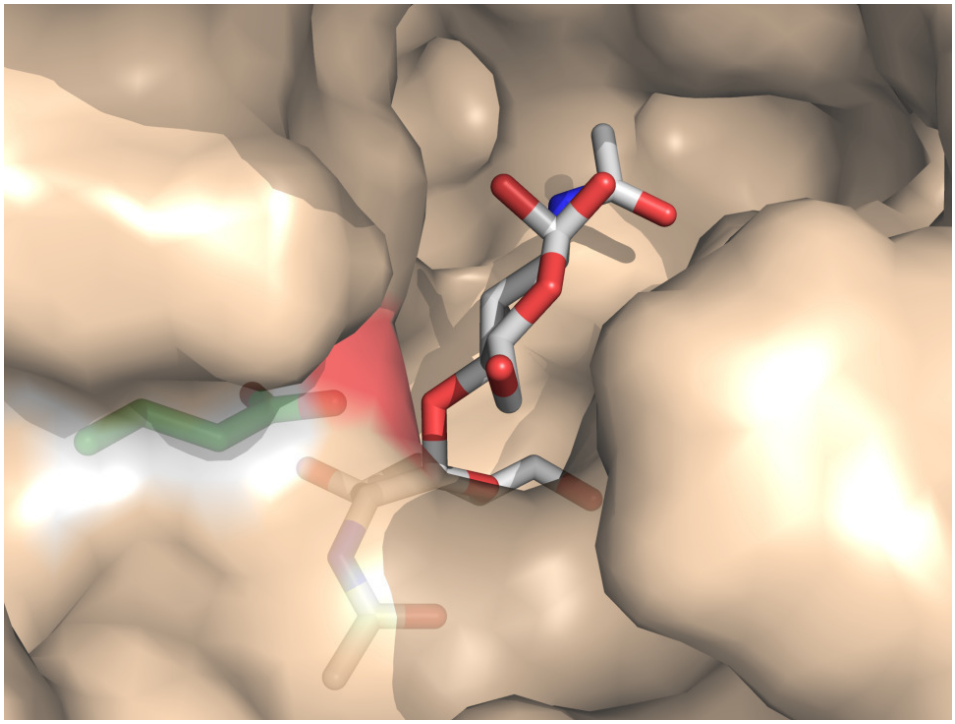


Figure 42. A zoom in picture of the active site of Chitobiase with the natural substrate of the enzyme, GlcNAc2 (Bacterial Chitobiase complexed with chitobiose (1QBB) (modified in PyMol by Gustav Vaaje-Kolstad). The non-reducing sugar residue is buried in the pocket, while the sugar bound in subsite +1 somewhat sticks out of the pocket, conceivably more freely bound than the sugar residue in subsite -1.

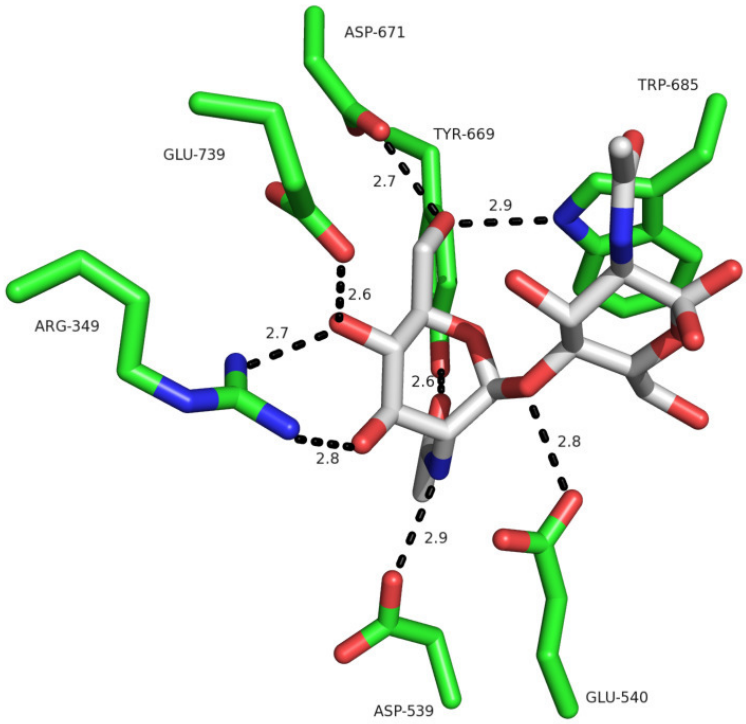


Figure 43. Active site residues of Chitobiase interacting with the natural substrate of the enzyme, GlcNAc2. The sugar bound in subsite -1 interacts with several enzyme residues, crucial for catalysis ((1QBB)modified in PyMol by Gustav Vaaje-Kolstad).

The leaving group of the 4-MU-GlcNAc substrate is somewhat larger than the leaving group of the natural substrate, $GlcNAc_2$. But as mentioned earlier, the hydrolysis of longer chitooligosaccharides, which results in leaving groups of $GlcNAc_3$ and $GlcNAc_2$ also occurs. It would be interesting to further investigate the hydrolysis of longer chitooligosaccharides, and to determine kinetic parameters of Chitobiase against these substrates. However, the kinetics of these substrates can be quite complicated. Digestion of $GlcNAc_3$ results in the products GlcNAc and $GlcNAc_2$, and it is probable that Chitobiase prefer to digest the $GlcNAc_2$ that will be arising before $GlcNAc_3$, and therefore strongly complicate kinetic characterization of the enzyme on this substrate. This was observed in some preliminary experiments performed on the activity of Chitobiase against $GlcNAc_3$ (A3) and $GlcNAc_4$ (A4) in this study (section 4.4). In degradation of A3, only minor amounts of A2 was formed, probably because further degradation to A1 by Chitobiase. In the degradation of A4, the products observed were A1 and A3, illustrating the preference of Chitobiase. Similar problems may possibly occur for $GlcNAc_4$ and longer chitooligosaccharides.

Interestingly, in a study by *Drouillard et al.* the affinity of Chitobiase for deacetylated inhibitors increased significantly with the chain length of the oligosaccharides (Drouillard, Armand et al. 1997). Therefore, and for the reasons mentioned earlier, it would be interesting to look at the activity of Chitobiase against longer chitooligosaccharides in more detail, to map the enzymes activity against these substrates, and to investigate if there exists subsites that may bind these oligosaccharides beyond the +1 subsite.

As described in the introductory part of the thesis (section 1.2.1) chitooligosaccharides with defined length and acetylation/deacetylation pattern is of interest for a variety of applications, ranging from agricultural to medical uses. Since the literature and the work presented here show that Chitobiase indeed can hydrolyse long chitooligosaccharides, it was attempted to incubate Chitobiase with a hexameric chitooligosccharide with a heterogenic acetylation pattern in order to see if there could be produced chitooligosaccharides with novel acetylation patterns that putatively can be of interest for various bioactivity assays. This was indeed accomplished (4.4) and a variety of chitooligosaccharides with varying length and

acetylation pattersn were generated. Since Chitobiase digests the substrate from the non-reducing end (Drouillard, Armand et al. 1997), it is probable that the chitooligosaccharides observed from degradation of the hexamer of chitosan by Chitobiase (section 4.4) in the MS spectrum have a deacetylated (D) residue in the non-reducing end, because Chitobiase is unable to cleave the substrate without an acetyl group. The sequences of the chitooligosaccharides from this experiment showed in (Table 9) was proposed on the basis of this hypothesis. If this is true, Chitobiase represents a valuable tool for production of certain CHOS.

5.3. Binding of chitooligosaccharides to α- and β-chitin

It is generally known that oligomers from insoluble polysaccahrides have low solubility and that they can bind to the surface of the crystalline polysaccharide. However, not much literature is available that shows experimental data for this. Thus, and experiment was designed in order to investigate if chitooligosaccharides attach to the chitin polymer and thus make the polymer more inaccessible for degradation by Chitinases (see below) If this hypothesis is true, the presence of Chitobiase, which has shown activity against dimer, trimer and tetramer of chitin (sections 4.3, 4.4, 5.2), may increase the overall chitin degradation rate because it removes these attached chitooligosaccharides.

The experiment performed in this thesis did however, not reveal binding of chitooligosaccharides to chitin. Only one concentration (100 μM) of chitooligosaccharides was tested, and it would be interesting to repeat this experiment with several concentrations of chitooligosaccharides, which may influence binding. Maybe the chitooligosaccharides bind to the polymers when present at a higher concentration and then prevents degradation of the chitin polymer. It would also be interesting to include higher chitooligosaccharides, such as $GlcNAc_5$ and $GlcNAc_6$.

Another explanation for the missing binding can be that chitooligosaccharides solved in water do not bind, while chitooligos generated by chitinases attach to the polymer because of dehydration. This problem may be overcome by use of other solvents.

5.4. Degradation of β-chitin

Previously, Vaaje-Kolstad *et al*, Brurberg *et al* and Suzuki *et al* have shown that the chitinases from *Serratia marcescens* act synergistically (Vaaje-Kolstad, Horn et al. 2005; Brurberg et al. 1996; Suzuki et al. 2002). In addition Vaaje-Kolstad *et al* showed that CBP21 increased the degradation efficiency (Vaaje-Kolstad, Horn et al. 2005). Based on these studies, it was interesting to investigate whether Chitobiase could act synergistically with the chitinases and CBP21, both since it would be feasible to believe that Chitobiase could aid in removing chitooligosaccharides bound to the surface of the chitin substrate that could act inhibitory on the chitinases and that maybe Chitobiase could attack an area of the substrate where the chitinases could not access readily (note that Chitobiase has a subdomain that resembles a carbohydrate binding domain (Figure 6); However, the results from the experiments did not reveal any synergistic effects with the chitinases, but the degradation rate of β-chitin by Chitobiase was increased by the presence of CBP21. This is conceivable, since CBP21 releases the tight packing of the chitin polymer by introducing chain breaks which results in increased substrate accesability, and produces oxidized ends and longer oligosaccharides, promoting further degradation by chitinases. (Vaaje-Kolstad, Westereng et al. 2010).

5.5. Analyzing possible interaction partners of Chitobiase

Chitobiase has a quite straightforward activity, hydrolysis of $GlcNAc_2$, raising the question why the enzyme is so large? The catalytic site is located on the third domain and the first domain is annotated as a putative substrate binding domain. The second and fourth domains are annotated as individual domains found in this family of enzymes (GH20), but with no determined function. Not all family 20 GHs show this multi-modular structure present in *S. marcescens* Chitobiase. For example, the family 20 β-hexosaminidase from *Streptomyces plicatus* is a two-domain protein of 55 kDa, which when compared to *S.marcescens* Chitobiase were found to lack two of the four domains that compose *S.marcescens* Chitobiase (Mark, Vocadlo et al. 2001) Thus, one possible function of these domains is to interact with other proteins/ enzymes that are involved in the chitin metabolism of *S. marcescens*. Therefore,

pulldown experiments were performed to investigate possible interaction partners of the Chitobiase.

The pulldown experiments only yielded proteolytic cleavage products of the Chitobiase, which indicates that a protease in the extract had cleaved the attached "bait" protein (= Chitobiase). The control experiment, which was performed by running the extracts through the column with no enzyme attached to the column, did not yield any extra protein bands. This indicates that there is a protease in the supernatant and the cell extract that cleaves the attached Chitobiase. One of the chitinases secreted by *S. marcescens*, ChiC naturally occurs in two variants, one comprising the full length protein (ChiC1) and one that is processed by a protease (or autoproteolysis) to yield the catalytic module (ChiC2) and the binding modules as separate proteins (Suzuki et al. 1999; Brurberg 2000). It is thus conceivable that also other secreted proteins from *S. marcescens* may be cut into various forms for a specific function. The peptides identifying the proteolytic products indicate cleavage with loss of the first domain. However, the peptides identified do not fully identify the domain, but in the sample representing the largest band from the gel (after Chitobiase) no peptides are identified from the first domain. This may be a functional cleavage, and should be further investigated.

The functions of the additional domains in Chitobiase thus still remain unknown. Our results do not indicate roles in protein-protein interactions, but more (protease-free) experiments should be done to verify this. Based on the Pfam database module annotation it appears that the first domain has structural similarity to a cellulose binding domain found in certain cellulases, indicating that Chitobiase may be able to bind to crystalline chitin (Both cellulose and chitin binding modules have affinities for both substrates, likely because the structure of chitin and cellulose are so similar). In this thesis, there was not revealed significant binding of Chitobiase to α- or β-chitin, upon incubation of α- and β- chitin polymers with Chitobiase. The experiment was performed by testing for decreased activity of Chitobiase against the 4-MU-GlcNAc substrate as a consequence of incubating the enzyme with the polymers. This would indicate binding of the enzyme to the polymer making it inaccessible for catalysis of the substrate. The control sample, comprising the same concentration of

enzyme in the same buffer as the sample containing buffer, chitin and enzyme, was incubated in room temperature with vertical rotation (20 rpm). The control sample and the samples incubated with the chitin polymers showed similar activity, thus indicating that the enzyme did not bind to the polymers. However, this was only conducted without varying the reaction conditions such as pH, temperature, and concentrations of enzyme and chitin polymers.

5.6. Secreted proteins of *Serratia marcescens* induced by cultivation on different carbon sources

The experiment performed was not fully optimized and should be considered a pilot study. SDS-PAGE gel electrophoresis was used to find possible differences in secretion of proteins from *S. marcescens* when cultivated on two different carbon sources. In the work presented here, a gel-based approach was utilized, which means that some information is probably lost in the running of the gel, in-gel trypsination etc. An optional and perhaps complementary approach would be to perform the digestion in solution, thereby avoiding some of the negative sides of the gel-based approach.

It is important to state that the identification of a protein in the gel lane from the cells using chitin as carbon source and not in the other gel lane does not exclude the possibility of the protein being expressed in both gels (as seen in table 10). It is simply an indication of the possible different expression of proteins, but as this is a pilot study, we cannot be absolutely certain that the changes do not simply arise from technical issues with the instruments or similar issues. In addition, it is important to note that MS analysis have a higher sensitivity than Coomassie staining. Hence, it is important to verify these results with other and additional experiments.

One problem with this study was the growth rate of *Serratia marcescens* observed on these two carbon sources, which were very unlike. This made it difficult to harvest the cultures in the same growth phases, which is important when comparing the secretomes. The reason why this is important is because we want to study the differences in the expression in proteins arising from differential carbon sources, and not simply arising from the bacteria growing in different metabolic phases.

Some of the proteins identified in table 10 were found with a discrepancy between the theoretical mass of the protein, and the size of the band excised from the SDS-PAGE gel. Among other possibilities, this could indicate that the proteins have been cleaved by proteases. As chitin almost exclusively exists in a protein/mineral/chitin composite in nature, the presence of proteases would be expected. This has been shown in previous studies where, in addition to the chitinolytic enzymes expected to be secreted when cultivated in presence of chitin, proteases secreted by *Serratia marcescens* was also detected (G.H. Jo 2008; Ohnishi et al. 1997; Suh et al. 1992). A serine protease and a metalloprotease were identified in the present study (table 10).

Among the identified proteins, we see the presence of chitinase C and chitinase A. The chitinases indicate increased enzyme activity. The observed elongation factor could be an indicator of increased transcriptional activity, but is also a common contaminant since it is very abundant in all cells. In addition to the elongation factor, another transcription-related protein was found (HTH type transcriptional regulator merD;) further indicating increased transcriptional activity. Why it is only found in the cells grown on chitin is not completely understood. All in all, the findings indicate an increased chitonolytic activity as seen by the expression of the chitinases.

As mentioned, *S. marcescens* is only capable of metabolizing the N-acetylglucosamine monomer. Therefore, the activity of Chitobiase, which is most likely located in the periplasm ((Toratani, Shoji et al. 2008), is essential for the bacteria when cultivated in presence of chitin. Interestingly, the secretome results show the presence of a chitobiose transporter system, as has also been described previously in the literature (Uchiyama et al. 2003; Toratani, Shoji et al. 2008).

An extensively studied and optimized in-depth study would be necessary to make any conclusions concerning the secretome of *S. marcescens* and its relationships to chitin metabolism. Further investigation on this subject may provide important information concerning the chitinolytic machinery of *S. marcescens*, as well as protein-protein interactions.

6. Conclusion and perspectives

The aim of this thesis was to clone and characterize Chitobiase from Serratia marcesens, to gain more insight into the function of the S. marcescens Chitobiase. Kinetic characterization was done by studying enzyme performance towards both artificial and natural substrates, as its activity towards the natural substrate shows remarkably little information in the literature. Another interesting part of this work was to elucidate possible "unknown" functions and interactions of the Chitobiase, especially concerning the large size and the presence of three domains additional to the catalytic domain.

Optimizing a purification protocol of IMAC purification followed the successful cloning. In the future, another method of purification may be combined with IMAC for higher yield of pure protein, as well as being less time consuming. The kinetic parameters gained important insight into the mechanism of Chitobiase, suggesting that hydrolysis of the oxazolinium ion and/or subsequent release of the product from subsite -1 may be the rate-limiting factor in this catalysis by Chitobiase. The enzyme was also shown to be active on longer oligosaccharides of chitin, $GlcNAc_3$ and $GlcNAc_4$. The activity of Chitobiase on these substrates should be further investigated, in order to determine whether Chitobiase has binding sites beyond the -1 and +1 sites. Based on the oligosaccharide degrading ability of Chitobiase, it was also shown that the enzyme indeed can aid in tailoring chitooligosaccharides with defined sequences, and thus Chitobiase is a powerful contribution to the enzyme "toolbox".

For further understanding of the large enzyme and its four distinct domains, cloning of individual domains from *S.marcescens* Chitobiase, followed by characterization of these would be interesting,e.g. activity analyses, binding analyses, and "pulldown" analyses. By creating such truncated versions of Chitobiase, it may be possible to reveal the roles of the three uncharacterized domains. Creation of truncated versions of Chitobiase in combination with optimized pulldown experiments may contribute to a better understanding of why this enzyme needs to be this large. Cells are energy efficient, and since they waste energy on producing such a large protein, it is probale that the domains additional to the catalytic domain are

crucial for Chitobiase's activity, perhaps for stabilization, substrate binding or binding to hitherto unknown interaction partners? There are still many unrevealed questions concerning this large multi-modular enzyme.

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