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Enzymatic Degradation of Chitosans

- A study of the mode of action of selected chitinases and chitosanases

Thesis for the degree of Philosophiae Doctor

Trondheim, January 2012

Norwegian University of Science and Technology
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To Kristiane, Anna Tomine and Ståle

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Ellinor B. Heggset
Trondheim, October 2011

Preface

The present study is part of the project; “Characterization and exploration of microbial enzymes for processing of chitin, chitosan and peptidoglycan” headed by Professor Vincent G. H. Eijsink. The project has been a collaboration between the Protein engineering and Proteomics group at the Norwegian University of Life Sciences at Ås and NOBIPOL at the Norwegian University of Science and Technology in Trondheim.

The work was carried out at the Department of Biotechnology at the Norwegian University of Science and Technology, under supervision of Professor Kjell Morten Vårum and co-supervised by Professor Vincent G. H. Eijsink. The work was financed by The Norwegian Research Council, grant number 164653/V40.

The thesis consists of a general introduction, objectives of the thesis, and results and discussion of the papers.

Summary

Chitin is a linear water-insoluble polymer consisting of β-(1→4) linked units of 2-acetamido-2-deoxy-D-glucopyranose (*N*-acetylglucosamine; GlcNAc; **A**-unit). It occurs as a structural component in the exoskeleton of crustaceans and insects, and in the cell walls of certain fungi and yeast. Chitosan is a derivative of chitin and can be prepared by deacetylation of chitin. Chitosans are a family of water-soluble, linear, binary polysaccharides composed of β-(1→4) linked units of 2-amido-2-deoxy-D-glucopyranose (Glucosamine; GlcN; **D**-unit) and **A**-units. Chitinases and chitosanases are enzymes that can be classified into different glycoside hydrolase (GH) families, and they are capable of converting chitin and chitosan into chitooligosaccharides by hydrolyzing the β-(1→4) linkage between the sugar units. When hydrolyzing chitosans, these enzymes differ in their mode of action as well as their productive binding preferences for **A**- and **D**-units, and as a consequence the chitooligosaccharide products will vary in chain length and composition.

The directionality of the degradation of a chitosan oligomer substrate with the two processive family 18 chitinases ChiA and ChiB from *Serratia marcescens* is analysed and discussed in paper II. An essentially monodisperse chitosan oligomer (degree of polymerization of 20) was prepared and tritium-labelled at the reducing end. The oligomer was hydrolyzed to low extents with ChiA and ChiB, and the product mixtures where separated and characterized according to size. The chromatograms showed a continuum of oligomers with a dominance of even-numbered oligomers, revealing an endo-processive mode of action for both enzymes, and that the enzymes degrade the substrate in opposite directions. ChiA and ChiB have chitin binding domains that extend the substrate binding cleft on the non-reducing and reducing side of the active site, respectively. The analysis of the results suggests that the chitin binding domains of the enzymes are involved in the initial binding to the substrate, and that ChiA degrades the substrate towards the non-reducing end while ChiB degrades the substrate in the opposite direction.

In paper III, the enzymatic properties of one of the two family 18 chitinases produced by humans, human chitotriosidase (HCHT) is studied by analyzing its degradation of chitosan substrates. HCHT has been suggested to play a role in the innate immune system, as a defense against chitin-containing pathogens. The information obtained when degrading chitosan is therefore important in an applied context, e.g. when designing inhibitors that are specific for certain chitinases or for understanding how chitosans are degraded in the human body. HCHT hydrolyzed a water-soluble and highly acetylated chitosan substrate ($F_A = 0.62$) according to an endo processive mode of action, with a degree of processivity of 2.5 cuts per formation of enzyme-substrate complex. Sequential characterization of the chitooligosaccharides obtained after degradation of the chitosan ($F_A = 0.62$) revealed a very strong, an absolute, and a relatively weak preference for **A**-units in subsites -2, -1 and +1, respectively. The absolute specificity for acetylated units in subsite -1 is a consequence of GH family 18 chitinases hydrolyzing the chitosans according to a substrate assisted mechanism.

In the last three papers (papers IV, V and VI), one chitinase and two chitosanases were characterized with respect to their mode of action, substrate binding preferences and chitooligosaccharide products.

The bacterial GH family 19 chitinase, ChiG from *Streptomyces coelicolor* A3(2), was found to operate according to an endo, non-processive mode of action, as determined from the rapid disappearance of the polymeric fraction and the initial production of a continuum of oligosaccharides, with a highly acetylated chitosan ($F_A = 0.64$) as substrate. In the initial, rapid degradation phase, **A**-units were productively bound in subsites -2, -1 and +1. However, in a second and slower phase **D**-units could also bind productively in subsite -1. After maximum conversion of the highly acetylated chitosan substrate, the dimers AD/AA and the trimer AAD were the dominant chitooligosaccharides. Since ChiG can productively bind both **A** -and **D**-units in subsite -1, but only **A**-units in subsite +1, quite different chitooligosaccharides were produced as compared to the GH family 18 chitinases.

The GH family 46 chitosanase, ScCsn46A, from *S. coelicolor* A3(2) degraded both a fully deacetylated chitosan (F_A = 0.008) and a chitosan with F_A = 0.32 according to an endo, non-processive mode of action. When degrading a chitosan (F_A = 0.32), **D**-units were productively bound in subsites -1 and +1 initially, but as the degradation proceeded oligomers with both acetylated and deacetylated reducing and non-reducing ends were produced. It was verified that the chitosanase could even cleave the glycosidic linkage between two acetylated units. As a result of the low subsite specificity towards acetylated and deacetylated units in the active site of ScCsn46A, the chitooligosaccharide products were complex with respect to composition and sequence.

SaCsn75A, a GH family 75 chitosanase from *S. avermitilis*, was found to degrade two chitosan substrates (F_A of 0.008 and 0.31) according to an endo, non-processive mode of action. Degradation of the chitosan with F_A = 0.31 revealed a fast initial degradation phase followed by a second slower phase. In the initial phase, **A**-units were productively bound in subsite -1, with an absolute specificity for **D**-units in subsites -2 and +1. In the slower degradation phase, **D**-units could also productively bind in subsite -1, with possibly both **A**- and **D**-units in subsite -2. Chitooligosaccharide products produced at low extents of degradation were composed of **D**-units with an acetylated reducing end. After extensive degradation of the F_A = 0.31 chitosan, the chitooligosaccharide products were predominantly fully deacetylated dimers and trimers, while the higher DP oligomers were dominated by acetylated reducing ends with increasing amounts of internal **A**-units.

Symbols and abbreviations

α	Degree of scission
AMAC	2-amino-acridone
A-unit	Acetylated (GlcNAc) unit
CAZY	Carbohydrate-Active Enzymes database
CBD	Chitin binding domain
ChiA	Chitinase A from *Serratia marcescens*
ChiB	Chitinase B from *S. marcescens*
ChiC	Chitinase C from *S. marcescens*
ChiG	Chitinase G from *Streptomyces coelicolor* A3(2)
CHOS	Chitooligosaccharides
CsnN174	Family 46 chitosanase from *Streptomyces* sp. N174
DP	Degree of polymerization
DP_n	Number average degree of polymerization
DPM	Disintegrations per minute
D-unit	Deacetylated (GlcN) unit
F_A	Fraction of acetylated units
GH	Glycoside hydrolase
GlcN	Glucosamine
GlcNAc	*N*-acetyl glucosamine
HCHT	Human chitotriosidase
HPLC	High pressure liquid chromatography
MALDI-TOF	Matrix assisted laser desorption/ionization time of flight
MS	Mass spectroscopy
[η]	Intrinsic viscosity
NMR	Nuclear magnetic resonance
P_A	Patterns of acetylation
PDB	Protein data bank
ppm	Parts per million
SaCsn75A	Family 75 chitosanase from *S. avermitilis*
ScCsn46A	Family 46 chitosanase from *S. coelicolor* A3(2)
SEC	Size exclusion chromatography

List of papers

Paper I
Aam BB, **Heggset EB**, Norberg AL, Sørlie M, Vårum KM & Eijsink VGH (2010). Production of Chitooligosaccharides and Their Potential Applications in Medicine. *Marine Drugs* **8**, 1482-1517.

Paper II
Heggset EB, Sikorski P, Aarstad OA, Eijsink VGH & Vårum KM (2011). Directionality in degradation of a chitosan oligomer substrate by Chitinase A and B from *Serratia marcescens*. Manuscript.

Paper III
Eide KB, Norberg AL, **Heggset EB**, Lindbom AR, Vårum KM, Eijsink VGH & Sørlie M (2011). Human Chitotriosidase Catalyzed Hydrolysis of Chitosan. Accepted for publication in Biochemistry.

Paper IV
Heggset EB, Hoell IA, Kristoffersen M, Eijsink VGH & Vårum KM (2009). Degradation of Chitosans with Chitinase G from *Streptomyces coelicolor* A3(2): Production of Chito-oligosaccharides and Insight into Subsite Specificities. *Biomacromolecules* **10**, 892-899.

Paper V
Heggset EB*, Dybvik AI*, Hoell IA, Norberg AL, Sørlie M, Eijsink VGH & Vårum KM (2010). Degradation of Chitosans with a Family 46 Chitosanase from *Streptomyces coelicolor* A3(2). *Biomacromolecules* **11**, 2487-2497.

Paper VI
Heggset EB, Tuveng TR, Hoell IA, Liu Z, Eijsink VGH & Vårum KM (2011). Degradation of Chitosans with a Family 75 Chitosanase from *Streptomyces avermitilis*. Submitted to Biomacromolecules.

* The authors contributed equally

List of related papers by the author, *not included* in the thesis

Hoell IA, Dalhus B, **Heggset EB**, Aspmo SI & Eijsink VGH (2006). Crystal structure and enzymatic properties of a bacterial family 19 chitinase reveal differences from plant enzymes. *Febs J* **273**, 4889-4900.

Karlsen V, **Heggset EB** & Sørlie M (2010). The use of isothermal titration calorimetry to determine the thermodynamics of metal ion binding to low-cost sorbents. *Thermochim Acta* **501**, 119-121.

Table of contents

1. Introduction

1.1 Chitin and Chitosan

1.1.1 Chemical structure and properties

Chitin is a linear homopolysaccharide consisting of (1→4)-linked units of 2-acetamido-2-deoxy-*β*-D-glucopyranose (*N*-acetylglucosamine/ GlcNAc/ **A**-unit). Since the **A**-units exist in the $^4C_1$ conformation and the β-(1→4) linkages are diequatorial, the chitin molecules are found in an extended-chain conformation. The consecutive sugar units are rotated 180° relative to each other (Figure 1.1).

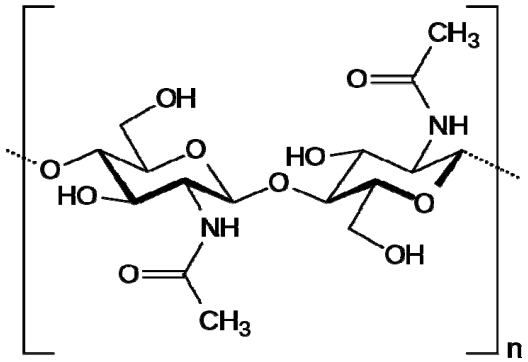

Figure 1.1. Structure of chitin.

Chitin is found in three different crystalline forms; α, β and γ, dependent on the arrangement of the individual chitin chains. In α-chitin, the most common chitin form, the chains are arranged in an antiparallel way (↑↓↑), and they are densely packed with both inter- and intramolecular hydrogen bonding. This explains the inability of α-chitin to swell in water and also why α-chitin is the most rigid chitin form (Carlstrom, 1957; Hackman and Goldberg, 1965; Minke and Blackwell, 1978). In β-chitin, the chains are packed in a parallel way (↑↑↑) (Rudall, 1963; Blackwell, 1969; Gardner and Blackwell, 1975), giving a looser packing which can incorporate small molecules into the crystal

lattice to form various crystalline complexes. The high degree of hydration and reduced packaging tightness gives more flexible and soft chitinous structures, as can be seen when comparing insect cuticle composed of β- versus α-chitin (Merzendorfer and Zimoch, 2003). γ-chitin, the third and most controversial form of chitin, consists of two parallel strands which alternate with a single antiparallel strand (↑↑↓) (Rudall, 1963).

Enzymatic degradation of crystalline substrates such as chitin and cellulose can be difficult to study. In addition, soluble intermediate oligosaccharide products are more readily available than the insoluble, crystalline substrate and, therefore, further degraded into the end products (typically mono-, di- and trimers) (Horn *et al.*, 2006a; Horn *et al.*, 2006b). Studies have shown that chitin has amorphous, unordered regions (Blackwell, 1988) which are believed to be better substrates for the enzymes, compared to the crystalline regions. Using soluble substrates, problems encountered when studying the enzymatic degradation of crystalline, insoluble substrates can be avoided (Horn *et al.*, 2006b).

Chitosan can be prepared from chitin by partial deacetylation, and are water-soluble, linear, binary polysaccharides consisting of (1→4)-linked units of 2-amido-2-deoxy-*β*-D-glucopyranose (Glucosamine/ GlcN/ **D**-unit) and **A**-units (Figure 1.2).

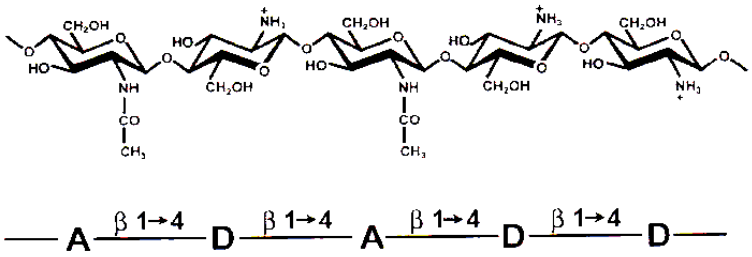

Figure 1.2. Molecular structure of a partially de-*N*-acetylated chitosan.

The name chitosan refers to a continuum of soluble polymeric chitin derivatives that can be described and classified according to the fraction of *N*-acetylated residues (F_A) or

degree of *N*-acetylation (DA), the degree of polymerization (DP) or the molecular weight (M_W), the molecular weight distribution (PD, for PolyDispersity), and the pattern of *N*-acetylation (P_A) or sequence.

The pK_a-value for chitosans has been found to be around 6.5 (Strand *et al.*, 2001), and at pH-values below 6.5, the polymer will be highly positively charged. The water-solubility at acidic pH and insolubility at basic pH values is a characteristic property of commercial high-molecular weight chitosans (F_A = 0-0.2). Chitosans with F_A-values between 0.4 and 0.6 have been found to be neutral soluble (Sannan *et al.*, 1976).

1.1.2 Production

In biomass, chitin is closely associated to minerals, proteins, pigments and lipids. Extraction of chitin from crustacean shells involves three basic steps (Hackman, 1954): demineralization, deproteinization and decoloration. Minerals are dissolved by addition of acid, and proteins are hydrolysed with alkali. To remove pigments and fat, extraction with an organic solvent can be performed. It is important to notice that the demineralization and deproteinization steps may introduce a risk of depolymerisation and de-*N*-acetylation of the chitin during the extraction procedure. It is necessary to optimize the extraction to minimize the degradation of the chitin and also to reduce the level of impurities to a satisfactory level for specific applications (Percot *et al.*, 2003). Several research groups have reported optimization of the extraction procedure (Percot *et al.*, 2003; Rødde *et al.*, 2008). For instance, Rødde and colleagues used a mild method (demineralization: cold 0.25 M HCl / 40 minutes; deproteinization: 1 M NaOH / 4 hours / 95 °C) compared to the method described by Hackman (demineralization: cold 2 M HCl / 48 hours; deproteinization: 1 M NaOH / 24 hours / 100°C) (Hackman, 1954; Rødde *et al.*, 2008). This optimized method resulted in a high molecular weight chitin, with a high degree of acetylation.

Chitosan can be produced from chitin by two fundamentally different methods, i.e. homogeneous (Sannan *et al.*, 1976) and heterogeneous deacetylation. In the homogeneous deacetylation process, the chitin is dissolved in an alkali solution at low

temperature and under excessive stirring. In the heterogeneous deacetylation process, the chitin remains insoluble in a hot alkali solution and the deacetylation proceeds in a two-phase system. Chitosans with F_A varying from 0 to about 65 % can be prepared by homogeneous deacetylation of chitin (Sannan *et al.*, 1976). There is little controversy regarding the distribution (pattern of *N*-acetylation; P_A) of the two monomers in these chitosans, which have been found to have a random distribution of the **A**- and **D**-units, i.e. according to Bernoullian distribution (Kurita *et al.*, 1977; Vårum *et al.*, 1991a; Vårum *et al.*, 1991b). There is, however, more controversy about the distribution of sugar units in chitosan prepared by heterogeneous deacetylation procedures. The heterogeneously deacetylated chitosans have been shown to be separated into a chitin-like, acid-insoluble fraction and a chitosan-like acid-soluble fraction, and a mixture of these two fractions can be misinterpreted as a block polymer (Ottøy *et al.*, 1996; Vårum and Smidsrød, 2005).

The chemical production of chitosan and chitosan oligomers is a harsh termochemical process. In principle, the use of chitin deacetylases (see also section 1.2.7) to produce chitosan offers an alternative method for chitosan production without these drawbacks (Tokuyasu *et al.*, 2000; Tokuyasu *et al.*, 2000; Tsigos *et al.*, 2000; Hekmat *et al.*, 2003; Blair *et al.*, 2006). These enzymes hydrolyze the *N*-acetyl linkage in chitin and convert GlcNAc to GlcN. So far no enzymatic method that provides a viable alternative to the current chemical production of chitosan has been found, and the major hurdle for this approach is the insoluble and crystalline nature of the chitin substrate.

1.1.3 Occurrence in nature

Chitin is found as a structural component in the cuticle of insect shells, in shells of crustaceans, and in the cell walls of fungi and yeasts (Gooday, 1990; Ruiz-Herrera and Martines-Espínoza, 1999; Merzendorfer and Zimoch, 2003), and chitin is the second most abundant polysaccharide in nature after cellulose.

The annual production of chitin in nature has been estimated to approximately 10^{10} – 10^{11} tons per year (Gooday, 1990). Despite this enormous production, chitin does not

accumulate in the environment, indicating that natural chitinolytic machineries are sufficiently efficient to handle the annual production of chitin. The amounts of chitin, chitosan and their derivatives that are used/produced in industrial processes (Sandford, 2002) have been estimated to be about 30 000 metric tons for chitin and about 10 000 metric tons for chitosan in 2007 (Chitin and Chitosan, A Global Strategic Business Report. MCP-2039; Global Industry Analysts Inc.). Most of the chitin is used as raw material for the production of glucosamine (the monomer GlcN). Glucosamine is used as pain relief of osteoarthritis and is the number one dietary supplement in the USA (Sandford, 2002).

Chitosan occurs in nature in the mycelia of the fungi *Mucor rouxii* (White *et al.*, 1979; Synowiecki and Al-Khateeb, 1997), in amounts ranging from 8.9 to 35% of the dry cell wall weight (White *et al.*, 1979; Arcidiacono *et al.*, 1989; Knorr *et al.*, 1989; Synowiecki and Al-Khateeb, 1997). The fungal chitosan was reported to be 5 to 10% acetylated (White *et al.*, 1979).

1.1.4 Production of chitooligosaccharides

Chitooligosaccharides (CHOS) are oligomers prepared from chitosan either chemically or enzymatically. Chemically, chitosan can be converted to CHOS by the use of acid hydrolysis. Vårum *et al.* (2001) studied acid hydrolysis of the glycosidic linkage (depolymerization) and of the *N*-acetyl linkage (de-*N*-acetylation) of a variety of partially *N*-acetylated chitosans in both concentrated and dilute acid. The acid-catalyzed degradation rates of chitosans were shown to depend on F_A, and the initial degradation rate constant was found to increase in direct proportion to F_A. Acid hydrolysis was found to be highly specific to cleavage of **A-A** and **A-D** glycosidic linkages, and these linkages were hydrolyzed with three orders of magnitude higher rates than the **D-D** and **D-A** linkages (Vårum *et al.*, 2001). This preference were explained by a combination of two factors: (i) the presence of a positively charged amino group (on the **D**-unit) makes the protonation of the glycosidic oxygen unfavorable and results in a slower rate of cleavage after a **D**-unit (Moggridge and Neuberger, 1938) and (ii) the presence of an acetamido group (on the **A**-unit) close to the glycosidic linkage may yield some degree

of substrate-assistance to the hydrolytic mechanism, resulting in an higher rate of cleavage of the glycosidic linkage following an **A**-unit (Piszkiew and Bruice, 1968). It has also been shown that the rate of depolymerization of the glycosidic linkages were more than ten times faster than the rate of deacetylation in concentrated acid, whereas the two rates were found to be equal in dilute acid. It was suggested that this is caused by these two processes having different reaction mechanisms with different rate-limiting steps (Vårum *et al.*, 2001).

Enzymatic hydrolysis of the $\beta(1\rightarrow4)$ glycosidic linkage with glycoside hydrolases like chitinases or chitosanases, is a promising approach to the production of CHOS. Here, the chemical composition (F_A), sequence (P_A) and DP of the resulting CHOS-mixture depend on the chitosan substrate and the specificity of the enzyme used. By optimizing the chitosan-enzyme combination and also considering the fact that the product profile changes considerably during the hydrolysis reaction, product mixtures can be enriched for certain CHOS (see paper IV, V and VI). In addition, chitin deacetylases could be used to modify the *N*-acetylation pattern of the CHOS, but this route has so far remained unexplored.

1.1.5 Purification and characterization of chitooligosaccharides

The CHOS produced after enzymatic (or chemical) degradation of chitosans contains a mixture of oligomers, which vary in DP, F_A and P_A. In the present work, Size Exclusion Chromatography (SEC) has been used for separation of CHOS and Nuclear Magnetic Resonance (NMR) has been used to characterize the CHOS in terms of F_A, P_A and DP. In addition, in paper III and V, Mass Spectrometry (MS) is used to obtain additional sequential information on the CHOS. In this section, a brief overview of these methods is given.

1.1.5.1 Size Exclusion Chromatography

The SEC system used to generate the data displayed in this thesis is composed of three Superdex^{TM} 30 columns, coupled in series. The separation is based on the size (DP) of

the CHOS, and recently, Sørbotten and colleagues (2005) described the separation of CHOS up to DP40, using the same system. In addition, oligomers from DP2 to DP20 can be separated individually, independent of F_A and P_A of the oligomer (Sørbotten *et al.*, 2005). To quantify the amounts (mass) of CHOS separated on the columns, a refractive index detector is used. In order to further characterize the separated CHOS with regard to DP, F_A and (if possible) P_A, NMR and MS can be used.

1.1.5.2 Nuclear Magnetic Resonance

^{1}H-NMR can be used to obtain information about the F_A and the DP_n of a chitosan sample, in addition to the DP and F_A of individual CHOS fractions. As an example, a ^{1}H-NMR spectrum (anomer region; from 4.5 to 5.5 ppm) of an isolated tetramer fraction obtained after initial degradation ($\alpha = 0.11$) of a $F_A = 0.65$ chitosan with ChiB from *Serratia marcescens* (Sørbotten *et al.*, 2005) is shown in Figure 1.3.

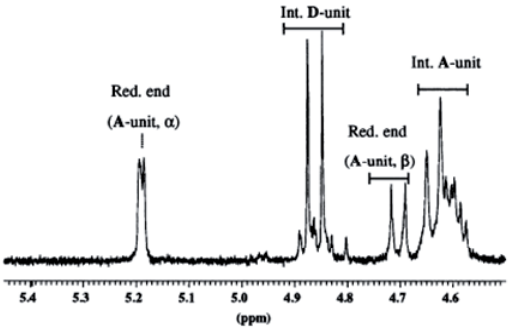

Figure 1.3. ^{1}H-NMR spectrum (anomer region) of an isolated tetramer fraction. The ^{1}H-NMR spectrum is from the tetramer fraction obtained after degradation of a $F_A = 0.65$ chitosan, degraded to a low degree of scission ($\alpha = 0.11$), by ChiB from *S. marcescens*. The reducing end of the α-anomer of an **A**-unit resonates at 5.19 ppm, while the β-anomer from the same unit resonates at 4.71 ppm if the neighboring residue is an **A** (seen in the spectrum) or at 4.74 ppm when the neighboring residue is **D** (not seen in the spectrum). The α-anomer of a **D**-unit resonates at 5.43 ppm and the β-anomer at 4.92 ppm. These are absent in the spectrum. Internal **D**-units resonate at 4.80 - 4.90 ppm, while internal **A**-units resonate at 4.55 – 4.68 ppm (Vårum *et al.*, 1991a; Ishiguro *et al.*, 1992). Figure from Sørbotten *et al.*, 2005.

For calculation of the F_A- and DP-value of the chitooligosaccharide, the following integrals of the peaks are used, as shown in equation 1 and 2, respectively.

$$F_A = \frac{I_{A\alpha} + I_{A\beta} + I_A}{I_{D\alpha} + I_{A\alpha} + I_{D\beta} + I_D + I_{A\beta} + I_A} \quad (1)$$

$$DP = \frac{I_{D\alpha} + I_{A\alpha} + I_{D\beta} + I_D + I_{A\beta} + I_A}{I_{A\alpha} + I_{A\beta} + (1.67 \times I_{D\alpha})} \quad (2)$$

where $I_{A\alpha}$ = Integral of the acetylated reducing end, α-anomer
$I_{A\beta}$ = Integral of the acetylated reducing end, β-anomer
I_A = Integral of the internal acetylated units
$I_{D\alpha}$ = Integral of the deacetylated reducing end, α-anomer
$I_{D\beta}$ = Integral of the deacetylated reducing end, β-anomer
I_D = Integral of the internal deacetylated units

The sequence of the (shorter) CHOS can thus be resolved if the oligomer mixture is not too complex. The identity of the reducing end of an oligomer (**A**- or **D**-unit) can be determined from the ^{1}H-NMR spectrum. In addition, if the reducing end sugar is an **A**-unit, it is possible to get information about the neighbouring sugar as the chemical shift of the β-anomer proton in **A**-units depends on whether the neighbouring unit is an **A** or **D**-unit (Figure 3 and Sørbotten *et al*., 2005). ^{13}C-NMR spectroscopy is used to reveal the identity of the non-reducing end of an oligomer, and in some instances the identity of its nearest neighbour may also be identified (see paper IV and Vårum *et al.,* 1996).

1.1.5.3 Mass Spectrometry

In cases were NMR has limitations due to insufficient resolution of the longer CHOS, MS can be an option for identification of the F_A, DP and P_A of oligomers (Bahrke *et al.*, 2002; Cederkvist *et al.*, 2006; Cederkvist *et al.*, 2008; Dybvik *et al.*, 2011). In the study by Bahrke and colleagues (2002), a method for sequencing CHOS up to DP12 was

developed using derivatization and matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) postsource decay (PSD) MS. In the method, the reducing ends of the CHOS were derivatized with reductive amination using 2-aminoacridone (AMAC) (Okafo *et al.*, 1997). Further, due to the AMAC derivatization, the sequencing of the oligomers was quite uncomplicated, since it favoured formation of Y-type ions (i.e. cleavage after the oxygen in the glycosidic linkage from the reducing end (Domon and Costello, 1988)). Paper V included in this thesis describes the use of AMAC derivatization and MALDI TOF/TOF MS^1/MS^2 analysis of CHOS.

1.1.6 Applications

Chitin and chitosan have several features that make them attractive for a variety of applications. They are of natural source, biodegradable and its unique cationic nature makes chitosan attractive in numerous of applications in agriculture (seed coating, fungus control agents), cosmetics (moisturizer in personal-care products), the food industry (packaging films, dietary fibre, fish feed additive), and water treatment (e.g. flocculation and metal contamination) (Muzzarelli and Tubertini, 1969; Roberts, 1992; Strand *et al.*, 2001; Tharanathan and Kittur, 2003; Kurita, 2006; Prashanth and Tharanathan, 2007; Karlsen *et al.*, 2010). Perhaps the most promising is the use of chitin and chitosan in biomedical, biotechnological and pharmaceutical applications. Chitosan has attractive safety profile, is well tolerated by human body, plants and animal cells, and show low toxicity. Here they can be used as for instance antimicrobial and antifungal agents (reviewed in Synowiecki and Al-Khateeb, 2003; Tharanathan and Kittur, 2003; Kurita, 2006; Xia *et al.*, 2011), as wound dressings (Berscht *et al.*, 1995; Degim *et al.*, 2002), gene and drug delivery vehicles (Schipper *et al.*, 1996; Köping-Höggård *et al.*, 2001) and chitosan gels (Kumar *et al.*, 2004).

There are, however, certain drawbacks related to the use of chitin and chitosan; their poor solubility at physiological pH, in addition to the high viscosity of high molecular weight chitosan solutions. To overcome these drawbacks, low molecular weight chitosan and CHOS are preferred in several applications as for instance as vectors in gene delivery (Köping-Höggård *et al.*, 2003; Köping-Höggård *et al.*, 2004), tumor

growth inhibition (Nam *et al.*, 2007; Shen *et al.*, 2009), as a drug against asthma (Donnelly and Barnes, 2004; Zhu *et al.*, 2004; Elias *et al.*, 2005; Kawada *et al.*, 2007), antibacterial agents (Rhoades *et al.*, 2006), antifungal effects (Oliveira *et al.*, 2008; Seyfarth *et al.*, 2008), as inhibitors of GH family 18 chitinases (Cederkvist *et al.*, 2008) and as inhibitors for chitinases in *Plasmodium* parasites, thereby preventing malaria (Shahabuddin *et al.*, 1993; Langer and Vinetz, 2001; Tsai *et al.*, 2001). In addition, chitooligosaccharides have been shown to play a role in plant defense against fungal pathogens. Chitinolytic enzymes from the plants are able to degrade the fungal cell wall, producing chitooligosaccharides that further act as elicitors, inducing defense responses in the plant tissues/cells such as the oxidative burst, structural changes in the cell wall (strengthening), extracellular pH increase and accumulation of pathogenesis-related (PR) proteins (e.g. chitinases) (Vander *et al.*, 1998; Rabea *et al.*, 2003; Silipo *et al.*, 2010).

Most of the research on bioactivities of CHOS has been performed with poorly defined mixtures of CHOS (varying in F_A, M_W, PD and P_A). In addition, the CHOS fractions referred to in the literature are produced from chitosans which have been characterized to only a limited extent. When using complex and uncharacterized mixtures of CHOS in bioassays, it is difficult to know which molecule/molecules are inducing the effects. The bioactivities that have been observed in several applications can therefore not be assigned to a particular CHOS. This is further discussed in paper I.

As described in the previous section, information about DP, F_A, and for some CHOS, even information about the P_A (sequence of **A**- and **D**-units) has been obtained. By using this kind of well-characterized CHOS-mixtures it can become possible to obtain a better understanding of the mechanisms underlying the bioactivities of the CHOS. As described in section 1.1.4, product mixtures can be enriched for certain CHOS by optimizing the chitosan-enzyme combination. In addition to the use of well-characterized chitosans, it is necessary to have a tool box of enzymes with various preferences for conversion of chitin and chitosan. The enzymes used in this project, chitinases and chitosanases, belong to the glycoside hydrolases, and they are further described in the following sections.

1.2. Glycoside hydrolases

The glycoside hydrolases (EC 3.2.1.x) are a group of enzymes (also called glycosidases) that hydrolyse the *O*-glycosidic linkage between two sugars.

1.2.1 Classification of glycoside hydrolases

Enzymes can be classified according to their substrate specificities, as recommended by the International Union of Biochemistry and Molecular Biology (IUBMB). IUBMB has provided the glycoside hydrolases an Enzyme Commission number, EC 3.2.1.x, where x represents the substrate specificity of the enzyme. However, there are some drawbacks to the use of this type of classification; enzymes that can act on several different substrates will not be appropriately accommodated, as is the case for the glycoside hydrolases which show broad, overlapping specificities. An additional drawback is the fact that this classification does not reflect the three-dimensional structure of the enzyme, and thus classifies structurally unrelated enzymes together, since they can show the same substrate specificity (Henrissat and Davies, 1997).

In 1991, Henrissat proposed a classification of the glycoside hydrolases based on the similarity in amino acid sequence. Since sequence and structure are related, helpful information about structure, and hence catalytic mechanism, can be obtained, based on the amino acid sequence. The Carbohydrate-Active Enzymes database (CAZy; www.cazy.org) provides a continuously updated list of the glycoside hydrolase (GH) families, and in resent years the number of GH-families has increased (Cantarel *et al.*, 2009). By October 2011, 128 families have been described. In addition, other families of carbohydrate-active enzymes such as glycosyltransferases, carbohydrate esterases, polysaccharide lyases and carbohydrate-binding modules (CBMs) are included in the database (Davies and Henrissat, 2002).

1.2.2 Mechanism of hydrolysis

The hydrolysis of the glycosidic linkage consists of a nucleophilic substitution at the anomeric carbon, and can lead to either retention or inversion of the anomeric configuration (Koshland and Stein, 1954; Sinnott, 1990). Both hydrolysis reactions take place through general acid catalysis, and thus they require a pair of carboxylic acids at the active site. One carboxylic acid is acting as a proton donor and the other carboxylic acid is acting as a proton acceptor (inverting mechanism) or as a nucleophile (retaining mechanism). In both mechanisms, the position of the proton donor is within hydrogen-bonding distance to the glycosidic oxygen.

The inverting mechanism (also called the single displacement mechanism) (Figure 1.4, panel (a)) is a "one-step" reaction, were the protonation of the glycosidic oxygen occurs simultaneously with a nucleophilic attack on the anomeric carbon by an activated water molecule. This water molecule is located between a carboxylic group and the anomeric carbon, and is activated by the carboxylic group that acts as a proton acceptor. The reaction proceeds through an oxocarbenium ion transition state. The distance between the two catalytic carboxylic residues is approximately 10 Å (McCarter and Withers, 1994). Because of this "long" distance, the water molecule can approach the anomeric carbon from this side, leading to an inversion of the anomeric configuration. Chitosanases belonging to glycoside hydrolase families 8, 46, 75 and 80 and family 19 chitinases use this inverting mechanism, which leads to α-anomeric hydrolysis products (Davies and Henrissat, 1995; Fukamizo *et al.*, 1995; Iseli *et al.*, 1996; Brameld and Goddard, 1998a; Adachi *et al.*, 2004; Cheng *et al.*, 2006; Cantarel *et al.*, 2009).

The retaining mechanism (also referred to as the double displacement mechanism) (Figure 1.4, panel (b)) is a two-step reaction, were the first step involves the protonation of the glycosidic oxygen (by the catalytic acid) and a congruent nucleophilic attack on the anomeric carbon atom by the nucleophile (the second carboxylic acid). This attack leads to breakage of the glycosidic linkage and the formation of a covalent linkage between the anomeric carbon and the catalytic nucleophile (Vocadlo *et al.*, 2001). Further, this intermediate is hydrolyzed by a water molecule that approaches the

anomeric carbon from a position close to that of the original glycosidic oxygen. This leads to retention of the anomeric carbon configuration. The distance between the two catalytic residues in the retaining mechanism is "short", only 5.5 Å (Davies and Henrissat, 1995). Both family 18 chitinases and chitosanases belonging to glycoside hydrolase family 5 and 7 use this retaining mechanism (Cantarel *et al.*, 2009).

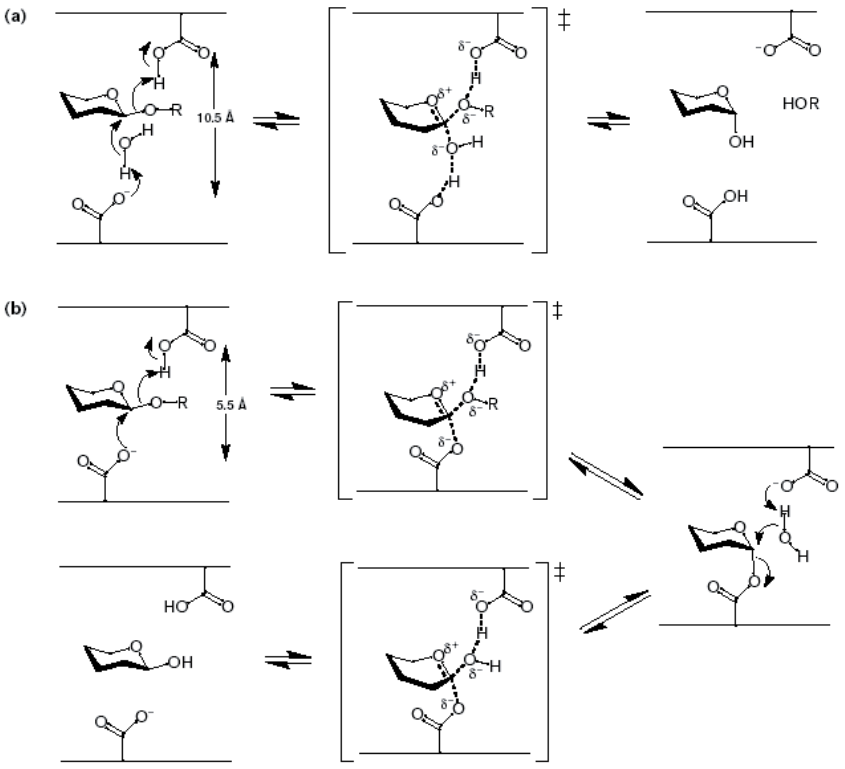

Figure 1.4. Mechanisms for glycoside hydrolysis. Panel (a) shows the inverting mechanism. Panel (b) shows the retaining mechanism (Rye and Withers, 2000). See text for further details.

Family 18 chitinases use a special variant of the double displacement mechanism, often referred to as the substrate-assisted double displacement mechanism (Figure 1.5). In this mechanism, the carbonyl oxygen atom from the *N*-acetylgroup of the sugar bound in subsite -1 acts as the nucleophile, leading to formation of an oxazolinium ion

intermediate (Terwisscha van Scheltinga *et al.*, 1995; Tews *et al.*, 1997; Brameld and Goddard, 1998b; van Aalten *et al.*, 2001). Therefore, productive substrate binding of chitosan and chitosan oligomers to family 18 chitinases requires that an acetylated sugar unit is bound in -1 subsite (Sørbotten *et al.*, 2005). On the other hand, CHOS that can bind with a **D**-unit in subsite -1 will act as an inhibitor of family 18 chitinases (Cederkvist *et al.*, 2008).

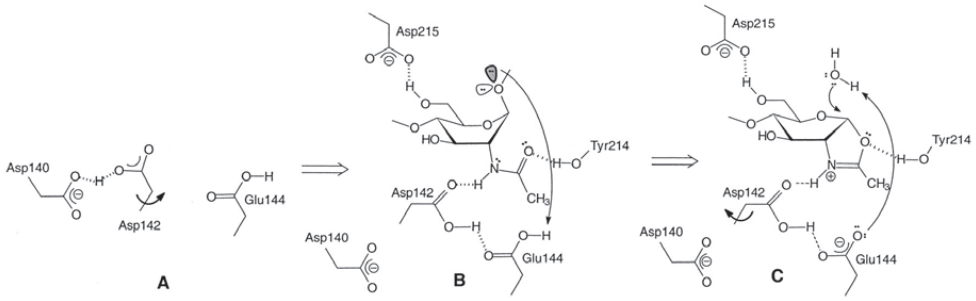

Figure 1.5. The substrate-assisted double displacement mechanism. Binding of an acetylated unit in subsite -1, causes distortion of the sugar ring into a boat conformation (in B). The oxazolinium ion intermediate (in C) is attacked by a water molecule, with an overall retention of the β-anomeric stereochemistry. Several amino acids function together in the substrate assisted catalysis, but the mechanism requires only one catalytic carboxylic group (Glu144), which acts as both the catalytic acid and base during the catalysis. The picture is taken from van Aalten *et al*., 2001.

Several family 18 chitinases and also hen egg white lysozyme catalyze transglycosylation reactions in addition to hydrolysis (Sharon and Seifter, 1964; Kravchenko, 1967; Chipman *et al.*, 1968; Aronson *et al.*, 2006; Lü *et al.*, 2009; Zakariassen *et al.*, 2009; Zakariassen *et al.*, 2011). Transglycosylation occurs if the oxazolinium ion intermediate bound to the retaining glycosyl hydrolases reacts with a hydroxyl group from an incoming carbohydrate instead of reacting with a water molecule (Fukamizo, 2000). However, reports on transglycosylation for family 19 chitinases are not found in the literature. As family 19 chitinases use the inverting mechanism, it is suggested that the more separated location of the two catalytical

residues can enable the water molecule to be located in the active site simultaneously with the oligomer substrate (Dennhart *et al.*, 2008).

1.2.3 Mode of action, processivity and substrate binding

Glycoside hydrolases can be classified according to different mode of action, as endo- or exo-enzymes. Endo-enzymes attack from a random point along the polymer chain, while exo-enzymes attack one of the chain ends of the polymer. Both the endo- and exo-mode of action can occur in combination with processivity (also known as multiple-attack). Processive enzymes will not release the substrate after a cleavage, but remain associated so several cleavages can take place as the substrate slides through the enzyme's active site (Robyt and French, 1967; Robyt and French, 1970). Processivity cannot be easily measured when degrading insoluble substrate such as e.g. chitin, but can be easily studied when degrading a soluble substrate such as chitosan (Sørbotten *et al.*, 2005; Horn *et al.*, 2006a; Horn *et al.*, 2006b; Eijsink *et al.*, 2008). This is further described in section 1.2.5.1, where a detailed discussion for the GH family 18 chitinases is included.

Non-processive endo-enzymes have an open substrate binding cleft which allows random binding to the substrate, and cleavage within the polymer chain followed by release of the substrate. The active site of exo-enzymes are rather pocket shaped, or tunnel-like when the enzyme hydrolyzes the substrate according to an exo- and/or a processive mode of action (Davies and Henrissat, 1995; Henrissat and Davies, 1997).

The glycoside hydrolases include several residues involved in binding of the substrate in addition to the catalytic residues which execute the catalytic cleavage. When binding a monomer (from a longer polymer chain) in a sugar binding subsite, a cluster of amino acids will be involved. The subsites in a glycoside hydrolase are per definition labelled –n to +n, where the –n subsites represents the binding of the non-reducing end of the sugar chain and +n subsites bind the reducing end of the sugar chain. Cleavage of the polymer chain will occur between sugars bound in subsites -1 and +1 (Davies *et al.*, 1997). The sugar productively bound in subsite -1 will, after hydrolysis, become the

new reducing end of one of the hydrolytic products, while the sugar bound in subsite +1 will be the new non-reducing end.

1.2.4 Occurrence of chitinolytic enzymes in nature

Since chitin is hard to degrade due to its physiochemical properties, some organisms contain a large repertoire of chitinolytic enzymes for more effective degradation. The chitinolytic machinery of the actinomycete *Streptomyces* is one example. *Streptomyces* are saprophytic soil bacteria that are considered to be the major producers of chitinases in soil (Saito *et al.*, 1999), producing several chitin-degrading enzymes to exploit chitin as a source of nutrients. Three genomes from the genus have been published; *S. coelicolor* A3(2) (Bentley et al., 2002), *S. avermitilis* (Ikeda et al., 2003) and *S. griseus* (Ohnishi et al., 2008). In the genome of *S. coelicolor* A3(2), 13 chitinase genes were found, encoding 11 family 18 chitinases and 2 family 19 chitinases (Saito *et al.*, 1999; Saito *et al.*, 2000; Kawase *et al.*, 2004). In addition, two genes putatively encoding GH family 46 chitosanases and one gene putatively encoding a GH family 75 chitosanase were found. The genome of *S. coelicolor* A3(2) also contains five genes putatively encoding GH5, but genes encoding enzymes belonging to GH family 7, 8 or 80 could not be found (www.cazy.org). The genome of *S. avermitilis* encodes several genes putatively encoding chitinases/chitosanases; 8 genes putatively encoding GH family 18 chitinases, 2 genes putatively encoding GH family 46 chitosanases and 2 genes putatively encoding GH family 75 chitosanases and 2 genes encoding enzymes in GH family 5. Genes encoding enzymes belonging to GH family 7, 8, 19 or 80 were not found in the genome of *S. avermitilis* (www.cazy.org).

Another well-characterized chitin-degrading microorganism in soil is the Gram-negative soil bacterium *Serratia marcescens*. *S. marcescens* produces three family 18 chitinases (ChiA, ChiB and ChiC), a chitobiase and a chitin binding protein (CBM family 33) which act synergistically in chitin degradation (Fuchs et al., 1986; Tews et al., 1996; Suzuki et al., 1998; Suzuki et al., 2002; Vaaje-Kolstad et al., 2005).

1.2.5 Chitinases

Chitinases (EC 3.2.1.14) catalyze the hydrolysis of the β-(1→4)-glycosidic linkages between the sugar units in chitin. In addition, they are capable of hydrolyzing chitosan, albeit to different extents depending on the degree of acetylation of the chitosan. Chitinases hydrolyze the **A**-**A** linkage, but not the **D**-**D** linkage, acting opposite as the chitosanases. Chitinases are found in GH family 18 and 19, and both families almost exclusively contains chitinases (Cantarel et al., 2009).

The chitinolytic enzymes have several different functions: (i) in chitin-producing organisms, they are essential for growth, (ii) bacteria produce these enzymes to digest chitin as a source of nitrogen and energy and (iii) plants use these enzymes as defense against plant pathogens (Kasprzewska, 2003; Merzendorfer and Zimoch, 2003).

1.2.5.1 GH family 18 chitinases

Family 18 chitinases are found in many organisms, including bacteria, fungi, insects, plants, mammals, archaea and viruses, and the family contains over 4000 entries in the CAZY database by October, 2011 (www.cazy.org). Many structures are known within the family 18 chitinases, for instance ChiA and ChiB from *S. marcescens* (Perrakis *et al.*, 1994; van Aalten *et al.*, 2000), hevamine from *Hevea brasiliensis* (rubber three) (Terwisscha van Scheltinga *et al.*, 1994) and the structure of the human chitotriosidase (HCHT) (Fusetti *et al.*, 2002).

The three-dimensional structures of the family 18 chitinases show that the common fold for the catalytic domain is an $(\alpha/\beta)_8$-barrel (also called TIM-barrel), composed of eight α-helices and a eight stranded β-sheet (Perrakis *et al.*, 1994; Terwisscha van Scheltinga *et al.*, 1994; van Aalten *et al.*, 2000; Fusetti *et al.*, 2002). This domain includes a highly conserved sequence motif, DXXDXDXE, where the last glutamate residue acts as the catalytic proton donor in the substrate-assisted mechanism family 18 chitinases use for hydrolysis (McCarter and Withers, 1994; Perrakis *et al.*, 1994; Terwisscha van Scheltinga *et al.*, 1994; Tews *et al.*, 1997; Brameld and Goddard, 1998a; van Aalten *et al.*, 2000). An insertion of a (α+β)-domain in the $(\alpha/\beta)_8$-barrel is found in some family

18 chitinases. This insertion is thought to form a "wall", leading to a deep catalytical cleft. In both ChiA and ChiB, this catalytical cleft contains six subsites, from -4 to +2 for ChiA and from -3 to +3 for ChiB (Perrakis *et al.*, 1994; van Aalten *et al.*, 2000), although substrate positioning studies by Norberg *et al*. suggests the possibility of an extra "+3" subsite in ChiA (Norberg *et al.*, 2011).

Apart from the catalytic domain, some of the family 18 chitinases contain an additional substrate binding domain which is presumed to promote activity on insoluble substrate (Horn *et al.*, 2006b). For example, ChiA from *S. marcescens* has a N-terminal chitin binding domain (fibronectin III-like) extending the substrate binding cleft at the non-reducing side (Figure 1.6). This suggests that ChiA degrades the substrate from the reducing end towards the non-reducing end (van Aalten *et al.*, 2000). In contrast, ChiB from *S. marcescens* has a C-terminal chitin binding domain (CBM5) on the reducing side of the active site (Figure 1.6), indicating that the substrate are degraded from the non-reducing side towards the reducing end of the substrate (van Aalten *et al.*, 2000; Hult *et al.*, 2005). This predicted opposite directionality according to the location of the chitin binding domain of ChiA and ChiB has been confirmed by degradation of end-labelled β-chitin using microscopy (Hult *et al.*, 2005). Additionally, a mutagenesis study by Zakariassen and colleagues also suggests opposite directionality (Zakariassen *et al.*, 2009). Paper II included in this thesis describe a method whereby the directionality of ChiA and ChiB when degrading a chitosan oligomer could be determined.

The third family 18 chitinase from *S. marcescens*, ChiC, is quite different from ChiA and ChiB. The crystal structure of ChiC is not known, but sequence alignments indicate that the catalytical domain resembles hevamine from *H. brasiliensis*. Both these enzymes lack the (α+β)-domain that form a "wall" in the substrate binding cleft of ChiA and ChiB, and as a consequence, ChiC and hevamine have a much more open substrate binding cleft (Terwisscha van Scheltinga *et al.*, 1994; Horn *et al.*, 2006c).

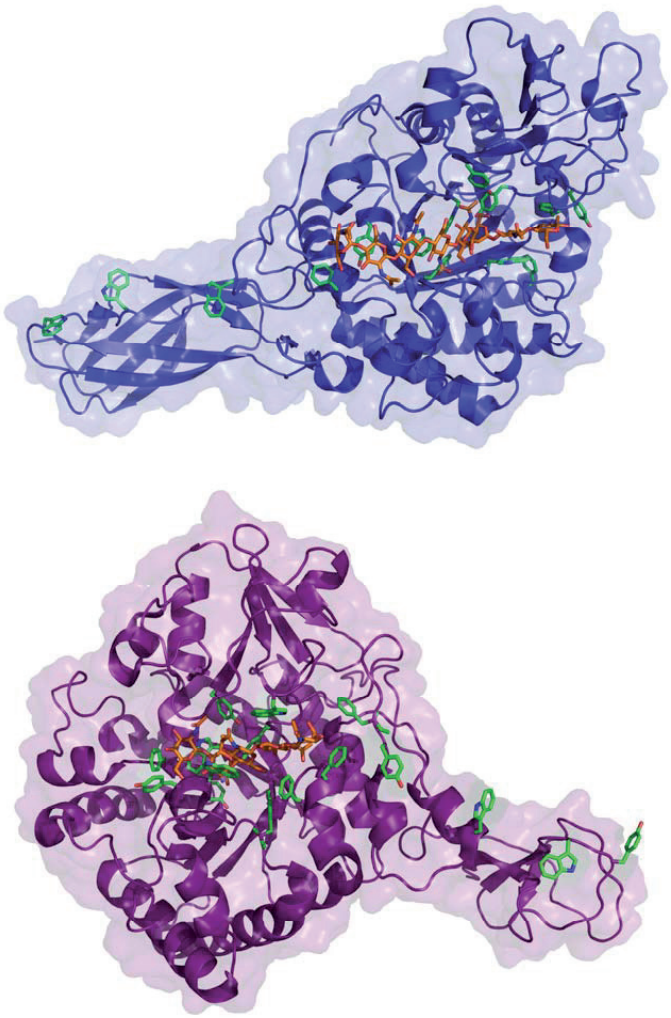

Figure 1.6. **Structures of ChiA and ChiB from *S. marcescens*.** ChiA (PDB: 1ehn, upper panel (Papanikolau *et al.*, 2001)) is shown in complex with a fully acetylated octamer, while ChiB (PDB: 1e6n, lower panel (van Aalten *et al.*, 2001)) is shown in complex with a fully acetylated pentamer. Aromatic side chains that line the substrate binding cleft of the catalytical domain and the chitin binding domain are drawn as sticks and highlighted in green. The picture was made with PyMOL (Schrödinger, 2010).

Several in-depth studies on the family 18 chitinases, ChiA, ChiB and ChiC, degrading well-characterized chitosans with different degree of acetylation have been performed (Sørbotten *et al.*, 2005; Sikorski *et al.*, 2006; Horn *et al.*, 2006a; Horn *et al.*, 2006b). When ChiA and ChiB degrade a highly acetylated chitosan ($F_A = 0.65$), a dominance of even-numbered CHOS could be observed in the initial degradation phase (Figure 1.7), which indicates a processive degradation pattern. When degrading chitin, the products of processive degradation are dimers (except for the first cleavage product, which may have any length), since productive binding to an acetylated unit only can occur for every second sugar due to 180° rotation of the sugars in the chain. When a processive chitinase degrades chitosan, there is a possibility of nonproductive binding of a **D**-unit in subsite -1, meaning that the enzyme will slide further, two sugar units at the time, until an **A**-unit is bound in subsite -1 (Horn *et al.*, 2006a). This may result in longer even-numbered oligomers, in contrast to the dimers produced when degrading chitin. At higher degree of scission (Figure 1.7), the dominance of even-numbered CHOS disappears, as the CHOS produced during the earlier degradation phases are rehydrolyzed.

The degree of processivity can be measured as described in Sikorski *et al*. (2006). Using a reducing end assay (Horn and Eijsink, 2004), the total fraction of chain ends produced in the enzymatic degradation (α_{total}) are observed. Simultaneously, the decrease in relative viscosity during the enzymatic degradation of the polymer solution is used to measure $\alpha_{polymer}$. A plot of the degree of scission obtained from the viscosity assay ($\alpha_{polymer}$) against the degree of scission from the reducing end assay (α_{total}) gives a linear relationship were the slope are directly related to the degree of processivity per formation of an enzyme-substrate complex (Sikorski *et al.*, 2006).

Compared to ChiA and ChiB, ChiC converts the chitosan into a continuum of CHOS, with both even- and odd-numbered CHOS produced. In addition, the polymer peak disappears early in the degradation reaction (Figure 1.7). These results taken together indicates that ChiC is an endo non-processive chitinase (Sikorski *et al.*, 2006; Horn *et al.*, 2006a). ChiA and ChiB were earlier proposed to be exo-enzymes, as they were reported to degrade β-chitin microfibrils from the chain ends (Uchiyama *et al.*, 2001;

Hult *et al.*, 2005). However, a study by Sikorski and colleagues in 2006 showed that they, together with ChiC, significantly reduced the number average degree of polymerization (DP_n) in the initial stage of the degradation of the chitosan, meaning that they are endo-acting enzymes (Sikorski *et al.*, 2006). One explanation for these discrepancies could be that ChiA and ChiB acts differently when degrading insoluble chitin substrate (acting exo-) versus soluble chitosan substrate (acting endo-). The crystallinity of the chitin, and the fact that the chitin chain ends might be more accessible as a substrate for the enzyme, could also have significance. This is further discussed in Sikorski *et al*. (2006). For ChiA and ChiB, the slow disappearance of the polymer peak can be explained from the processivity, performing many cuts per chitosan chain. In contrast, ChiC acts non-processive, hydrolyzing several chitosan chains with few cuts, which cause a rapid disappearance of the polymer peak.

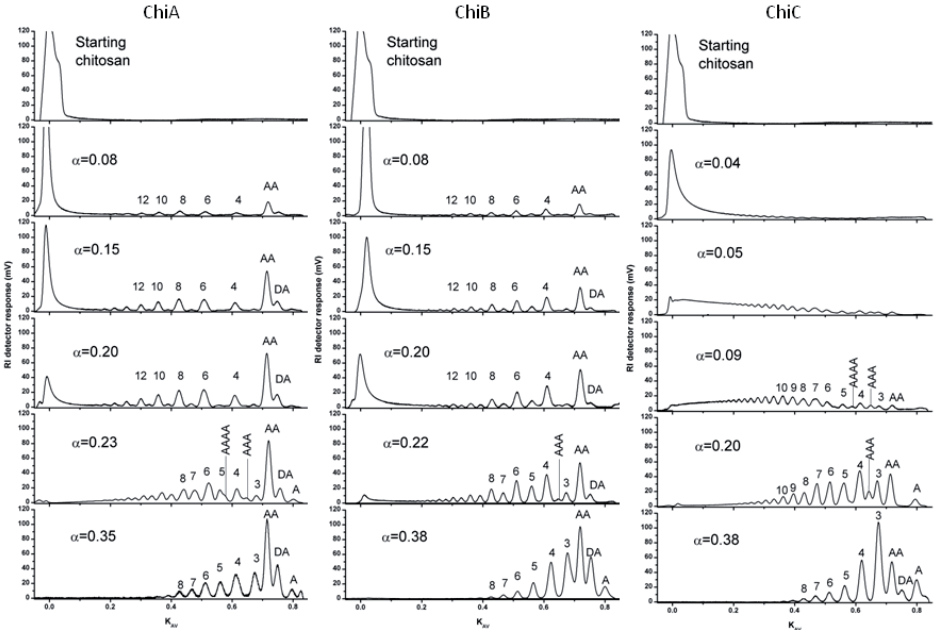

Figure 1.7. Size-distribution of CHOS obtained after degradation of a highly acetylated chitosan (F_A = 0.65) by ChiA, ChiB and ChiC from *S. marcescens*. The peaks are labeled with numbers, which indicates the DP of the oligomer, or with the sequence of the oligomer. Undegraded chitosan and chitosan fragments with DP > 40 elutes in the void volume. The lower panels show the product spectrum obtained after maximum degree of scission (α-value). The figure is from Horn *et al*., 2006a.

The presence of aromatic residues in both the catalytic cleft and the substrate binding cleft of ChiA and ChiB have been thought to be important in processive attack since the aromatic residues provide "fluid" binding that is important to enable the polymer chain to slide through the substrate binding cleft during the processive mode of action (Divne *et al.*, 1998; Varrot *et al.*, 2003; Horn *et al.*, 2006b; Zakariassen *et al.*, 2009). Site directed mutagenesis of residue Trp97 in the +1 subsite of ChiB gave a mutant which showed loss of processivity and reduced efficiency in degrading crystalline chitin. However, the degradation rates for soluble and more accessible substrates, such as the fully acetylated hexamer and chitosan were increased for the non-processive W97A mutant (Figure 1.8) (Horn *et al.*, 2006b). Thus, it seems like processivity reduces enzyme efficiency for certain substrates, and the results from Horn *et al*. (2006b) demonstrate that the beneficial effect of processivity on substrate accessibility comes at a cost of enzyme speed when using soluble, and therefore more accessible, substrates, such as chitosan. This knowledge can be important in the development of enzyme technology for efficient degradation of lignocellulose biomass for novel production of bioethanol (Horn *et al.*, 2006b; Eijsink *et al.*, 2008). In a similar site directed mutagenesis experiment, Zakariassen and colleagues showed that the aromatic residues in ChiA were important for its processivity and that the processive mechanism was essential for an efficient degradation of crystalline chitin (Zakariassen *et al.*, 2009).

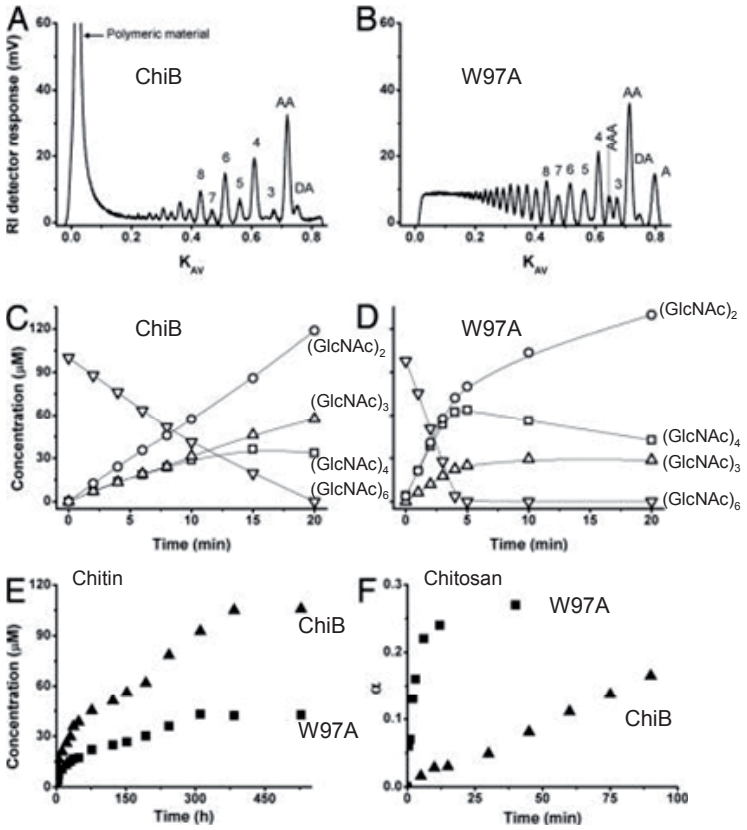

Figure 1.8. Comparison of degradation of substrates by the use of wild-type ChiB and the W97A mutant. The upper panel show the size-distribution of CHOS produced after degradation of a chitosan (F_A = 0.65) to a degree of scission (α-value) of 0.14 with ChiB (A) and W97A (B). The mid panel show the degradation of the fully acetylated hexamer for ChiB (C) and W97A (D). The various oligomers are indicated by ▽ $(GlcNAc)_6$; □ $(GlcNAc)_4$; △ $(GlcNAc)_3$; ○ $(GlcNAc)_2$. In the lower panel, (E) shows the degradation of chitin with ChiB (▲) and W97A (■). In (F), the time course of the degradation of a chitosan (F_A = 0.65) with ChiB (▲) and W97A (■) are shown with the degree of scission (α; as determined from ^{1}H-NMR spectra), as a function of the degradation time. The figure is taken from Horn *et al.*, (2006b).

According to the substrate-assisted mechanism, family 18 chitinases have an absolute requirement for binding acetylated units in subsite -1 (sugar unit that will give the identity of the new reducing end after cleavage). Sequence analysis of the CHOS produced after initial and extensive degradation of a chitosan (F_A = 0.65) showed that every oligomer had acetylated reducing ends, as expected (Table 1.1). In subsite -2, all three enzymes had a strong, although not absolute, preference for **A**-units, producing CHOS with –AA at their reducing end. For ChiC, this preference was almost absolute, only producing a small amount of the dimer DA in the end of the degradation (α = 0.38, Table 1.1). The non-reducing end could be both acetylated and deacetylated, indicating that ChiA, ChiB and ChiC did not show any preferences for **A**- or **D**-units in subsite +1 (Horn *et al.*, 2006a).

Table 1.1. Composition and (partial) sequence of dimer, trimer and tetramer fractions at different degree of scission during degradation of a highly acetylated chitosan (F_A = 0.65) by ChiA, ChiB and ChiC. The table is from Horn *et al.* (2006a).

Enzyme	α	Dimer	Trimer	Tetramer
ChiA	0.15	81 % AA 19 % DA	81 % DAA 19 % ADA	100% -AA
	0.35	64 % AA 36 % DA	51 % DAA 28 % ADA 21 % DDA	56 % -AA 44 % -DA
ChiB	0.11	86 % AA 14 % DA	71 % DDA 29 % AAA	100 % -AA
	0.38	66 % AA 34 % DA	95 % DAA 3 % DDA 2 % ADA	75 % -AA 25 % -DA
ChiC	0.20	100 % AA	66 % DAA 34 % AAA	100 % -AA
	0.38	81 % AA 19 % DA	100 % DAA	100 % -AA

When ChiB degrades chitosans with varying degree of acetylation, a size-distribution of higher CHOS lengths for the substrates with lower F_A have been observed (Figure 1.9). Since ChiB has preferences for acetylated units binding in the active site, the longer CHOS produced after degradation of the chitosans with low F_A consists of longer, uncleavable stretches of consecutive D-units (Sørbotten *et al.*, 2005). Interestingly, enzyme-substrate interactions in ChiB involve several aromatic residues that stack with the hydrophobic faces of the sugar (van Aalten *et al.*, 2001). Such contacts are not dependent on the acetyl group, and therefore ChiB can degrade highly deacetylated chitosans.

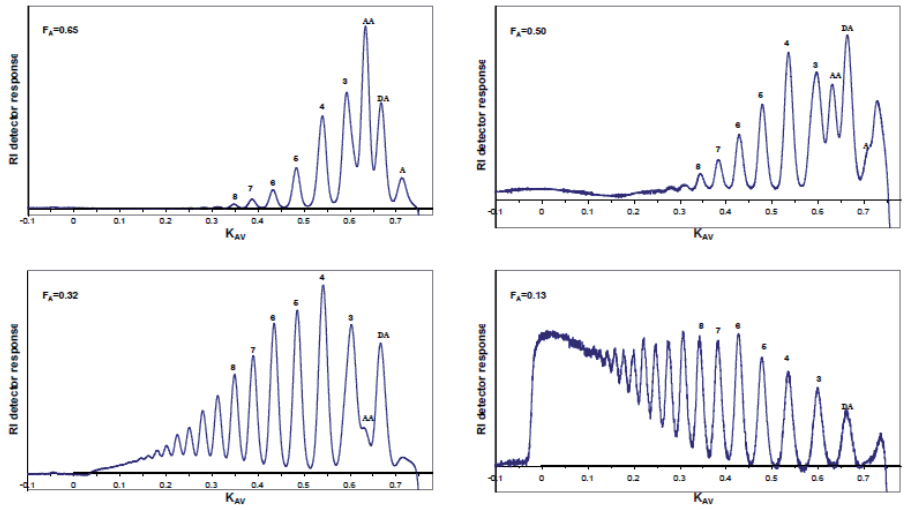

Figure 1.9. Size-distribution of CHOS obtained after extensive degradation of chitosans with varying degree of acetylation by ChiB. Chitosans with F_A of 0.65, 0.50, 0.32 and 0.13 were degraded to maximum α-values of 0.37, 0.34, 0.22 and 0.11, respectively. Peaks are labeled with DP-values or with the oligomer sequence. The figure is taken from Sørbotten *et al*. (2005).

These in-depth studies on degradation of chitosans with ChiA, ChiB and ChiC show the importance in varying the enzyme concentration, the degree of acetylation of the chitosan and the extent of cleavage, for production of different mixtures of CHOS (Sørbotten *et al.*, 2005; Horn *et al.*, 2006a).

Although humans do not contain chitin, two active family 18 chitinases are produced in the human body; human chitotriosidase (HCHT) and acidic mammalian chitinase (AMCase) (Bussink *et al.*, 2007). In addition, two other family 18 chitinase-like proteins called chi-lectins have been reported. The chi-lectins have, however, been found to lack enzymatic activity (Renkema *et al.*, 1998; Sun *et al.*, 2001). The structures of HCHT and AMCase have been reported (Fusetti *et al.*, 2002; Olland *et al.*, 2009). Both chitinases are produced as two-domain proteins, consisting of a catalytical domain and a C-terminal chitin binding domain. The active sites are formed as an open cleft covered by aromatic residues. It has been shown that an isoform of HCHT, lacking the chitin binding domain, is sufficient for chitinolytic activity (Bussink *et al.*, 2006). HCHT has been discovered as a marker for e.g. Gaucher disease and has been found in human macrophages (Hollak *et al.*, 1994). Although the physiological function is unknown, a role in the innate immune system as a defense against pathogens containing chitin has been suggested (Hollak *et al.*, 1994; van Eijk *et al.*, 2005). Gorzelanny and colleagues report on the increased stimulation of macrophages by oligomers produced when HCHT degrades chitosan substrates (Gorzelanny *et al.*, 2010). In asthmatic patients, AMCase expression is increased and therefore, inhibition of the acidic chitinase has been suggested as a therapeutic strategy to prevent asthma (Donnelly and Barnes, 2004; Zhu *et al.*, 2004; Elias *et al.*, 2005). However, inhibition of HCHT is believed to be unfavorable due to its fungistatic effects and thus, potential inhibitors of HCHT should be avoided when inhibitors of AMCase are developed. A study of the degradation of chitosans by HCHT is included in this thesis (Paper III).

1.2.5.2 GH family 19 chitinases

Family 19 chitinases are commonly found in plants, bacteria and viruses. For many years, it was generally believed that the family 19 chitinases occurred in only plants, but in 1996, the first bacterial family 19 chitinase, chitinase C (ChiC), was found in *Streptomyces griseus* HUT6037 (Ohno *et al.*, 1996). Subsequently, several bacterial family 19 chitinases have been identified as bacterial genomes have been sequenced.

There are six crystal structures of plant family 19 chitinases reported to date; a endo-chitinase from barley (Hart *et al.*, 1993; Song and Suh, 1996), a chitinase from jack bean (Hahn *et al.*, 2000), a chitinase from rice (Kezuka *et al.*, 2010), a chitinase from mustard greens (Ubhayasekera *et al.*, 2007), a chitinase from papaya (Huet *et al.*, 2008) and a chitinase from Norway spruce (Ubhayasekera *et al.*, 2009). In 2006, the structures of two bacterial family 19 chitinases was solved; One from *S. griseus* HUT6037 (ChiC) (Kezuka *et al.*, 2006) and the other from *S. coelicolor* A3(2) (ChiG) (Hoell *et al.*, 2006).

The three-dimensional structures of the family 19 chitinases are composed of two lobes with high α-helical content. The substrate binding cleft is estimated to lie between the two lobes as a deep cleft, which is composed of two α-helices and a three-stranded β-sheet (Hart *et al.*, 1995; Monzingo *et al.*, 1996; Fukamizo, 2000). This structure is also common for GH family 46 chitosanases (see section 1.2.6.1), and for lysozymes in GH family 22, 23 and 24, although they do not exhibit a significant amino acid sequence similarity (Holm and Sander, 1994; Monzingo *et al.*, 1996; Fukamizo, 2000; Lacombe-Harvey *et al.*, 2009).

The family 19 chitinases use an inverting mechanism when degrading a substrate, producing α-anomeric hydrolysis products (Davies and Henrissat, 1995; Iseli *et al.*, 1996). The two putative amino acid residues involved in the catalysis are structurally well conserved between family 19 chitinases from bacteria and plants (Hoell *et al.*, 2006), and site-directed mutagenesis experiments have confirmed their importance in catalysis (Andersen *et al.*, 1997; Hoell *et al.*, 2006). Interestingly, it is found that family 19 chitinases lack aromatic residues in the catalytic cleft, compared to family 18 chitinases and cellulases (Ubhayasekera, 2011). As described in the previous section, the enzyme-substrate interactions for ChiB involve several aromatic residues that stack with the hydrophobic faces of the sugars (van Aalten *et al.*, 2001). Such contacts, which do not necessarily depend on the presence of a *N*-acetyl group, are absent in the interaction between family 19 chitinases and their substrates, since substrate binding in the catalytic clefts is dominated by specific hydrogen bonds that often involve the *N*-acetyl group (Huet *et al.*, 2008).

Comparison of the structure of plant and bacterial family 19 chitinases reveal that the bacterial enzymes lack three loops and a C-terminal extension (Figure 1.10) (Hoell *et al.*, 2006; Kezuka *et al.*, 2006). In addition, a region referred to as the 161-166 loop (Figure 1.10) has a different conformation than in plant chitinases. It has been suggested that the lack of these loops in bacterial enzymes reduces the length of the substrate binding cleft to four sugar binding subsites (-2, -1, +1, +2), compared to six for plant family 19 chitinases (Hoell *et al.*, 2006). Loop B (also referred to as loop II in the literature) is proposed to be located at the reducing end site (+3/+4) of the putative substrate binding cleft, and it is assumed that its residue Trp72 participates in substrate binding (Mizuno *et al.*, 2008; Fukamizo *et al.*, 2009). According to Mizuno and colleagues, the 161-166 loop contains two amino acids (Gln162 and Lys165) which are purported to have significance for sugar binding in subsites -3 and -4 (Mizuno *et al.*, 2008). Studies of the product profile after ChiG degradation of fully acetylated CHOS, support the assumption that bacterial family 19 chitinases have a truncated substrate binding cleft (Hoell *et al.*, 2006).

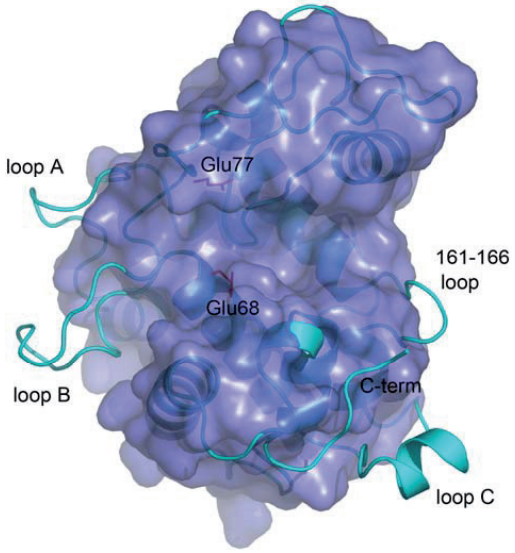

Figure 1.10. Structural superposition of a bacterial family 19 chitinase, ChiG from *S. coelicolor* A3(2), and the barley family 19 chitinase. Superposition of the barley enzyme (PDB: 2BBA; cyan) and the surface of ChiG (PDB: 2CJL; purple). Glu68 and Glu77 are the catalytic acid and the catalytic base of ChiG, respectively (shown in red). Compared to the plant enzyme, the bacterial enzyme lacks three loops (loop A, B and C) and a C-terminal extension (Hoell *et al.*, 2006).

There is lack of structural information on the enzyme-substrate complexes, and therefore little is known about how the family 19 chitinases interact with their substrates (Hoell *et al.*, 2010). Huet and colleagues recently reported on a complex with a family 19 chitinase from *Carica papaya* preferentially binding two acetylated monomers in subsites -2 and +1. In addition, they modelled a complex with $(GlcNAc)_4$ as substrate, developed using the experimental data of the **A**-units binding in subsite -2 and +1 (Huet *et al.*, 2008). This complex provides additional information on substrate binding, resulting in an increased knowledge on sugar binding in family 19 chitinases.

There are few reports on the degradation of chitosans by family 19 chitinases. Mitsutomi *et al*. (1995) reports on the extensive degradation of a chitosan with a degree of acetylation of 47% using the family 19 chitinase ChiC from *S. griseus* HUT 6037. The CHOS produced were isolated and characterized, and the chitinase was found to have an absolute specificity for **A**-units in subsites -2 and +1 (Mitsutomi *et al.*, 1995). A family 19 chitinase from rice (Sasaki *et al.*, 2006) has also been studied. It was found to operate according to a non-processive endo mode of action and with strong preferences for acetylated units in subsites -2 and +1 and with preference for acetylated units in the -1 subsite. An in-depth study of a family 19 chitinase, ChiG from *S. coelicolor* A3(2), degrading fully water-soluble chitosan substrates is presented in paper IV.

1.2.6 Chitosanases

Chitosanases (EC 3.2.1.132) are enzymes that hydrolyze the β-(1→4) linkages in chitosan, and they have been found in GH families 5, 7, 8, 46, 75 and 80. GH5 contains a variety of enzymatic activities, including chitosanases, cellulases, licheninases, mannanase and xylanases. Chitosanase activity has been detected in only a few cases, e.g. with the chitosanase II from *S. griseus* HUT6037 (Tanabe *et al.*, 2003). Some enzymes in GH5 seem to be bifunctional, acting both on chitosan and cellulose (Xia *et al.*, 2008). The same has been reported for the GH family 7; GH7 is a cellulase family and in a very few cases chitosanase activity has been detected as a side activity of these enzymes (Ike *et al.*, 2007; Xia *et al.*, 2008). In GH8, enzymes annotated as chitosanases occur more frequently (next to e.g. cellulases and xylanases), and this family seems to

contain a few true chitosanases, e.g. ChoK from *Bacillus* sp. K17 (Adachi *et al.*, 2004; Sakihama *et al.*, 2004) and a chitosanase from *Bacillus* sp. No.7M (Izume *et al.*, 1992; Vårum *et al.*, 1996), which both show subclass II specificity (see below). Another GH family 8 chitosanase studied is a chitosanase from *Bacillus circulans* WL-12 (Mitsutomi *et al.*, 1998). The other three families, GH46, GH75 and GH80, exclusively contain chitosanases. The best studied chitosanases are those belonging to family 46 (Marcotte *et al.*, 1996; Saito *et al.*, 1999; Lacombe-Harvey *et al.*, 2009), and even three-dimensional structures have been solved for members of the family (Marcotte *et al.*, 1996; Saito *et al.*, 1999). Families GH75 and GH80 have only a few members and there is no structural information and only limited functional information for these enzymes.

The different GH families have been examined regarding the molecular mechanism of hydrolysis. Family 5 and 7 enzymes are retaining (Wang *et al.*, 1993; Divne *et al.*, 1994), while enzymes from family 8, 46 and 75 use the inverting mechanism (Fukamizo *et al.*, 1995; Adachi *et al.*, 2004; Cheng *et al.*, 2006). The chitosanases from family 80 are inferred to be inverting (www.cazy.org), as suggested from resemblance to the family 46 chitosanases due to a common molecular pattern (Tremblay *et al.*, 2000).

Chitosanases have been classified into three subclasses (I-III) according to the substrate specificity towards chitosan (Fukamizo *et al.*, 1994). All three subclasses hydrolyze the linkage between two **D**-units, and chitosanases belonging to subclass I and III are also able to cleave the glycosidic linkage between **A**-**D** and **D**-**A**, respectively (Fukamizo *et al.*, 1994; Mitsutomi *et al.*, 1996). Cleavage of the linkage between two **A**-units separates chitinases from chitosanases.

Chitosanases have a metabolic function, since they hydrolyze high-molecular weight chitosans into shorter CHOS that can be transported inside the cell and used as carbon and nitrogen source. Another function of the chitosanases is the protection of microbial organisms against the antimicrobial activity of chitosan. Since neither low-molecular weight chitosan nor CHOS show inhibitory effects, chitosanases could function as protectors, hydrolyzing the high-molecular weight chitosan into these short-chain

chitosan forms (Blanchard *et al.*, 2003; Rabea *et al.*, 2003; Lacombe-Harvey *et al.*, 2009; Ghinet *et al.*, 2010).

Below, the GH family 46 and 75 chitosanases are discussed in more detail.

1.2.6.1 GH family 46 chitosanases

GH family 46 chitosanases are found in bacteria and viruses, and two crystal structures have been reported; (i) the structure of a chitosanase (CsnN174) from *Streptomyces* sp. N174 (Marcotte *et al.*, 1996) and (ii) the structure of a chitosanase (MH-K1) from *Bacillus circulans* MH-K1 (Saito *et al.*, 1999).

The CsnN174 chitosanase was reported purified in 1992, by Boucher and colleagues. Subsequently, the sequence of the gene was determined (Masson *et al.*, 1994). The structure of CsnN174 (see Figure 1.11) was found to consist of two globular domains, with a high α-helical content. In between the two lobes a large substrate binding and catalytical cleft is formed, which consists of two α-helixes and a three-stranded β-sheet (Marcotte *et al.*, 1996). This structural core is similar for the "lysozyme superfamily", i.e. for the CsnN174 chitosanase (and the rest of the members of the GH46), the family 19 chitinases (as described in section 1.2.5.2) and the lysozymes in GH family 22, 23 and 24, although they do not exhibit a significant amino acid sequence similarity (Holm and Sander, 1994; Monzingo *et al.*, 1996; Fukamizo, 2000; Lacombe-Harvey *et al.*, 2009).

The CsnN174 chitosanase was found to hydrolyze the substrates according to an endo mode of action, and in addition, degradation products were found to be in the α-anomer form, meaning that CsnN174 use the inverting mechanism (Boucher *et al.*, 1992; Fukamizo *et al.*, 1995). Fukamizo and colleagues studied the CsnN174 hydrolysis of $(GlcN)_6$, revealing a large amount of $(GlcN)_3$, and lesser amounts of $(GlcN)_2$ and $(GlcN)_4$ produced (Fukamizo *et al.*, 1995). This indicates that the chitosanase has a substrate binding cleft consisting of six subsites from -3 to +3, with cleavage occurring in the middle (Fukamizo *et al.*, 1995; Tremblay *et al.*, 2001). The N-terminal region of

CsnN174 shows significant homology with other GH46 chitosanases, and includes five invariant carboxylic residues (Masson *et al.*, 1994; Boucher *et al.*, 1995). Site-directed mutagenesis of these carboxylic residues identified Glu22 and Asp40 as the putative catalytical amino acids essential for catalytical activity (Boucher *et al.*, 1995). The catalytic residue Glu22 (the general acid) is located in close proximity to an Arg205 side chain. Arg205 is located in close distance to an Asp145, which again is close to an Arg190 residue. Site-directed mutagenesis showed that Arg205, Asp145 and Arg190 are important for catalytic activity, participating in an interaction network at the catalytic cleft (Fukamizo *et al.*, 2000). Interestingly, a similar interaction network of charged residues can be observed near the catalytic acid in family 19 chitinases (Fukamizo *et al.*, 2000; Hoell *et al.*, 2010). The catalytic residue Asp40 has been confirmed to be the general proton acceptor in the active site of CsnN174, although mutational studies supposed that residue Glu36 could be an alternative general proton acceptor (Lacombe-Harvey *et al.*, 2009). The same study also showed the importance of residue Thr45, which is thought to play a role in the positioning of the attacking water molecule in the catalysis. Studies regarding the importance of amino acid residues in the binding of CHOS to CsnN174 have been performed. Asp57 has been shown to participate in substrate binding in subsite -2, presumably through both electrostatic and hydrogen bonding interactions (Tremblay *et al.*, 2001; Katsumi *et al.*, 2005). In addition, it has been suggested that residue Glu197 interacts with the sugar binding in subsite -1 (Katsumi *et al.*, 2005).

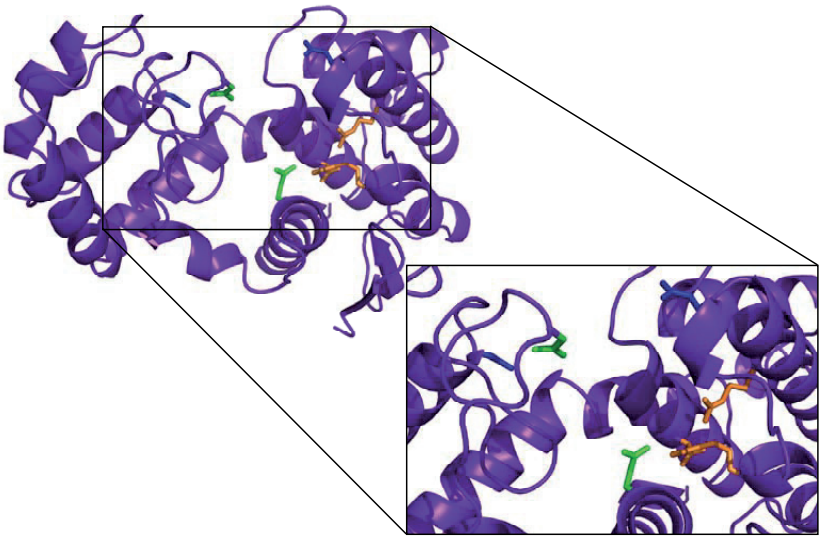

Figure 1.11. Structure of chitosanase N174 from *Streptomyces* sp. N174 with a detailed overview of important residues in the active site. The picture shows a cartoon of chitosanase N174 (PDB: 1CHK (Marcotte *et al.*, 1996)). The catalytical amino acids Glu22 and Asp40 are colored green. The residues Asp145, Arg190 and Arg205 (colored orange) are located on three different α-helixes, and participate in an interaction network with Glu22 (the catalytic acid). The residues which are colored blue, Asp57 and Glu197, are important for substrate binding in subsite -2 and -1, respectively. Both the catalytical amino acids, the residues involved in the interaction network and those involved in the sugar binding in subsite -2 and -1 are conserved in the GH family 46 chitosanase, ScCsn46A, studied in paper V. The pictures were made with PyMOL (Schrödinger, 2010).

When CsnN174 degraded a partially acetylated chitosan (F_A = 0.25-0.35), fully deacetylated CHOS together with partially deacetylated CHOS were produced (Fukamizo *et al.*, 1995). The reducing end of the partially deacetylated CHOS could be both acetylated and deacetylated, while the non-reducing end of the CHOS was always deacetylated. The results showed that the chitosanase hydrolyze the **A-D** and the **D-D** linkage, meaning that it belongs to subclass I.

Degradation experiments with the family 46 chitosanase (MH-K1) from *Bacillus circulans* MH-K1 show a different selectivity when hydrolyzing a F_A = 0.54 chitosan.

This chitosanase hydrolyze the linkage between **D-D** and **D-A** (Mitsutomi *et al.*, 1996), which classifies it into a subclass III chitosanase. Saito and colleagues discuss the clear difference in cleavage specificity between the two chitosanases MH-K1 and N174, according to an artificial substrate model (Saito *et al.*, 1999). The substrate binding model shows that some steric hindrance in the active site cleft may explain the specificity (Saito *et al.*, 1999). In addition, the comparison of the structure of the two chitosanases reveals differences in the size and shape of the cleft, and it was also found that the active site of MH-K1 was less open than the active site of CsnN174. Paper V describes the characterization of a GH family 46 chitosanase from *S. coelicolor* A3(2) degrading chitosan substrates.

1.2.6.2 GH family 75 chitosanases

By October 2011, the GH75 family contains only 47 entries in the CAZy database (www.cazy.org), and the family has not been studied to the same extent as GH family 46. Structural information is lacking, and there is very little functional information for these enzymes.

The family 75 chitosanases are found in fungi such as *Aspergillus* sp. and *Fusarium* sp., and also in prokaryotes (primarily) of the genus *Streptomyces*. One of the most studied family 75 chitosanases is the endo-chitosanase from *Aspergillus fumigatus* (Cheng and Li, 2000; Cheng *et al.*, 2006). Studies have shown that the *Aspergillus* chitosanase uses an inverting mechanism when degrading chitosan. In addition, site-directed mutagenesis revealed that Asp160 and Glu169 are the amino acid residues essential for catalytical function (Cheng *et al.*, 2006). Based on the substrate specificity towards a 60% acetylated chitosan, this chitosanase was shown to cleave **A-D** and **D-D** linkages, meaning that the enzyme belongs to subclass I (Cheng *et al.*, 2006). Another family 75 chitosanase that has been studied is one from *Fusarium solani* that was found to be inverting, with the two conserved amino acid residues, Asp175 and Glu188, directly involved in the catalytic function (Shimosaka *et al.*, 2005). In addition, Chen and colleagues reported (in 2005) on a GH 75 chitosanase from *Paecilomyces lilacinus*, which used an endo-mode of action when hydrolyzing chitosans and fully deacetylated

CHOS. The family 75 chitosanase from *P. lilacinus* showed no significant difference in activity when degrading chitosans with F_A < 0.21, but was 70% less active when degrading chitosans with increased F_A (F_A from 0.33 to 0.62) (Chen *et al.*, 2005). In paper VI, a study of a GH family 75 chitosanase from *S. avermitilis* degrading different chitosan substrates is reported.

1.2.7 Other enzymes modifying chitin and chitosan

In addition to the chitinases and chitosanases, several other groups of enzymes are found to modify chitin and chitosan. Some of them are briefly discussed in this section.

β-*N*-acetylglucosaminidases (also known as chitobiases) are involved in the degradation of chitobiose [$(GlcNAc)_2$], the major degradation product of chitinases, into the GlcNAc monomer (Toratani *et al.*, 2008). The chitobiases are found in GH family 20, and there exist crystal structures for several members of the family, e.g. the chitobiase from *S. marcescens* (Tews et al., 1996; Vorgias et al., 1996; Toratani et al., 2008).

In GH family 2, there are a group of enzymes known as exo-*β*-D-glucosaminidases (or GlcNases), which catalyze the cleavage of the *β*-D-glucosaminidine groups from the non-reducing end, producing GlcN monomers (Cote *et al.*, 2006). These enzymes are reported from both fungi and bacteria, and a common property of all the GlcNases studied so far is that they do not possess any detectable β-*N*-acetylglucosaminidase (chitobiase) activity (Nanjo *et al.*, 1990; Zhang *et al.*, 2000; Cote *et al.*, 2006; Fukamizo *et al.*, 2006; Ike *et al.*, 2006). GlcNases in fungi are believed to act in synergy with chitosanases, for an efficient hydrolysis of the deacetylated portion of chitin in the cell wall (Zhang *et al.*, 2000). Fukamizo *et al*. (2006) reports on the synergism between endo-chitosanases and a GlcNase from *Amycolatopsis orientalis*.

Lysozyme (EC 3.2.1.17) is known to degrade the peptidoglycan cell wall of Gram-positive bacteria composed of alternating residues of (1→4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid. In addition, it has been shown that the enzyme can hydrolyse chitin and chitosan (Amano and Ito, 1978; Nordtveit *et al.*,

1994). In 1978, Amano and Ito reported on the oligomers formed upon extensive degradation of a partially deacetylated chitosan (F_A = 0.32) using lysozyme (Amano and Ito, 1978). They identified that the fully *N*-acetylated monomer, dimer, trimer and tetramer were produced, in addition to the partially *N*-acetylated oligomers AAAD (1.7%), ADAA (2.4%), ADAD (3.2%), AAD (0.8%) and AD (6.1%). Later, Vårum *et al.* (1996) studied lysozyme degradation of a highly *N*-acetylated chitosan (F_A = 0.68) and found an absolute specificity for productive binding of **A**-units in subsite C, D and E (subsite -2 to +1, according to Davies and colleagues (1997)). These results were quite different from the findings of Amano and Ito, regarding the specificity of subsite D (i.e. subsite -1). Vårum and colleagues therefore degraded a more deacetylated chitosan (F_A = 0.35), also showing a clear, but not dominant specificity for **A**-units (93%) in subsite D (Vårum *et al.*, 1996). One reason for the variance could be that Amano and Ito fractionated their chitosan after the degradation with lysozyme, characterizing only the low-molecular weight fraction, while Vårum and colleagues characterized the whole chitosan fraction.

There are several reports on unspecific enzymes as for instance papaine, pectinases and cellulases that degrade chitosans (Terbojevich *et al.*, 1996; Kittur *et al.*, 2003; Sashiwa *et al.*, 2003). However, since the enzyme preparations are rather crude and also derived from sources which are known to produce chitinolytic enzymes, there is some doubt concerning which enzymes that in fact catalyze the degradation reactions (described in paper I).

Chitin deacetylases (EC 3.5.1.41) are enzymes that catalyze the hydrolysis of *N*-acetamido linkages in chitin to produce chitosan. These enzymes are members of the carbohydrate esterase family 4 (CE4), as defined by the CAZY database (Cantarel *et al.*, 2009). Other enzymes included in the CE4-family are rhizobial NodB chitooligosaccharide deacetylases, peptidoglycan N-acetylglucosamine deacetylases, acetyl xylan esterases and xylanases (Caufrier *et al.*, 2003). Chitin deacetylases from several fungi have been characterized, e.g. *M. rouxii* (Araki and Ito, 1975; Kafetzopoulos et al., 1993), *Aspergillus nidulans* (Alfonso *et al*., 1995), *Colletotrichum lindemuthianum* (Tsigos and Bouriotis, 1995) and *Saccharomyces cerevisiae*

(Christodoulidou *et al*., 1996), and recently, the occurrence of deacetylases in insects and marine bacteria was reported (Zhao *et al.*, 2010). In 2006, the first structure of a chitin deacetylase, *Cl*CDA, from *C. lindemuthianum*, was solved (Blair *et al.*, 2006). The biological role of the fungal chitin deacetylases is proposed to be involvement in cell-wall formation and remodelling, and in plant-pathogen interactions (Davis and Bartnicki-Garcia, 1984; Tsigos and Bouriotis, 1995; Tsigos *et al.*, 2000). The enzymatic modification pattern of the chitin deacetylases may be divided into the three main types that have traditionally been used to describe enzymes that act on polymeric substrates; (i) multiple-chain (or random attack), (ii) multiple attack and (iii) single-chain mechanisms (Robyt and French, 1967; Martinou *et al.*, 1998). The multiple-chain attack involves a single deacetylation, i.e. the hydrolysis of only one **A**-unit, resulting in a binary heteropolysaccharide with a random distribution of **A**- and **D**-units. Multiple attack and single-chain mechanism catalyze the hydrolysis of several **A**-units, generating block structures (Martinou *et al.*, 1998; Tsigos *et al.*, 2000). Also, in the de-esterification of pectins (Grasdalen *et al.*, 1996) and epimerisation of alginate (Campa *et al.*, 2004), the latter methods of modification have been described.

2. Objectives of the thesis

This thesis is part of a project which aims to use chitinases and chitosanases to convert the low-value material chitin and its derivative chitosan into high-value chitooligosaccharides with possible bioactivity.

The main objectives of this thesis are:
(1) A fundamental characterization of enzymes that degrade chitin and chitosan using well-defined substrates and detailed characterization of the products.
(2) Use chitinases and chitosanases to develop processes for conversion of well-characterized chitosans with varying degree of acetylation into chitooligosaccharides with defined lengths and sequences.

This is accomplished through the six papers included in this thesis:
Paper I is a review describing production, purification, characterization and applications of chitooligosaccharides. The review is included as a part of this thesis to give an overview of the field, and will not be discussed further.
Paper II describes a method used to study the direction in which the two already well-characterized family 18 chitinases, ChiA and ChiB from *S. marcescens*, degrade a chitosan oligomer substrate. The involvement of chitin binding domains is also discussed.
The four following papers cover characterization of four different enzymes relevant for chitosan hydrolysis; **Paper III** describes the characterization of the human chitotriosidase, a GH family 18 chitinase. **Paper IV and V** give detailed characterization of a GH family 19 chitinase (ChiG) and a GH family 46 chitosanase (ScCsn46A), both from the bacteria *S. coelicolor* A3(2). In **paper VI**, a GH family 75 chitosanase, SaCsn75A, from *S. avermitilis* is characterized.

3. Results and Discussion

3.1 Directionality of ChiA and ChiB from *S. marcescens* when degrading a chitosan oligomer substrate (Paper II)

The two family 18 chitinases, ChiA and ChiB from *S. marcescens* have previously been shown to act in an endo processive mode of action when degrading chitosan substrates (Sikorski *et al.*, 2006). In paper II, the directionality of ChiA and ChiB when degrading a chitosan oligomer substrate is described.

3.1.1 Preparation of the chitosan oligomer substrate

An essentially monodisperse chitosan oligomer with DP of 20 was prepared by acid hydrolysis of a high molecular weight chitosan. Figure 3.1(a) shows the SEC chromatogram of the acid-hydrolysed chitosan. The chitosan was further fractionated to obtain the oligomer fraction with a DP of 20, and the SEC chromatogram of this fractionated oligomer is shown in Figure 3.1(b). Subsequently, the fractionated oligomer was tritium-labelled at the reducing end ('DP20'), making it possible to study the directionality when ChiA and ChiB from *S. marcescens* degrade this substrate.

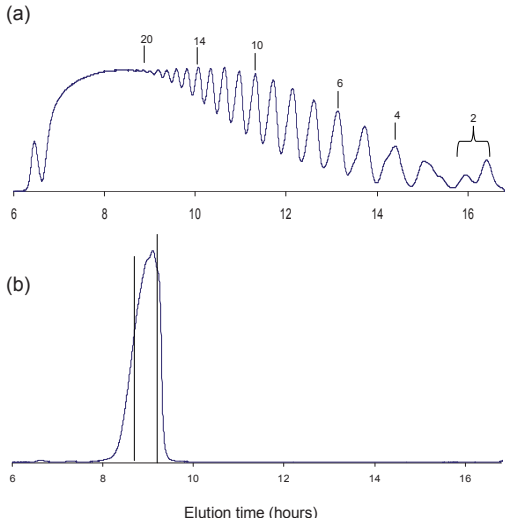

Figure 3.1. Size exclusion chromatograms showing the (polydisperse) acid-hydrolysed chitosan (a), and the fractionated sample after collecting the fractions with DP around 20 (b). To get an even more monodisperse oligomer fraction with DP of 20, the high- and low-molecular weight shoulders of the sample in (b) were not included in the DP 20 fraction as indicated by the two vertical lines.

3.1.2 Directionality of ChiA and ChiB from *S. marcescens*

The chitosan substrate ('DP20') was hydrolyzed to a low degree with ChiA and ChiB, and the degradation products were separated according to size (Figure 3.2). The results show a dominance of even-numbered chitooligosaccharides, as expected from a processive degradation. ChiA has earlier been shown to hold a higher processivity than ChiB (Sikorski *et al.*, 2006), and this was confirmed as the even-numbered oligomers were more dominant for ChiA than ChiB.

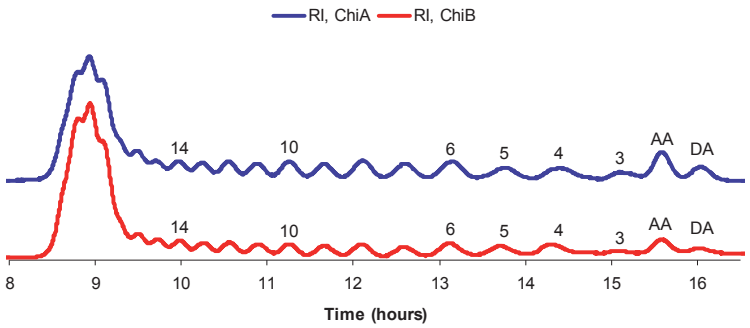

Figure 3.2. Size exclusion chromatograms of the ^{3}H-labeled DP 20 degraded with ChiA and ChiB. The chromatograms obtained after degradation with ChiA and ChiB are shown in blue and red, respectively. RI: Refractor Index response.

The results in Figure 3.2 also support the assertion that ChiA and ChiB are acting in an endo processive mode of action (Sikorski *et al.*, 2006), as the size-distribution of oligomers produced are rather continuous. If ChiA and ChiB degraded the substrate according to an exo processive mode of action, a significantly more bimodal distribution of oligomers would have been expected at such low extent of degradation.

The fractionated oligomers (with tritium-labelled ends) obtained after degradation with ChiA and ChiB were counted and DPM (disintegration per minute) calculated (Figure 3 in paper II). The results showed that ChiA-degradation of 'DP20' contains more low-molecular weight oligomers with labelled ends as compared to ChiB-degraded 'DP20' which has relatively more high-molecular weight oligomers with labelled ends. The results obtained from the RI-detector (SEC) and from DPM were recalculated in order to obtain the molar concentration and the molar concentration of labelled ends for each oligomer (DP 2-20). The results are shown in Figure 3.3.

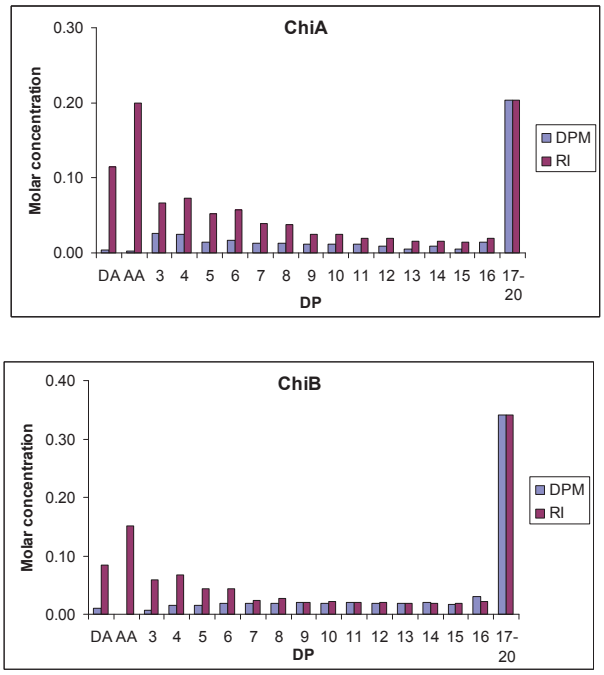

Figure 3.3. Plot of molar concentration of oligomers and molar concentration of labelled chain ends as a function of DP. The data has been scaled (described in the experimental section in paper II), so both data sets can be compared.

The data in Figure 3.3 show that ChiA and ChiB degrade the substrate in opposite directions. The results further suggest that ChiA moves towards the reducing end (as a dominance of shorter labelled oligomers is observed) and ChiB towards the non-reducing end of the substrate (dominance of longer labelled oligomers). Quite surprisingly, these directions of the processive degradation of ChiA and ChiB are opposite to those previously supposed. ChiA and ChiB have chitin binding domains (CBD) that extend the substrate binding cleft on the non-reducing and reducing side of the active site, respectively. Therefore, ChiA is proposed to degrade the substrate from the reducing end towards the non-reducing end, while ChiB is thought to degrade the substrate from the non-reducing side towards the reducing end (van Aalten *et al.*, 2000). This predicted opposite directionality of ChiA and ChiB has been supported by degradation of end-labelled β-chitin using microscopy (Hult *et al.*, 2005), and from site

directed mutagenesis studies (Horn *et al.*, 2006b; Zakariassen *et al.*, 2009). In ChiA and ChiB, the aromatic residues Trp167 and Trp97, respectively, were found to be important for processivity, as site directed mutagenesis of these two residues gave loss of processivity. Trp167 is located in subsite -3 in ChiA, while Trp97 is located in subsite +1 in ChiB. Therefore, these aromatic residues are suggested to interact with the polymeric part of the substrate during the processive mode of action (Horn *et al.*, 2006b; Zakariassen *et al.*, 2009).

When the results discussed above are taken into consideration, a directionality of ChiA and ChiB towards the reducing and non-reducing end, respectively, is difficult to accept. An alternative explanation was therefore proposed; could the substrate binding domain contribute to where the initial endo-binding to the 'DP20' substrate will take place? In ChiB, the distance from the catalytic residue to the most remote of the exposed aromatic residues in the CBD is 55 Å, corresponding to the length of approximately 10 sugar units (van Aalten *et al.*, 2000). Therefore, if the CBD is involved in the initial endo-binding to the oligomer substrate, the active site of the enzyme will be shifted 5 sugar units towards the non-labelled (non-reducing) end. Similar arguments are valid for ChiA and the Fibronectin like 3 domain (Fn3), but then the initial endo-binding of the active site are shifted towards the labelled end of the substrate. This is schematically shown in Figure 3.4. Thus, if the CBD of both ChiA and ChiB are involved in the initial endo-binding of the chitosan substrate, the interpretation visualized in Figure 3.4 is correct, showing that ChiB degrades the substrate towards the reducing end and ChiA degrades it towards the non-reducing end. Since this approach supports the earlier conclusions (van Aalten *et al.*, 2000; Hult *et al.*, 2005; Zakariassen *et al.*, 2009), we believe that the chitin binding domain takes part in the initial binding to the substrate.

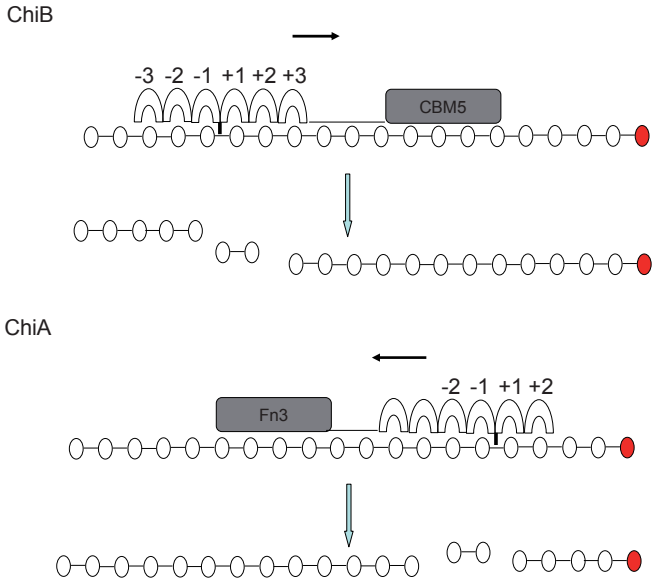

Figure 3.4. Endo-enzymes, with the significance of chitin binding domain in initial binding. The part of the enzyme that binds to the substrate, CBM5 for ChiB and Fn3 for ChiA, is assumed to force the enzyme to bind closer to the non-reducing end (ChiB) and to the reducing end (ChiA).

Processivity of enzymes involved in the modification of polysaccharides have been reported for e.g. amylases (Robyt and French, 1967; Robyt and French, 1970), cellulases (Rouvinen *et al.*, 1990; Varrot *et al.*, 2003), carrageenases (Michel *et al.*, 2003), alginate epimerases (Campa *et al.*, 2004) and pectinesterases (Grasdalen *et al.*, 1996). The preparation of labelled oligomers from other polysaccharides can therefore be used as a valuable tool in the study of directionality and/or in the involvement of for instance polysaccharide binding modules.

3.2 Degradation of chitosans with the human chitotriosidase (Paper III)

In paper III, one of the two family 18 chitinases produced by humans, human chitotriosidase (HCHT), is characterized, and novel insights into its enzymatic properties derived from a detailed analysis on its action on chitosans are described.

HCHT has been suggested to play a role in the innate immune system, as a defense against chitin-containing pathogens (Hollak *et al.*, 1994; van Eijk *et al.*, 2005). The information obtained from the degradation of chitosans is therefore useful in an applied context, e.g. when designing inhibitors that are specific for certain chitinases or for understanding how chitosans are degraded in the human body.

3.2.1 Degradation of a highly acetylated chitosan ($F_A = 0.62$)

Determination of endo/exo mode

To determine whether HCHT shows endo or exo activity when degrading chitosan, a study of the extent of decrease in relative viscosity was performed. As previously discussed by Sikorski *et al*. (2006), exo enzymes will reduce the viscosity of the chitosan solution much more slowly than endo acting enzymes. Structural studies of HCHT (Figure 1 in paper III) show that the substrate binding cleft is deep (as for ChiA and ChiB), indicating exo and/or processive mode of action (Davies and Henrissat, 1995; Henrissat and Davies, 1997). However, HCHT was found to degrade the chitosan substrate ($F_A = 0.62$) in an endo mode of action, as the relative viscosity of the chitosan solution decreased rapidly when the enzyme was added (Figure 5 in paper III). As a control experiment hydrolysis of chitosan by acid was performed as a model of endo degradation in addition to ChiB which has previously been shown to act in an endo mode of action when degrading chitosan (Sikorski *et al.*, 2006). In crystalline substrates, the polymer ends and amorphous regions are the most accessible, and therefore preferred by the enzymes. Soluble substrates, such as chitosan, are much more accessible and contain internal sugar units that are available for substrate binding. These internal sugar units will be in large excess in relation to the number of chain ends

available, and therefore, enzymes which ordinary tend to act as exo enzymes will show endo activity when acting upon chitosan. In the case of ChiB, exo activity has been reported when degrading crystalline β-chitin (Hult *et al.*, 2005). For HCHT, there is no information on the mode of action on crystalline chitin available.

Processivity

HCHT produced a high ratio of even-numbered CHOS in the very early degradation (α-value below 0.01) of a highly acetylated chitosan ($F_A = 0.62$). This is indicative of a processive mode of action. The size-distribution of CHOS produced is shown in the insert chromatogram in Figure 3.5. This characteristic product profile from SEC has earlier been confirmed for other processive family 18 chitinases degrading chitosan (Horn *et al.*, 2006a). Furthermore, Gorzelanny and colleagues also detected an initial dominance of even-numbered products for HCHT, using electrophoresis and MS (Gorzelanny *et al.*, 2010). A slow disappearance of the polymer peak (at $\alpha > 0.13$) was observed when chitosan was hydrolyzed with HCHT (Figure 3.5). This is often believed to be an indication of exo activity, but in a study of Sikorski *et al*. it was shown to be a typical characteristic of enzymes acting in an endo processive mode of action, as e.g. ChiA and ChiB (polymer peak disappears at $\alpha \approx 0.20$ (Sikorski *et al.*, 2006)). This is also the case for HCHT, which hydrolyzes the chitosan according to an endo processive mode of action. For HCHT, the degree of processivity was calculated to an average of 2.5 cuts per formation of enzyme-substrate complex (Figure 3.6). In comparison, for ChiA and ChiB the degree of processivity were 9.1 and 3.6 cuts per formation of enzyme-substrate complex, respectively (Sikorski *et al.*, 2006). This indicates a moderate processivity for HCHT when hydrolyzing chitosan. The presence of aromatic residues in the substrate binding cleft have previously been shown to be important in processive attack (Divne *et al.*, 1998; Varrot *et al.*, 2003; Horn *et al.*, 2006b; Zakariassen *et al.*, 2009). HCHT resembles ChiA when it comes to the pattern of aromatic residues in the substrate binding cleft. Since ChiA are thought to hydrolyze the substrate from the reducing end towards the non-reducing end (paper II and Zakariassen *et al*., 2009), it can be expected that HCHT shows the same directionality because of the similarity in aromatic residues present.

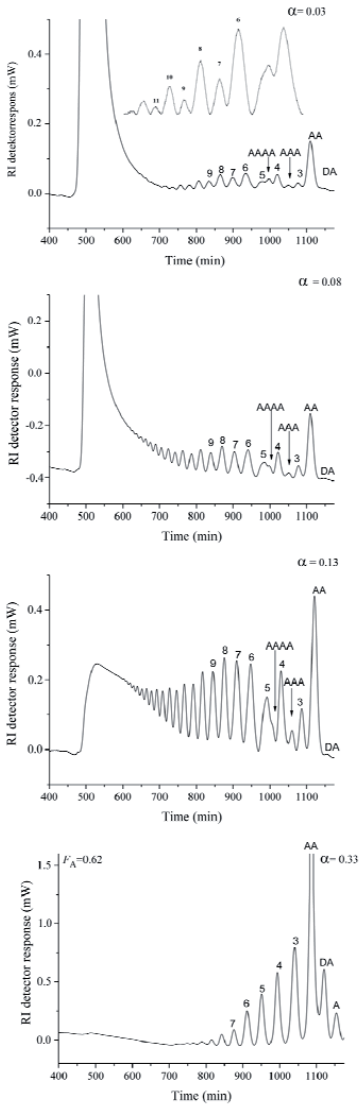

Figure 3.5. Size-distribution of oligomers obtained after HCHT degradation of chitosan (F_A = 0.62). α-values are varying from 0.03 to 0.33, and the insert in the top chromatogram shows the oligomers produced at α-values below 0.01. Peaks are labelled with DP-value or with the sequence of the oligomer.

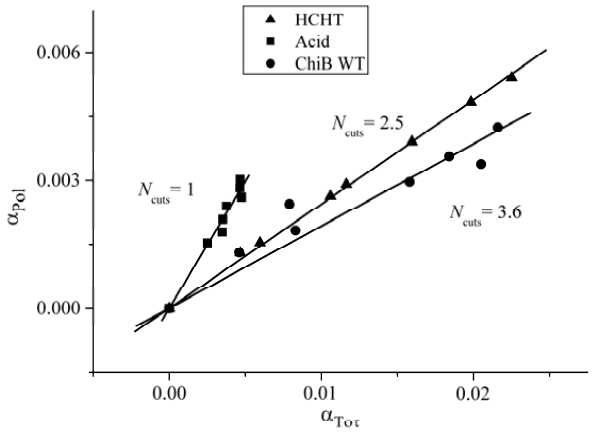

Figure 3.6. Degree of scission of the polymer fraction (α_{pol}) as a function of the total degree of scission (α_{tot}). The degree of processivity (N_{cuts}) was calculated for HCHT, ChiB and acid hydrolyzing a highly acetylated chitosan (F_A = 0.62). See paper III for further descriptions.

Chemical composition of chitooligosaccharides

Since family 18 chitinases such as HCHT use the substrate-assisted double displacement mechanism, a *N*-acetyl group from an **A**-unit bound in subsite -1 is required for catalysis. Therefore, all CHOS produced contained –**A** on their reducing ends, as identified from MALDI-TOF/TOF mass spectrometry. In addition to the absolute preference for **A**-units binding in subsite -1, there was a strong preference for **A**-units in subsite -2 (i.e. the neighbour position to the new reducing end) and an initial preference, although weak, for **A**-units in subsite +1 (i.e. the sugar binding in subsite +1, which gives the identity of the new non-reducing end). Table 2 in paper III shows an overview of the sequences for the isolated trimer to the isolated hexamer fractions. The kinetics of the degradation reaction suggested two different phases (Figure 2 in paper III), indicating that the rate of degradation slowed down as the most preferred substrate (i.e. when **A**-units are bound in subsites -2, -1 and +1) were depleted.

3.2.2 Degradation of chitosans with varying F_A

In addition to the highly acetylated chitosan (F_A = 0.62), three other chitosans (F_A of 0.49, 0.35 and 0.18, respectively) were extensively degraded to maximum degree of scission (to α-values of 0.33, 0.26, 0.24 and 0.15, respectively; Figure 3.7). The efficiency of HCHT decreased as the fraction of acetylated units in the substrate decreased as expected due to the preferences for **A**-units (described above). However, it has previously been shown that ChiB is more capable of hydrolyzing chitosans with lower degrees of acetylation, than ChiG, a family 19 chitinase (further discussed in Paper IV). This has been explained by the fact that family 18 chitinases have several aromatic residues in the substrate binding cleft that stack with the hydrophobic faces of the sugars (van Aalten *et al.*, 2001). Such enzyme-sugar interactions do not depend on the existence of *N*-acetyl groups, which is the case for family 19 chitinases (see paper IV and discussion in section 3.3.2). Both the ability of HCHT to degrade chitosans with low F_A-values (Figure 3.7) and the structural data (Figure 1 in paper III), suggest that HCHT resembles ChiB, using aromatic amino acids in the interaction with the substrate.

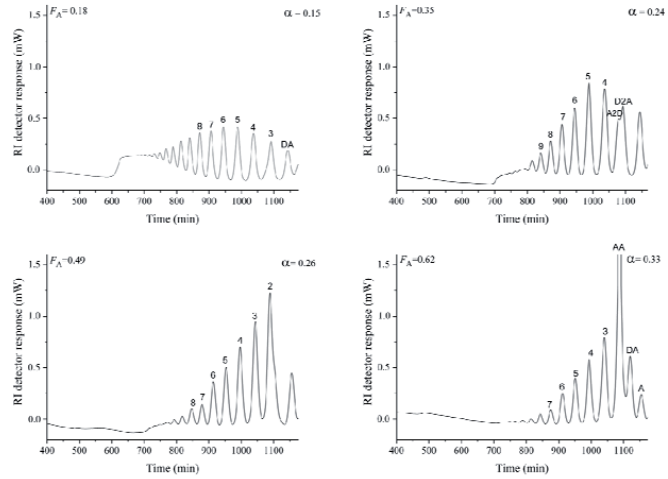

Figure 3.7. Size-distribution of CHOS obtained after extensively degradation of chitosans with varying degree of acetylation. Chitosans with F_A of 0.18, 0.35, 0.49 and 0.62 were degraded to maximum α-values of 0.15, 0.24, 0.26 and 0.33, respectively.

3.3 Degradation of chitosans with ChiG from *S. coelicolor* A3(2) (Paper IV)

The crystal structure and enzymatic properties of the GH family 19 chitinase ChiG from *S. coelicolor* A3(2) has been reported (Hoell *et al.*, 2006). However, degradation of chitosan substrates has not been studied. In paper IV in this thesis, we focus on the enzymatic properties of ChiG, when degrading chitosans of varying degrees of acetylation.

3.3.1 Degradation of a highly acetylated chitosan (F_A = 0.64)

Subsite specificities and kinetics of the degradation reaction

Progress in the degradation of a highly acetylated chitosan (F_A = 0.64) with ChiG, was followed by ^{1}H-NMR analyzes. The ^{1}H-NMR spectra are shown in Figure 3.8. The identity of the new reducing ends produced (**A** or **D**), provides information about the binding preferences in subsite -1. In addition, when an acetylated unit is bound in subsite -1, information about the neighboring sugar (bound in subsite -2) is also obtained. The kinetics of the degradation reaction was clearly biphasic (as could be seen when the degree of scission was plotted as a function of the incubation time, Figure 2 in paper IV): in the initial, rapid phase (i.e. at low α-values), the new reducing ends were exclusively acetylated. However, in the second, slower phase (at α-values ≥ 0.15), deacetylated reducing ends could also be observed (Figure 3.8). This shows that ChiG can bind both **A**- and **D**-units productively in subsite -1, and there is no absolute requirements for **A**-units in subsite -1, in contrast to the family 18 chitinases (Horn *et al.*, 2006a). In the neighbour position to the new reducing end (i.e. the sugar unit binding in subsite -2), only acetylated units were bound productively, as shown by the absence of the resonance for an acetylated reducing end with a deacetylated sugar in the neighbor position (–DA ; 4.74 ppm). When the reducing end is deacetylated, it is not possible to discriminate between the identities of the neighboring sugar unit, since the β-resonance for a **D** is only partially resolved from the resonance for the internal **D**-units. However, characterization of the produced CHOS and the observation for

acetylated reducing ends, suggests that only **A**-units are bound in subsite -2, independent of the identity of the reducing end.

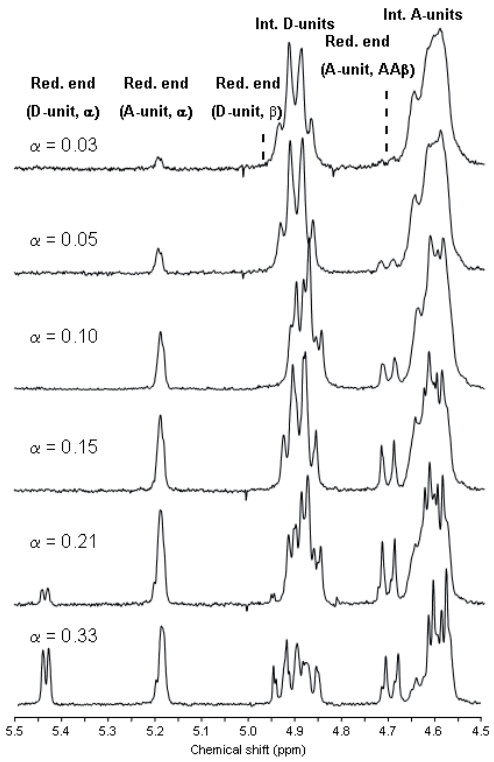

Figure 3.8. ^{1}H-NMR spectra of the anomer region of chitosan (F_A = 0.64) after degradation with ChiG to α-values between 0.03 and 0.33. Spectra were recorded at 300 MHz at 90° C and pD 4.2 and resonances were assigned according to previously published ^{1}H-NMR spectra of degraded chitosan (Vårum *et al.*, 1991a; Vårum *et al.*, 2001; Sørbotten *et al.*, 2005). The α-anomer of a **D** resonates at 5.43 ppm and the β-anomer at 4.92 ppm. The α-anomer of the **A**-unit resonates at 5.19 ppm, whereas the β-anomer resonates at 4.71 ppm, if the neighboring residue is an **A** or at 4.74 ppm if the neighboring residue is a **D** (not detected). The internal **D**-units resonate at 4.80-4.95 ppm, while the internal **A**-units resonate at 4.55-4.68 ppm (Vårum *et al.*, 1991a; Ishiguro *et al.*, 1992). The β-resonance for a **D** is only partially resolved from the resonance for the internal **D**-units.

The new non-reducing end of the hydrolysis products (i.e. the sugar unit which has been bound in subsite +1 prior to the cleavage) was found to be exclusively acetylated, as identified by ^{13}C-NMR spectroscopy (Figure 3 in paper IV).

Other reports on (extensive) degradation of chitosans by other family 19 chitinases show that they also have preferences for binding **A**-units in subsites -2 and +1 (Mitsutomi *et al.*, 1995; Sasaki *et al.*, 2006), in agreement with the results obtained with ChiG. Furthermore, Huet *et al*. recently reported on a complex with a family 19 chitinase from *Carica papaya* preferentially binding two **A**-units in subsites -2 and +1 (Huet *et al.*, 2008).

Chitooligosaccharides: size-distribution and chemical composition

The size-distribution of the CHOS produced during degradation of the 64% acetylated chitosan was studied (Figure 3.9). ChiG was found to act as a non-processive endo chitinase, as a continuum of CHOS was produced with no dominance of even-numbered CHOS. This degradation pattern is quite different from those observed for ChiA and ChiB, but similar to ChiC (see Figure 1.7 in Introduction) (Horn *et al.*, 2006a). As revealed from the crystal structure (Hoell *et al.*, 2006), ChiG has an open active site cleft, which is common for non-processive endo activity (Davies and Henrissat, 1995; Henrissat and Davies, 1997). This is also suggested to be the case for ChiC since it lacks the (α+β)-domain that form a "wall" in other (processive) family 18 chitinases (Horn *et al.*, 2006c).

Due to the differences in subsite specificities, ChiG yields oligomers with quite different sequences (Table 3.1) than those obtained by ChiA, ChiB and ChiC. When ChiG degrades the F_A = 0.64 chitosan extensively, the trimer AAD is produced (Figure 3.9 and Table 3.1). On the other hand, ChiB yield the trimer DAA, when hydrolyzing the same chitosan substrate (Sørbotten *et al.*, 2005; Horn *et al.*, 2006a).

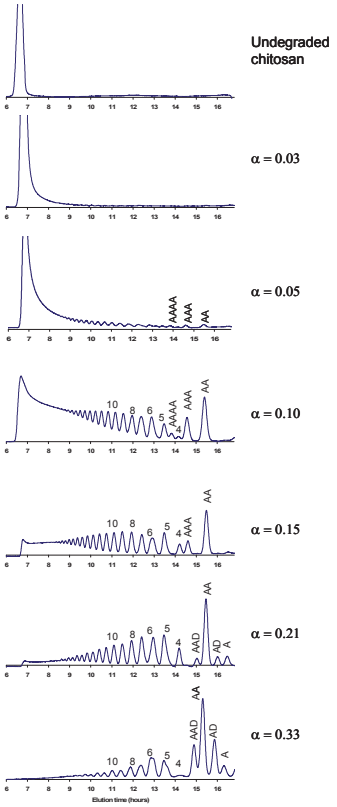

Figure 3.9. SEC chromatograms showing the size-distribution of CHOS obtained after degradation of a highly acetylated chitosan (F_A = 0.64) with ChiG. α-values are varying from 0 (undegraded substrate) to 0.33. Peaks are labeled with DP-values or with the sequence of the oligomer.

Table 3.1. Sequence of isolated chitooligosaccharide fractions obtained after ChiG degradation of a 64% acetylated chitosan to α-values of 0.10 and 0.33. ND: not determined, due to too small amounts obtained.

	Monomer	Dimer		Trimer	Tetramer	Pentamer
α = 0.10	-	AA		AAA	AAAA ADAA	AADAA/ ADAAA
α = 0.33 (max)	A	AA	AD	AAD	ND	ADDAA ADDAD

3.3.2 Degradation of chitosans with varying F_A

Extensive degradation of chitosans with F_A of 0.13, 0.32, 0.50 and 0.64 to α-values of 0.04, 0.12, 0.23 and 0.33, respectively, shows that the efficiency of ChiG was dramatically decreased with decreasing F_A. Therefore, **A**-units are important for efficiently degradation of the chitosan substrate (Figure 3.10). A similar experiment was performed for HCHT (paper III), and for ChiB (Figure 1.9 in the Introduction section and Sørbotten *et al*., 2005). The results for HCHT and ChiB showed that the family 18 chitinases were capable of hydrolyzing chitosans with lower degrees of acetylation. Both HCHT and ChiB have several aromatic residues that stack with the hydrophobic faces of the sugars (see paper III and van Aalten *et al.,* 2001), and such enzyme-sugar interactions do not depend on the existence of *N*-acetyl groups. However, in family 19 chitinases, substrate binding in the catalytical cleft is dominated by specific hydrogen bonds which often involve *N*-acetyl groups (Huet *et al.*, 2008). This may explain the low efficiency of ChiG against the more deacetylated chitosans.

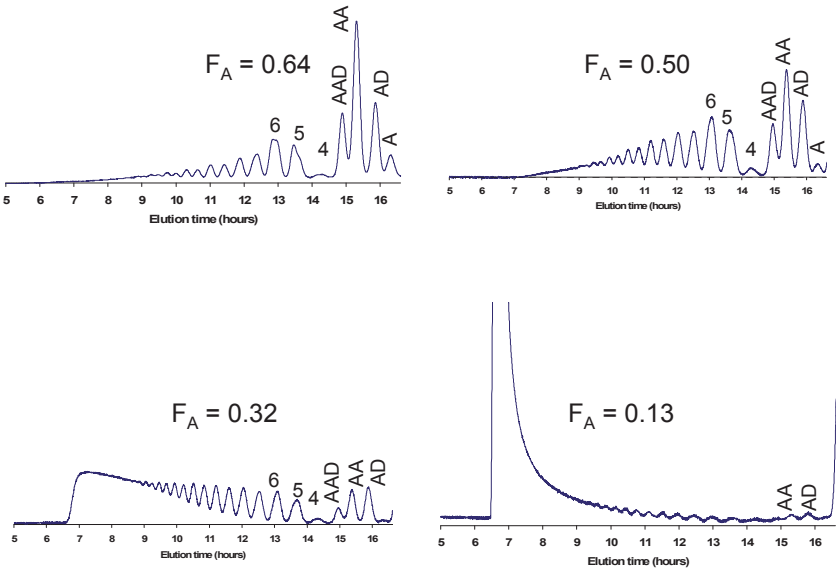

Figure 3.10. Size-distribution of CHOS obtained after extensively degradation of chitosans with varying degree of acetylation. Chitosans with FA of 0.13, 0.32, 0.50 and 0.64 were degraded to maximum α-values of 0.04, 0.12, 0.23 and 0.33, respectively.

3.3.3 Comparison of family 18 and family 19 chitinases

Although ChiG degraded the more deacetylated chitosans inefficiently, a highly acetylated chitosan ($F_A = 0.64$) was degraded 2.4 times faster than with ChiB (specific activities of 2280 and 970 min^{-1}, respectively). On the other hand, ChiB has been found to degrade β-chitin much more complete than ChiG (Vaaje-Kolstad *et al.*, 2005). Other family 19 chitinases, like ChiF from *S. coelicolor* A3(2) and ChiC from *S. griseus* HUT6037, have also showed low hydrolytic activity towards crystalline chitin (Watanabe *et al.*, 1999; Kawase *et al.*, 2006). The presence of aromatic residues in the substrate binding cleft of ChiB has previous been shown to increase the efficiency in the degradation of crystalline chitin, while reducing the efficiency in degradation of chitosan (Horn *et al.*, 2006b).

The differences in subsite specificity between the family 18 and family 19 chitinases are remarkable, and a suggestion could be that they have different roles in chitin turnover in nature. Some organisms produce several chitinases, and the enzymes are thought to have different functional roles in the organism (Seidl *et al.*, 2005; Itoh *et al.*, 2006; Kawase *et al.*, 2006). The diversity between the two families, as well as within them, is reflected in e.g. mode of action (endo versus exo, processive versus non-processive) and in the differences in domain structure, and these "factors" effect enzyme efficiency in a substrate-dependent manner.

3.4 Characterization of a GH family 46 chitosanase from *S. coelicolor* A3(2) (Paper V)

Several in-depth studies have been reported on chitinases degrading chitosans. On the other hand, chitosanases have not been studied to the same extent. In paper V, a study on the degradation of chitosans by a novel GH family 46 chitosanase, ScCsn46A, from *S. coelicolor* A3(2) is described.

3.4.1 Degradation of a 32% acetylated chitosan

Subsite specificities and kinetics of the degradation reaction

A chitosan (F_A = 0.32) was degraded to different degrees of scission, and the identities of the new reducing ends and the neighboring units next to the new reducing end were identified by the use of ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectra are shown in Figure 3.11. Initially, at α-values below 0.10, the new reducing ends were almost exclusively deacetylated, while new acetylated reducing ends appeared upon more extensive degradation (α-values above 0.10). Thus, ScCsn46A has a preference for productively binding **D**-units in subsite -1, although the enzyme can also hydrolyze the glycosidic linkage following an **A**-unit. The neighboring sugar next to the acetylated reducing end, i.e. the sugar unit bound productively in subsite –2, is exclusively deacetylated (signal at 4.74 ppm; -DA(β)). Even upon prolonged degradation (α = 0.44), the resonance corresponding to an acetylated reducing end with an acetylated neighboring unit cannot be identified (i.e. –AA(β), expected at 4.71 ppm (Sørbotten *et al*., 2005)). When a deacetylated unit is productively bound in subsite -1, it is not possible from the ^{1}H-NMR spectra to discriminate between the identities of the neighboring sugar next to the reducing end **D** (as described in paper IV).

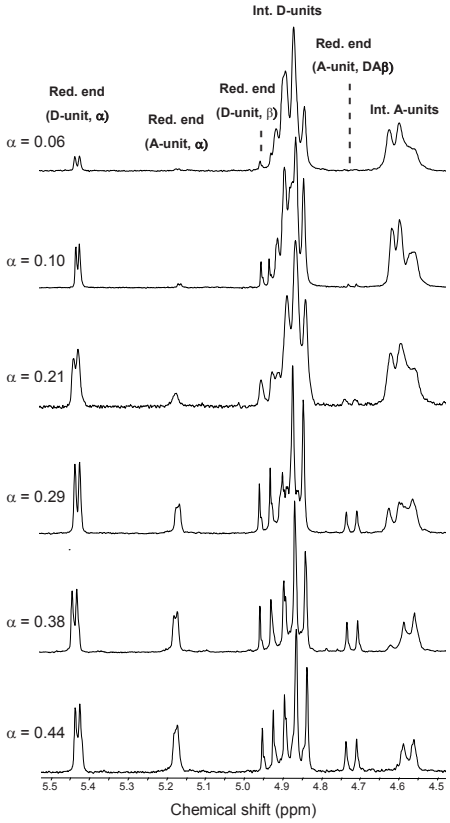

Figure 3.11. ^{1}H-NMR spectra of the anomer region of chitosan (F_A = 0.32) after degradation with ScCsn46A to α-values between 0.06 and 0.44. For further information, see legend to Figure 3 in paper V.

To identify potential preferences in subsite +1, ^{13}C-NMR spectroscopy was performed for both partially degraded (α = 0.10) and more extensively degraded chitosan (α = 0.29) (Figure 5 in paper V). The partially degraded chitosan showed a clear preference for **D**-units in the +1 subsite, as almost exclusively **D**-units was identified in the ^{13}C-NMR spectrum. In the case of the more extensively degraded chitosan, the spectrum revealed that a small, but significant fraction of acetylated non-reducing ends was present, demonstrating that ScCsn46A has no absolute specificity towards **D**-units in the +1 subsite.

The kinetics of the degradation of the F_A 0.32 chitosan showed three different phases (Figure 4 in paper V); An initial rapid phase ($\alpha \leq 0.10$), a slower second phase (until $\alpha \approx 0.30$) and a third, even slower phase ($\alpha \approx 0.34$). Prolonged incubation with additional enzyme resulted in the maximum obtainable α-value of 0.44. The initial fast product formation rate corresponded to the cleavage of the glycosidic linkage between two **D**-units, i.e. subsites -1 and +1 preferred to bind deacetylated units, initially. However, as the chitosan substrate was further degraded, the degradation rate decreased. As detected from ^{1}H- and ^{13}C-NMR spectroscopy, this coincides with the identification of both acetylated and deacetylated reducing and non-reducing ends. Therefore, productive binding of acetylated units in subsite -1 and/or +1 appears to be less preferred as it slows down the degradation rate. This indicates that ScCsn46A has low subsite specificities towards acetylated and deacetylated units binding in the active site, resulting in the formation of complex mixtures of CHOS. Compared to other characterized chitinolytic enzymes (see paper III, IV and VI in this thesis, in addition to Sørbotten *et al*., 2005; Horn *et al*., 2006a), ScCsn46A has a much lower subsite specificity (summarized in Table 3.3). Surprisingly, it was verified that even the linkage **A-A** was cleaved, as ScCsn46A hydrolyzed the fully acetylated chitin hexamer (Figure 3.12), although more than 5 orders of magnitude slower than the degradation of a fully deacetylated chitosan.

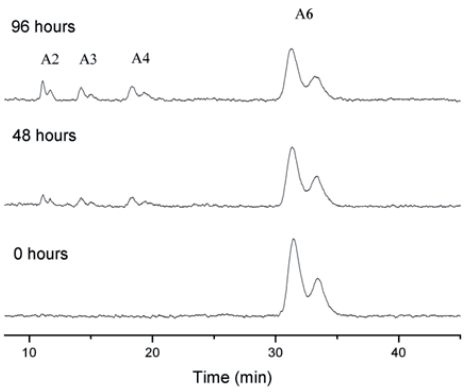

Figure 3.12. HPLC analysis of CHOS produced after degradation of a fully acetylated hexamer with ScCsn46A. The chromatograms show that the fully acetylated dimer, trimer and tetramer are produced after 48 and 96 hours of incubation.

Chitooligosaccharides: size-distribution and chemical composition

The 32% acetylated chitosan was degraded to α-values from 0.06 to 0.44, and the chitosan samples were separated according to size. The polymer peak gradually disappeared (absent in the chromatogram of α = 0.29, see Figure 6 in paper V), and subsequently, a continuum of oligomers were produced with no preference for even-numbered oligomers. This indicates that ScCsn46A hydrolyze the substrate according to a non-processive, endo mode of action. As the chitosan was extensively degraded, the dimers (DD and DA) appeared as the dominant products.

Oligomer fractions obtained after degradation of the 32% acetylated chitosan to low α (α = 0.10) and high α (α = 0.44) were collected and characterized to determine the sequence of the oligomers. Limitations in the use of ^{1}H-NMR spectroscopy (due to the sequential complexity of the oligomeric products) required the additional use of AMAC derivatized oligomers analyzed by mass spectroscopy (MS) to obtain more sequential information (Table 2 and Figure 7 in paper V) (Bahrke *et al.*, 2002; Cederkvist *et al.*, 2008). A drawback by the use of AMAC derivatized oligomers analyzed by MALDI-TOF/TOF-MS^1/MS^2 is lack of quantitative information. Thus, there is no information on the quantitative amounts of the oligomers identified by this method.

3.4.2 Degradation of a chitosan, F_A = 0.008

Fully deacetylated chitosan (F_A = 0.008) was also degraded to varying degrees of scission, and the size-distribution profile of the oligomers showed a continuum of both even and odd-numbered oligomers indicating a non-processive, endo mode of action (Figure 2 in paper V). This confirmed the results from the degradation of the F_A = 0.32 chitosan. When the fully deacetylated chitosan was degraded to increasing extents of degradation, fully deacetylated oligomers were produced with good yields. Thus, by controlling the degree of scission, the yield of a specific oligomer with a given length can be optimized. At α = 0.59 (maximum degree of scission) the dominant degradation product was the dimer DD, with considerable amounts of monomer present.

Fully deacetylated chitosan substrates have been found to be very resistant to acid hydrolysis, as the rate of hydrolysis of a glycosidic linkage following a **D**-unit are at least two orders of magnitude lower in comparison to the rate of hydrolysis of a glycosidic linkage following an **A**-unit (Vårum *et al.*, 2001; Einbu *et al.*, 2007; Einbu and Vårum, 2008). Therefore, enzymatic degradation of a fully deacetylated chitosan with ScCsn46A is clearly preferable instead of acid degradation for the production of fully deacetylated oligomers.

3.4.3 Degradation of chitosans with varying F_A

The low subsite specificity of ScCsn46A was also shown when degrading chitosans with widely varying degrees of acetylation (F_A = 0.008, 0.32, 0.46 and 0.63). The results showed that ScCsn46A could degrade all chitosan substrates extensively (to α-values of 0.59, 0.44, 0.37 and 0.32, respectively), although to a decreasing degree of scission as F_A of the chitosans increased (Figure 3.13). The oligomers produced after extensively degradation of the F_A = 0.63 chitosan were analyzed by MS, and the results showed that they were highly acetylated, with increasing F_A as the DP of the oligomers increased (Table 3 in paper V). The non-reducing end of the oligomers with DP > 3 was almost exclusively acetylated, while the reducing ends could be both deacetylated and acetylated.

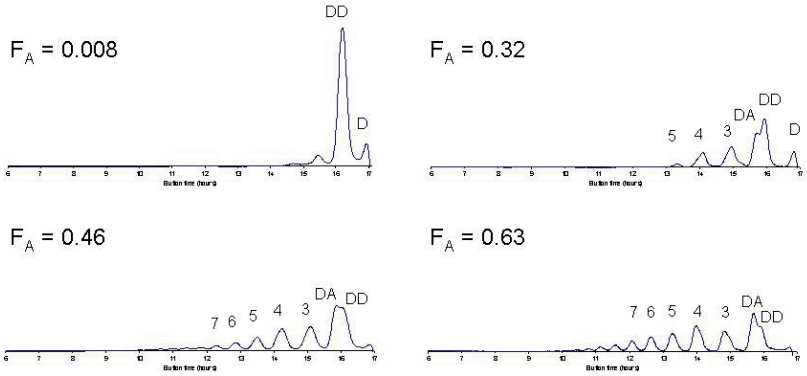

Figure 3.13. Size-distribution of oligomers obtained after extensively degradation of chitosans with varying degree of acetylation. Chitosans with F_A of 0.008, 0.32, 0.46 and 0.63 were degraded to α-values of 0.59, 0.44, 0.37 and 0.32, respectively. Peaks are labeled with DP-values or with the sequence of the oligomer.

3.5 Characterization of a GH family 75 chitosanase from *S. avermitilis* (Paper VI)

Paper VI describes the first in-depth study of a GH family 75 chitosanase, SaCsn75A from *S. avermitilis*, giving new insight into the enzyme's specificity and its ability to produce various mixtures of chitooligosaccharides.

3.5.1 Degradation of a 31% acetylated chitosan

Subsite specificities and kinetics of the degradation reaction

To obtain information on the subsite specificities in subsites -1 and -2 for SaCsn75A, ^{1}H-NMR spectroscopy was performed. Figure 3.14 shows the ^{1}H-NMR spectra of the F_A = 0.31 chitosan, degraded to α-values from 0.04 to 0.27. Initially, at α-values below 0.07, the new reducing ends were almost exclusively acetylated, and the neighbouring sugars were always deacetylated, as no resonance for –AA(β) (signal at 4.71 ppm; Sørbotten *et al*., 2005) was detected in the spectra. Upon more extensive degradation of the chitosan, new deacetylated reducing ends appeared (α > 0.07), and in the spectrum for the extensively degraded chitosan (α = 0.27) the oligomers were composed of about equal amounts of acetylated and deacetylated reducing ends. Thus, SaCsn75A has a clear initial preference for binding acetylated units in subsite -1, with an absolute specificity for binding deacetylated units in subsite -2. As the degradation reaction proceeds deacetylated units are also productively bound in subsite -1. The kinetics of the degradation showed a biphasic degradation profile (Figure 3 in paper VI); an initial rapid phase obtained at α-values below 0.11 (i.e. the productive binding of (mainly) **A**-units in subsite -1), and a slower second phase up to α-values around 0.20 (i.e. when **D**-units are productively bound in subsite -1 as well). The specific activity for the initial degradation phase was calculated to be 6.5 min^{-1}, which is very different to the initial specific activity of 325 min^{-1} for ScCsn46A degrading the same chitosan substrate (see paper V). Thus, SaCsn75A is ineffective when degrading a F_A = 0.31 chitosan, as compared to ScCsn46A.

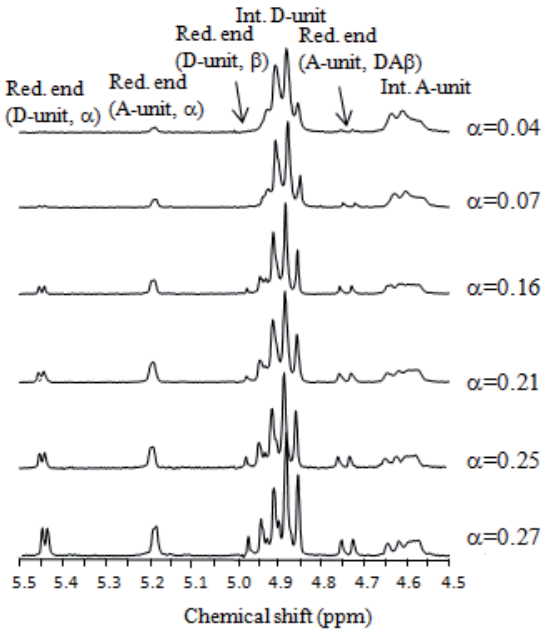

Figure 3.14. Anomer region of the ^{1}H-NMR spectra of a F_A = 0.31 chitosan after degradation with SaCsn75A to α-values between 0.04 and 0.27. For further information, see legend to Figure 2 in paper VI.

^{13}C-NMR spectroscopy was performed to obtain information on the identity of the new non-reducing ends. The spectra (Figure 4 in paper VI) revealed almost exclusively deacetylated units at the new non-reducing ends, both after initial and extensive degradation (i.e. at α-values of 0.07 and 0.27) of the F_A = 0.31 chitosan. This shows that SaCsn75A has an absolute preference for deacetylated units in subsite +1.

Chitooligosaccharides: size-distribution and chemical composition

The size-distributions of oligomers resulting from the degradation of the 31% acetylated chitosan to different α-values are shown in Figure 3.15. SaCsn75A was found to operate according to a non-processive endo mode of action, as earlier described for both ChiC (Horn *et al*., 2006a), ChiG (paper IV) and ScCsn46A (paper V). Interestingly, at the lower degrees of scission ($\alpha \leq 0.16$; Figure 3.15), a relative low occurrence of dimers and trimers could be observed, quite different from the relatively high concentrations of the same oligomers when ScCsn46A degraded the same chitosan substrate (paper V). This difference in oligomer distribution can be explained by the initial difference in the preferences for **A**- and **D**-units in subsite -1. Since SaCsn75A initially prefers **A**-units in -1 subsite, the probability of finding another **A**-unit two or three units away, which is necessary to form the dimer/trimer, is relatively low with a substrate dominated by **D**-units ($F_A = 0.31$). In contrast, since ScCsn46A prefers **D**-units in subsite -1, there is higher probability of finding another **D**-unit two or three units away with the same chitosan substrate. Upon more extensive degradation with SaCsn75A, an increasing amount of shorter oligomers appeared. The size-distribution of oligomers at $\alpha = 0.27$ showed that the dominating oligomers were dimers, trimers and tetramers, although oligomers with DP > 10 were also present.

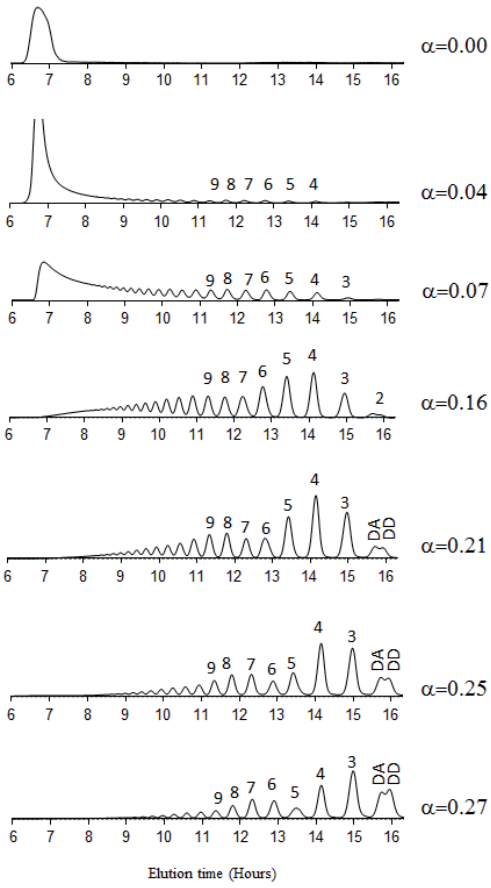

Figure 3.15. Size-distribution of CHOS after SaCsn75A degradation of a chitosan F_A = 0.31. SEC chromatograms showing the size-distribution of oligomers obtained after degradation of chitosan (F_A = 0.31) to α-values from 0.04 to 0.27. A chromatogram of undegraded chitosan is shown at the top of the figure. Peaks are labelled with the DP-value or with sequence of the oligomer.

Individual CHOS fractions obtained after initial (α = 0.07) and extensive (α = 0.27) degradation of the chitosan (F_A = 0.31) were collected and analyzed by ^{1}H-NMR spectroscopy. An overview of the chemical composition and probable sequence of the oligomers (DP2-DP5) is shown in Table 3.2. The ^{1}H-NMR spectra of the discussed CHOS fractions are shown in Figure 6 and 7 in paper VI. The main oligomers obtained

after degradation to α = 0.07 consisted of deacetylated units with an acetylated reducing end. However, a high presence of the trimer DDD was observed, in addition to increasing amounts of internal **A**-units for the higher DP oligomers. After extensively degradation, the dimer and trimer fractions were dominated by DD and DDD, while the higher DP oligomers were dominated by acetylated reducing ends. Moreover, the higher DP oligomers contained increasing amounts of consecutive **A**-units (Table 3.2). Due to the absolute subsite specificity towards **D**-units in subsite +1, together with the same absolute specificity in subsite -2 when there is an **A**-unit in subsite -1, these sequences are not cleavable by SaCsn75A.

Table 3.2. Composition of isolated oligomers. Chemical composition and sequence of isolated oligomers obtained after degradation of a chitosan with $F_A = 0.31$, degraded to a degree of scission (α) of 0.07 and 0.27. Dominating compounds are shown in bold. The relative abundance in compounds in each of the oligomer fractions is either given in percent (dimer) or may be partly deduced from the overall F_A-values for the fractions that are included in the Table. IA; Insignificant Amounts.

α-value	Dimer		Trimer	Tetramer	Pentamer
0.07	IA		$F_A = 0.19$ **DDA** **DDD**	$F_A = 0.23$ **DDDA** DDDD DDAD* DADD*	$F_A = 0.21$ **DDDDA** DDDDD DADDD* DDADD* DDDAD* DADAD*
0.27 (max)	**DD** 52%	**DA** 48%	$F_A = 0.15$ **DDD** **DDA**	$F_A = 0.23$ **DDDA** DDDD	$F_A = 0.30$ **DDDDA** DDDDD DAADD* DDAAD* DAADA*

* Indicates species with internal **A**-units that do not necessarily all occur, but all the given sequences are compatible with the experimental data.

3.5.2 Degradation of chitosans with varying F_A

Chitosans with widely varying degrees of acetylation (F_A = 0.008, 0.11, 0.31, 0.52, and 0.63) were degraded to maximum α-values of 0.42, 0.34, 0.27, 0.21, and 0.14, respectively. Figure 3.16 shows the size-distribution of the CHOS obtained. The results showed that the size-distribution of oligomers shifted towards higher oligomer lengths when the degree of acetylation increased. For the chitosan with F_A = 0.63, much of the chitosan eluted in the void volume (DP>50). However, for the slightly more deacetylated chitosan with F_A = 0.52, almost no oligomers with DP above 40 were produced. In addition, relatively high yields of oligomers with DP below 10 could be obtained. The chemical composition of these fractions revealed a high content of internal **A**-units and an increased content of the diad AA, reflecting an increased occurrence of non-cleavable stretches of consecutive **A**-units.

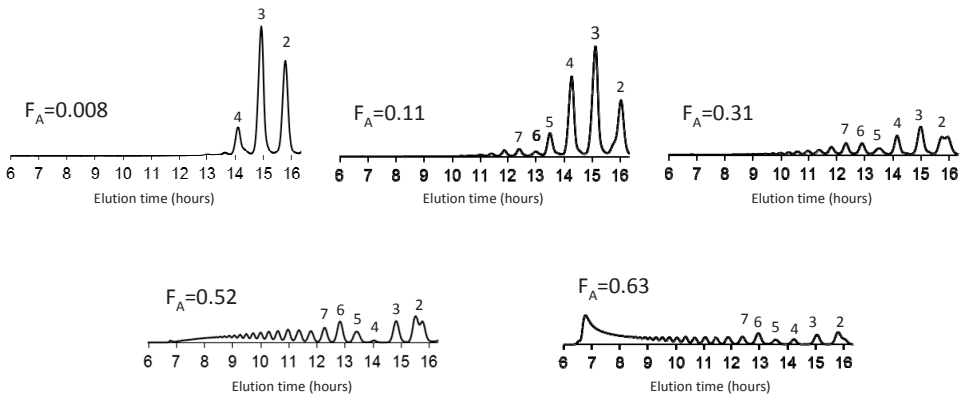

Figure 3.16. Size-distribution of CHOS after extensively degradation of various chitosans using SaCsn75A. SEC chromatograms showing the size-distribution of oligomers obtained after extensively degradation of chitosans with F_A of 0.008, 0.11, 0.31, 0.52 and 0.63 to α-values of 0.42, 0.34, 0.27, 0.21 and 0.14, respectively. Peaks are labeled with DP-values.

3.6 Conclusions

The directionality for the two processive chitinases ChiA and ChiB was studied with an end-labelled and essentially monodisperse chitosan oligomer with DP 20, showing that the enzymes move in opposite directions. The results suggest that their chitin binding domains are involved in the initial endo-binding of this substrate, and that ChiA moves towards the non-reducing end, while ChiB moves in the opposite direction.

Two chitinases and two chitosanases have been characterized with regard to their substrate binding preferences and how they convert chitosans into various types of chitooligosaccharide mixtures. The large variety in properties of the chitinolytic enzymes characterized as a part of this thesis is summarized in Table 3.3.

Table 3.3. Summary of properties of the chitinolytic enzymes which are discussed in this thesis. In cases where both **A**- and **D**-units bind productively to a subsite, the preferred sugar is printed in upper case.

Enzyme	GH family	Extra CBM[a]	Mechanism	Endo/ Exo	Degree of processivity	Subsite specificity		
						-2	-1	+1
Chitinases								
ChiA	18	Yes – 1	Retaining	Endo[c]	N_{cuts} = 9.1	**A/D**	**A**	**A/D**
ChiB	18	Yes – 1	Retaining	Endo[c]	N_{cuts} = 3.4	**A/D**	**A**	**A/D**
HCHT	18	Yes – 1[b]	Retaining	Endo	N_{cuts} = 2.5	**A/D**	**A**	**A/D**
ChiG	19	No	Inverting	Endo	No	**A**	**A/D**	**A**
Chitosanases								
ScCsn46A	46	No	Inverting	Endo	No	**D/A**	**D/A**	**D/A**
SaCsn75A	75	No	Inverting	Endo	No	**D/A**	**A/D**	**D**

[a] The GH family 18 chitinases contain extra chitin binding domains; ChiA contains a Fn3 domain and ChiB contains a chitin binding domain classified as CBM5 (Figure 1.6).

[b] HCHT can contain a chitin binding domain, but it is not necessary for chitinolytic activity. In the present work, HCHT was produced without its chitin binding domain.

[c] ChiA and ChiB has been shown to act in an endo mode of action when degrading chitosan substrates (Sikorski *et al.*, 2006), although they have been reported to show exo-activity when degrading crystalline chitin (Uchiyama *et al.*, 2001; Hult *et al.*, 2005).

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Review

Production of Chitooligosaccharides and Their Potential Applications in Medicine

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Abstract: Chitooligosaccharides (CHOS) are homo- or heterooligomers of *N*-acetylglucosamine and *D*-glucosamine. CHOS can be produced using chitin or chitosan as a starting material, using enzymatic conversions, chemical methods or combinations thereof. Production of well-defined CHOS-mixtures, or even pure CHOS, is of great interest since these oligosaccharides are thought to have several interesting bioactivities. Understanding the mechanisms underlying these bioactivities is of major importance. However, so far in-depth knowledge on the mode-of-action of CHOS is scarce, one major reason being that most published studies are done with badly characterized heterogeneous mixtures of CHOS. Production of CHOS that are well-defined in terms of length, degree of *N*-acetylation, and sequence is not straightforward. Here we provide an overview of techniques that may be used to produce and characterize reasonably well-defined CHOS fractions. We also present possible medical applications of CHOS, including tumor growth inhibition and inhibition of T_H2-induced inflammation in asthma, as well as use as a bone-strengthener in osteoporosis, a vector for gene delivery, an antibacterial agent, an antifungal agent, an anti-malaria agent, or a hemostatic agent in wound-dressings. By using well-defined CHOS-mixtures it will become possible to obtain a better understanding of the mechanisms underlying these bioactivities.

Keywords: chitooligosaccharide (CHOS); chitinase; chitosanase; chitosan; application

1. Introduction to Chitin, Chitosans and Chitooligosaccharides (CHOS)

Chitin is a linear polysaccharide consisting of $\beta(1\rightarrow4)$ linked *N*-acetyl-*D*-glucosamine (GlcNAc; **A**) residues. It is often considered the second most abundant polysaccharide in nature, after cellulose, and occurs mainly as a structural component in the cell walls of fungi and yeasts and in the exoskeletons of insects and arthropods (e.g., crabs, lobsters and shrimps). Chitin is insoluble in water and exists mainly in two crystalline polymorphic forms, α and β. α-Chitin consists of sheets of tightly packed alternating parallel and antiparallel chains [1] and is found in the exoskeleton of arthropods, in insects and in fungal and yeast cell walls. In β-chitin the chains are arranged in parallel [2]. β-chitin occurs less frequently in nature than α-chitin, but can be extracted from squid pens.

Chitosan can be prepared from chitin by partial deacetylation, and is a heteropolymer of GlcNAc and D-glucosamine (GlcN; **D**) residues. Unlike chitin, chitosan is soluble in dilute aqueous acid solutions. The name chitosan refers to a continuum of soluble polymeric chitin derivatives that can be described and classified according to the fraction of *N*-acetylated residues (F_A) or degree of *N*-acetylation (DA), the degree of polymerization (DP) or the molecular weight (M_W), the molecular weight distribution (PD, for PolyDispersity), and the pattern of *N*-acetylation (P_A) or sequence. Chitosan exhibits a variety of interesting physicochemical and biological properties. This, in combination with its non-toxicity, biocompatibility and biodegradability, makes chitosan suitable for use in numerous applications in agriculture, cosmetics, water treatment and medicine [3–7].

Chitooligosaccharides (CHOS) are oligomers prepared from chitosan either chemically or enzymatically. Chitosan can be converted to CHOS by acid hydrolysis or by enzymatic hydrolysis with glycosyl hydrolases like chitinases or chitosanases. The F_A, M_W, PD and P_A of the resulting CHOS-mixture depend on the chitosan and the specificity of the enzyme used. As described below, product mixtures can be enriched for certain compounds by optimizing the chitosan-enzyme combination.

There is ample literature concerning the biological effects of chitosans, but relatively little is known about the effects of CHOS [6,8], and the mechanisms behind observed bioactivities are generally poorly understood. CHOS are primarily thought to interact with proteins that either act on chitin (e.g., chitinases) or that are supposed to bind to and detect chitin. When it comes to medicine, there are indications that CHOS may be developed as drugs against asthma [9–12], antibacterial agents [13], ingredients in wound-dressings [14,15] and vectors in gene-therapy [16,17]. Furthermore, according to the literature, CHOS may reduce metastasis and tumor-growth in cancer [18–20], increase bone-strength in osteoporosis [21,22] and could be used to inhibit chitinases in *Plasmodium* parasites and thereby prevent malaria [23]. Several other potential effects of CHOS have been described, including immune modulatory effects [24], anti-fungal activities [25,26] and a lowering effect on serum glucose levels in diabetics [27]. In the context of this review, it should be noted that some of the biological effects reported for chitosan in fact may be due to CHOS, which emerge when chitosan is degraded by naturally occurring hydrolytic enzymes.

So far, most of the research on bioactivities of CHOS has been done with badly defined mixtures containing CHOS of various F_A, M_W, PD and P_A. Moreover, the CHOS fractions appearing in the literature are derived from various sources of chitosan that are not always traceable and that have been characterized to only a limited extent. Clearly, when using complex mixtures of CHOS in bioassays, it is difficult to know which molecule/molecules are causing the effects. Furthermore, reproducibility can be an issue. All in all, while interesting biological activities have been observed, there is little information on the molecular properties that confer bioactivity to a particular CHOS.

In this review we provide an overview of some of the most promising applications of CHOS. Furthermore, we describe current knowledge on how well-defined mixtures of CHOS can be prepared and characterized. It is possible to prepare CHOS from chitosan by using different physical methods, like hydrothermal [28], microwave [29], ultrasonication [30] and gamma-rays [31], but these methods are not optimal for creating well-defined CHOS-mixtures and will not be dealt with in this review. Chemical methods using acid [32,33], H_2O_2 [34] or $NaNO_2$ [35], can yield CHOS and one of these methods is described briefly below. We will, however, focus our review on the enzymatic production of CHOS, and on further separation and purification methods for producing well-defined mixtures.

2. Chitosan, the Starting Material for CHOS Production

It is possible to isolate chitosan directly from the cell walls of certain fungi, but commercially available chitosans are usually prepared from chitin in a heterogeneous deacetylation process. Chitosans will in this paper be defined as proposed by Roberts [36], *i.e.*, according to their solubility at acidic pH-values. This distinguishes clearly between chitins and chitosans, although some controversy may exist when discussing chitin oligomers (*N*-acetyl-CHOS) and chitosan oligomers (CHOS). To avoid this problem, we restrict the terms chitin and chitosan to polymers with more than 100 sugar units.

Chitosans can be prepared from chitin by two fundamentally different methods, *i.e.*, homogeneous [37] and heterogeneous deacetylation. In the homogeneous deacetylation process, the chitin is dissolved in an alkali solution during the deacetylation process (at low temperature and using excessive stirring). In the heterogeneous deacetylation process, the chitin is kept insoluble in a hot alkali solution, meaning that this is a two-phase process. Chitosans with F_A varying from 0 to about 65% can be prepared by homogeneous deacetylation of chitin [37]. There is little controversy regarding the distribution (P_A) of the two monomers in these chitosans, which have been found to have a random distribution of the GlcNAc and GlcN units, *i.e.*, according to Bernoullian distribution [38–40]. There is, however, more controversy about the distribution of sugar units in chitosan prepared by heterogeneous deacetylation procedures. Weinhold *et al.* [41] studied the *N*-acetylation patterns of 32 different chitosans of various F_A, using a previously described NMR-method [39], most of them prepared by heterogenous deacetylation, and found a close to random distributions for all samples. Although this recent study indicates that the P_A in chitosans prepared by heterogenous deacetylation is more random than previously assumed, it should be kept in mind that the NMR-method is only capable of determining an average distribution, meaning that certain block patterns may in fact exist.

The annual production of chitin in nature has been estimated to approximately 10^{10}–10^{11} tons per year [42]. The amounts of chitin, chitosan and their derivatives that are used/produced in industrial

processes [43] have been estimated to be about 30 000 metric tons for chitin and about 10,000 metric tons for chitosan in 2007 [44]. Most of the chitin is used as raw material for the production of the monosaccharide GlcN, which is the number one dietary supplement in the USA, used for pain relief of osteoarthritis [43].

3. Enzymes Acting on Chitin and Chitosan

3.1. Chitinases and chitosanases

Enzymatic depolymerization of chitin and chitosan involves chitinases and chitosanases, respectively (Figure 1). These enzymes hydrolyze the glycoside bonds between the sugars and are thus glycoside hydrolases (GH). Such GH are classified in the Carbohydrate-Active enZYmes database (CAZy) [45–48] that provides a continuously updated list of the GH families and, since a few years, also other families of carbohydrate-active enzymes such as glycosyl transferases and carbohydrate esterases. The CAZy classification is based on the amino acid sequence, which gives very useful information since sequence and structure, and hence mechanism, are related. It should be noted that the CAZy system does not take substrate and product activities into account *a priori*. The same applies to enzyme properties such as exo- *versus* endo-binding, processivity, and the presence of additional modules that may improve substrate-binding. All these properties are not taken into account either. The CAZy classification is purely based on amino acid sequence similarities and, indeed, several GH classes contain enzymes acting on a variety of substrates. Many GH enzymes have one or more carbohydrate binding domains in addition to their catalytic domain (Figures 1 and 2). These are referred to as carbohydrate-binding modules (CBMs) and are also classified in the CAZy database.

Chitinases occur in families GH18 and GH19 and both these classes almost exclusively contain these enzymes. Chitinases have the unique ability to hydrolyze **A**-**A** bonds and this property discriminates these enzymes from chitosanases. However, as described below, chitinases are perfectly capable of hydrolyzing chitosan, albeit to different extents. Chitinases do not hydrolyze **D**-**D** bonds.

Enzymes with chitosanase activity have been found in GH families 5, 7, 8, 46, 75 and 80. GH7 is a cellulase family and in a very few cases chitosanase activity has been detected as a side activity of these enzymes. GH5 contains a variety of enzymatic activities, including chitosanases, cellulases, licheninases, mannanase and xylanases. Again, chitosanase activity has been detected in only a very few cases, and the activity seems to be a side activity of cellulases. In GH8, enzymes annotated as chitosanases occur more frequently (next to e.g., cellulases and xylanases), and this family seems to contain a few true chitosanases [49]. The other three families, GH46, GH75 and GH80, exclusively contain chitosanases. Families GH75 and GH80 have only a few members and there is no structural and only very little functional information available for these enzymes. The best studied chitosanases, by far, are those belonging to family 46 [50–52] (Figure 1).

Based on their substrate specificity towards chitosan, chitosanases have been classified into subclasses I, II and III [53]. Chitosanases in subclass I can hydrolyze **A**-**D** and **D**-**D** linkages, subclass II enzymes can hydrolyze **D**-**D** linkages only, whereas subclass III enzymes can hydrolyze **D**-**A** and **D**-**D** linkages. Subclass I enzymes have been found in both families 46 and 75 [54,55]. In family 46, subclass III enzymes have also been found [52].

Figure 1. Structures of the enzymes discussed in detail in this review. Figure 1a and 1b show, respectively, ChiA and ChiB from *Serratia marcescens*. Figure 1c shows hevamine, a plant family 18 chitinase whose structure is thought to resemble the (unknown) structure of the catalytic domain of ChiC from *Serratia marcescens*. Figure 1d shows ChiG from *Streptomyces coelicolor* A3(2). Figure 1e shows CsnN174, a family 46 chitosanase from *Streptomyces* sp. N174, which, judged from sequence similarity, is highly similar to Csn88 from *Streptomyces coelicolor* A3(2). The side chains of the catalytic acid and of the catalytic base/nucleophile are shown.

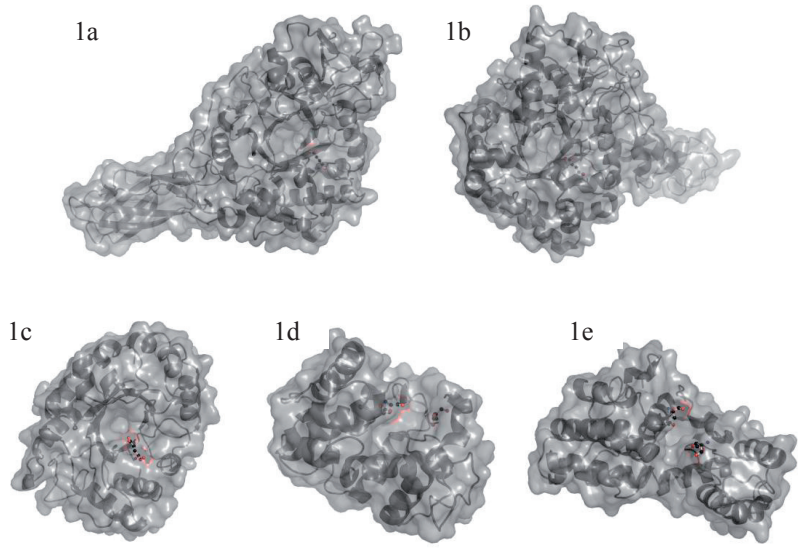
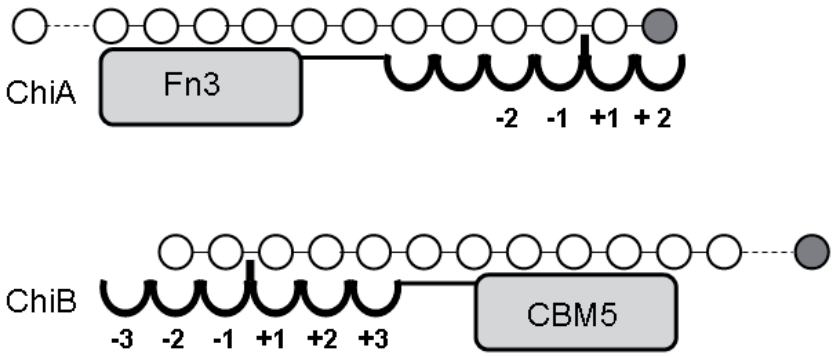


Figure 2. Schematic drawing of subsites, chitin binding domains and proposed orientation of polymeric substrates in ChiA and ChiB. Fn3, Fibronectin type 3 domain (substrate-binding); CBM5, chitin binding module. Dotted lines indicate that the polymer substrates are much longer than shown in the figure. Reducing end sugars are shown in grey. Figure and legend are from Horn *et al.* [56], and is reproduced with permission from Wiley-Blackwell.



3.2. Catalytic mechanism

The hydrolysis of the glycosidic linkage is a nucleophilic substitution at the anomeric carbon, and can lead to either retention or inversion of the anomeric configuration [57,58]. Both hydrolysis reactions take place through general acid catalysis, and require a pair of carboxylic acids at the enzyme's active site. One carboxylic acid is acting as a proton donor, facilitating leaving group departure, and the other acts as a base (inverting mechanism) or as a nucleophile (retaining mechanism). In both mechanisms, the position of the proton donor is within hydrogen-bonding distance of the glycosidic oxygen. The inverting mechanism (also called the single displacement mechanism) is a "one-step" reaction, where the protonation of the glycosidic oxygen occurs simultaneously with a nucleophilic attack on the anomeric carbon by an activated water molecule. This water molecule is located between a carboxylic group and the anomeric carbon and it is activated by the carboxylic group that acts as a base. Since the water molecule approaches the anomeric carbon from the side of the catalytic base, this mechanism leads to inversion of the anomeric configuration. Chitosanases belonging to families GH46, GH75 and GH80 and family 19 chitinases use the inverting mechanism [45,47,49,54,59–61].

The retaining mechanism (also referred to as the double displacement mechanism) is a two-step reaction, were the first step involves the protonation of the glycosidic oxygen (by the catalytic acid) and a congruent nucleophilic attack on the anomeric carbon atom by the nucleophile (the second carboxylic acid). This attack leads to breakage of the glycosidic linkage and the formation of a covalent linkage between the anomeric carbon and the catalytic nucleophile [62]. Subsequently, this intermediate is hydrolyzed by a water molecule that approaches the anomeric carbon from a position close to that of the original glycosidic oxygen, leading to retention of the anomeric carbon configuration.

Family 18 chitinases use a special variant of the double displacement mechanism, referred to as the substrate-assisted double displacement mechanism. Here, the carbonyl oxygen atom from the *N*-acetylgroup of the sugar bound in subsite -1 act as the nucleophile, leading to formation of an oxazolinium ion intermediate. Because of this involvement of the *N*-acetylgroup in catalysis, productive substrate-binding of chitosan and chitosan oligomers to family 18 chitinases requires that a GlcNAc is bound in the -1 subsite [63–67].

Chitinases and chitosanases can have endo- or exo-activity, where both the endo- and exo-mode of action can be combined with processivity. Processive enzymes will not release the substrate after one cleavage, but remain associated with the substrate so that a new cleavage can take place as the polymer substrate slides through the substrate-binding cleft (Figure 2). Processivity is difficult to analyze when degrading insoluble substrates such as chitin, but can be studied when using chitosan as substrate [56,64,68,69]. Whereas processivity generally is considered to be favorable for the hydrolysis of crystalline substrates, processivity has been shown to reduce enzyme efficiency towards soluble and more accessible polymeric substrates such as chitosan [69,70]. Thus, for the industrial production of CHOS, the use of non-processive enzyme variants may be beneficial in some cases.

3.3. Human chitinases

Even though chitin and chitin synthases have not been found in humans, we produce two family 18 chitinases with chitinolytic activity [71]. In addition, two highly homologous proteins named chi-lectins and a homologous protein called oviductin are expressed. These proteins lack enzymatic activity while having retained the typical features of family 18 enzymes, including carbohydrate binding to the active site [71].

One of the human chitinases, called chitotriosidase (HCHT), is synthesized and secreted as a 50-kDa two-domain protein in human macrophages [72]. A significant portion of produced enzyme is routed to lysosomes and processed into a 39-kDa isoform, lacking the C-terminal chitin-binding domain [73]. HCHT was first discovered as a marker for Gaucher disease [72], but has later been associated with several diseases like malaria [74], fungal infections such as candidosis [75], sarcoidosis [76,77], cardiovascular risk [78], coronary artery disease [79], primary prostate cancer and benign prostatic hyperplasia [80], nonalcoholic steatohepatitis [81], multiple sclerosis [82], and Niemann-Pick disease [83]. The other chitinase, acidic mammalian chitinase (AMCase), is also synthesized as a two-domain 50 kDa protein containing a 39 kDa N-terminal catalytic domain and a C-terminal chitin-binding domain. AMCase is found in the stomach [84], in tears [85], sinus mucosa [86], and lungs [12,84]. Chitinases play important roles in the antiparasite responses of lower life forms [87–89]. Both HCHT and AMCase are believed to play similar roles in the human immune defense system, being a part of antiparasitic responses [10,87,90].

The two chi-lectins are associated with numerous diseases. YKL-40 (alternatively called HCgp39 or CHI3L1) is observed in elevated levels for patients with severe asthma [91], cardiovascular disease and diabetes [92], cancer [93], peritoneal endometriosis [94], morbid obesity [95], osteoarthritis [96], and liver fibrosis [97]. The other chi-lectin YKL-39 (or CHI3L2) has been observed up-regulated in osteoarthritic chondrocytes [98] and osteoarthritic cartilage [99]. The loss of hydrolytic activity in these chi-lectins is due to replacement of the catalytic acid (E) and the adjacent aspartic acid (D) in the conserved DxxDxDxE motif with Ala and Leu or Ser and Ile for YKL-40 and YKL-39, respectively. The chi-lectins have retained their ability to bind CHOS [100] and this may be a feature underlying some of the alleged biological effects of CHOS.

3.4. Inhibition of family 18 chitinases with CHOS

Family 18 chitinases are of particular interest, because they are abundant in nature, are crucial in the life cycles of numerous plague and pest organisms, and because they occur in humans themselves. Because of the substrate-assisted catalytic mechanism of GH18 enzymes, catalysis requires that a GlcNAc is bound to the -1 subsite. CHOS that preferably bind in such a way that a GlcN ends up in the crucial -1 subsite will act as an inhibitor. Sugar binding to the -1 subsite leads to an energetically unfavorable distortion [101] which involves the *N*-acetylgroup [66,67] and which may amount to an unfavorable ΔG as high as ~8 kcal/mol [102]. Although this has not yet been substantiated by experimental data, it seems plausible that binding of GlcN in the -1 subsite in fact could be energetically less unfavourable than binding of a GlcNAc. Thus, binding of a GlcN would be non-productive, but perhaps stronger than binding of a GlcNAc. This shows that the idea of developing

partially deacetylated CHOS as inhibitors for family 18 chitinases is worth pursuing. Indeed, the validity of this idea has been substantiated by an early study by Peter and co-workers [103] as well as by more recent work [104,105].

For chitinase B of *Serratia marcescens*, the -2 subsite has a strong preference for an GlcNAc [67]. The oxygen atom in the acetamidogroup of the -2 sugar forms a bifurcated hydrogen bond with Trp ^{403} and Gln ^{407}, whereas the methyl group packs tightly in an apolar environment provided by the side chains of Tyr ^{292} and Ile ^{337}. Thus, the -2 subsite of this enzyme seems optimized for strong binding of an GlcNAc, which is not surprising taking into account that the positive effects of binding sugars in subsites adjacent to the -1 subsite is needed to "pull" the -1 sugar in its distorted conformation [106]. Indeed, one has observed non-productive binding by a **DADAA** oligomer bound from subsites -3 to +2 in chitinase B [104].

CHOS being based on the substrate, hold a tremendous advantage in being very specific inhibitors towards chitinases, and hence not likely to interfere with other enzymatic systems. Moreover, the binding strength of the CHOS based inhibitor, an important parameter, can be tuned simply by increasing the chain length of the CHOS or by coupling additional groups to the reducing end.

3.5. Lysozyme

In addition to its natural substrate, the glycosidic linkage of certain bacterial cell wall peptidoglycans, lysozyme may also hydrolyze chitin and chitosans [107]. In very early work, Amano and Ito [107] studied oligomers formed upon lysozyme degradation of an F_A = 0.32 chitosan, and identified the fully *N*-acetylated trimer and tetramer together with the partially *N*-acetylated oligomers **AAD**, **DAA**, **AAAD**, **ADAA** and **ADAD** among the oligomeric products. Later, Vårum *et al.* [108] studied lysozyme degradation of a highly *N*-acetylated chitosan (F_A = 0.65). In this study, NMR-spectroscopy of the degradation products was used to determine the identities of the newly formed reducing and non-reducing ends. This methodology, which has later been used to characterize chitinases (see below), provided insight into the cleavage specificities of the enzyme, *i.e.*, its preference for cleaving **A-A**, **A-D**, **D-A** and/or **D-D** linkages in chitosans. Some information on the identity of the nearest neighbors to the new reducing and non-reducing ends could also be obtained.

4. CHOS Production—Enzymatic Methods

So far, there are no robust enzymatic methods for the production of chitosan that could provide an alternative to the current chemical production methods. In principle, chitin deacetylases could be used to produce chitosan [109–113]. These enzymes hydrolyze the *N*-acetyl linkage and convert GlcNAc to GlcN. However, the insolubility and crystallinity of the chitin substrate forms a major hurdle for this approach. Chitin deacetylases could also be used to modify the *N*-acetylation pattern of CHOS, but this route has so far remained unexplored.

Although there are routes for chemical conversion of chitosan to CHOS [7] (see below), even CHOS with specific DP and P_A, enzyme technology probably is the most promising approach. The specificity of chitosan-degrading enzymes has traditionally been studied by extensive enzymatic degradation of the polymer and subsequent isolation and characterization of the resulting oligomers. More recently, studies with chitinases have shown that the kinetics of the degradation reactions is such

that product profiles change considerably during the hydrolysis reaction. Because the enzymes have very different binding affinities for different sequences on the substrate, reactions show multiphasic kinetics, and the product mixtures obtained at the end of each of these phases differ considerably. Another important issue is processivity; degradation processes may change during a reaction, from initial mainly processive hydrolysis of polymeric chains to non-processive hydrolysis of intermediate products as the polymeric material becomes exhausted. All in all, this means that the choice of the starting chitosan, the choice of the enzyme, and the choice of the processing time all affect the outcome of the enzymatic conversion process and that there are ample opportunities to manipulate this outcome [114]. This is illustrated by several studies on enzymatic degradation of chitosans [56,60,107,114–117], some of which are discussed in detail below. Structures of the enzymes discussed below are shown in Figure 1, whereas Table 1 shows some key properties.

Table 1. Some properties of the five enzymes that are specifically discussed in this review.

Enzyme	GH fam	Extra CBM[1]	Mechanism	Endo/Exo	Processivity	Subsite specificity		
						-2	-1	+1
Chitinases:								
ChiA	18	Yes (1)	Retaining	Endo/exo[2]	Yes	A/D	A	A/D
ChiB	18	Yes (1)	Retaining	Endo/exo[2]	Yes	A/D	A	A/D
ChiC	18	Yes (2)	Retaining	Endo	No	A/D	A	A/D
ChiG	19	No	Inverting	Endo	No	A	A/D	A
Chitosanase:								
Csn88	46	No	Inverting	Endo	No	D/A	D/A	D/A

[1] ChiA and ChiB are compact two domain enzymes containing an Fn3 domain and a chitin-binding domain classified as CBM5 in addition to their catalytic domain, respectively (Figures 1 and 2). The crystal structures of complete ChiA and ChiB are known (Figure 1). In ChiC, the catalytic domain is connected to an Fn3-like domain and a chitin-binding domain classified as CBM12 by a proline- and glycine-rich linker, which tends to be proteolytically cleaved *in vivo*. It has so far not been possible to determine the crystal structure of intact ChiC.

[2] It has been shown that ChiA and ChiB primarily act as endo-processive enzymes on chitosan [118]. This is probably also the case on chitin, although there may be more exo-activity in this case [119]. In any case, the endo-/exo- difference is of little relevance for enzymes that act processively.

4.1. Degradation of chitosan by family 18 chitinases

The degradation of chitosan by the family 18 chitinases, ChiA, ChiB and ChiC, from *Serratia marcescens* has been studied in much detail [56,64,69,118]. Figure 3 shows the size-distribution of oligomers obtained upon degradation of a highly *N*-acetylated chitosan (F_A = 0.65) to various extents of degradation (α). For ChiA and ChiB, the product profiles obtained during the initial phase of the degradation show a dominance of even-numbered oligomers, which is indicative of processive action [56,64,68]. This product pattern is due to the fact that enzyme-ligand complexes where there is a GlcN bound to the -1 subsite is not productive (in the case of family 18 chitinases). If the enzyme is processive, the enzyme will slide by two sugar units at the time, until a productive complex is formed

[the primary condition being that there is an GlcNAc bound in the -1 subsite (see Sørbotten *et al.* [64] and Eijsink *et al*. [68] for a more detailed discussion)]. Consequently, while the first product of an enzyme-substrate association may have any length, every subsequent product will be even-numbered. In the case of chitin, all these products would be dimers; in the case of chitosan, these products may be longer even-numbered oligomers. Later during the reaction, the dominance of even-numbered oligomers disappears because there are no longer substrate molecules left and the enzyme is primarily involved in rebinding and further cleavage of oligomers from the preceding processive phase (see Figure 3). For example, an oligomer such as **ADADAA** that could emerge during processive degradation by ChiB can be converted by this same enzyme to **ADA** and **DAA** upon rebinding in a mode that was not explored during the processive phase, where the substrate moves by two sugars at the time (see Horn *et al.* [56] and Eijsink *et al.* [68] for a more extensive discussion and explanation).

Figure 3. Degradation of chitosan (F_A 0.65) by ChiA, ChiB and ChiC from *Serratia marcescens*. The pictures show chromatograms from size-exclusion chromatography. The peaks are marked by numbers which indicate the lengths (DP) of the oligomers they contain, or, in the case of peaks containing only one known compound, by the sequence of the oligomer. The annotation of the peaks is based on the use of standard samples, as well as NMR analyses. The α-values denote the degree of scission [full conversion of the chitosan to dimers only (DP_n = 2) would give an α = 0.5 (α = 1/DP_n)]. The lower panels represent the maximum obtainable α-values. Undegraded chitosan and fragments with a DP > 40 elute in the void volume of the column. The figure is from Horn *et al.* [56], and is reproduced with permission from Wiley-Blackwell. Additional product profiles at very low α for ChiA and ChiB that clearly reveal processivity have been published in Sikorski *et al.* [118].

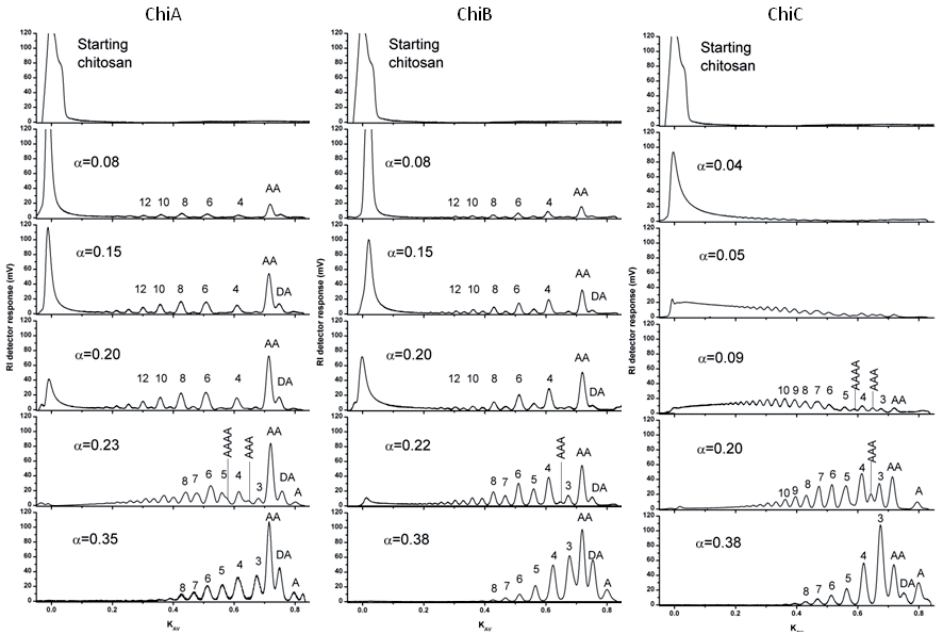


Figure 3 shows a totally different product pattern for ChiC that is characteristic for an endo-acting, non-processive enzyme. ChiC converts chitosan to a continuum of oligomers of different sizes and the polymer peak disappears early in the degradation reaction. Also, there is initially no accumulation of dimers or other even numbered oligomers. This all indicates that ChiC attacks the polymeric substrate chains in random positions, without processivity. Indeed, this "endo" activity could be confirmed by viscosity measurements during the hydrolysis reaction [118]. The contrast with ChiA and ChiB is perhaps best illustrated by the void peak, which disappears much more slowly in the case of processive enzymes. These latter enzymes perform many cuts per chain instead of a few cuts in every chain as in the case of ChiC.

Analysis of the sequences of the products (Table 2) showed considerable differences between the enzymes as well as differences over the time course of the degradation reactions. In all three enzymes, productive binding requires a GlcNAc in subsite -1, explaining why all products have a GlcNAc at their reducing ends. The enzymes did not have any detectable preferences for GlcNAc versus GlcN in the +1 subsite, and this is reflected in the oligomers having both *N*-acetylated and deacetylated non-reducing ends. All three chitinases showed a strong, but not absolute, preference for GlcNAc in subsite -2, meaning that oligomers preferentially have an *N*-acetylated unit next to the reducing end. The kinetics of the reaction with ChiB illustrate this (Figure 4; Table 2): in the initial rapid phase of the reaction almost all oligomeric producs have **–AA** at their reducing ends; during the second, much slower phase, oligomeric products ending at **–DA** appear to a larger extent [56, 64]. The preference for a GlcNAc in subsite -2 was strongest and in fact almost absolute for ChiC; all oligomeric products end with **–AA**, at any point during the reaction (Table 2).

Table 2. Composition of dimer, trimer and tetramer fractions at different α-values during degradation of chitosan (F_A = 0.65) by ChiA, B and C. Data from Horn *et al.* [56]. Reproduced with permission from Wiley-Blackwell.

Enzyme	α	Dimer	Trimer	Tetramer
ChiA	0.15	81% AA 19% DA	81% DAA 19% ADA	100% -AA
	0.35	64% AA 36% DA	51% DAA 28% ADA 21% DDA	56% -AA 44% -DA
ChiB	0.11	86% AA 14% DA	71% DDA 29% AAA	100% -AA
	0.38	66% AA 34% DA	95% DAA 3% DDA 2% ADA	75% -AA 25% -DA
ChiC	0.20	100% AA	66% DAA 34% AAA	100% -AA
	0.38	81% AA 19% DA	100% DAA	100% -AA

Figure 4. Time course of the degradation of a chitosan with F_A 0.65 by ChiB from *Serratia marcescens*. The graph shows the degree of scission (α) as a function of time; the biphasic kinetics is clearly visible. The slow phase continues until α reaches a value of about 0.37. Figure from Sørbotten *et al.* [64]. Reproduced with permission from Wiley-Blackwell.

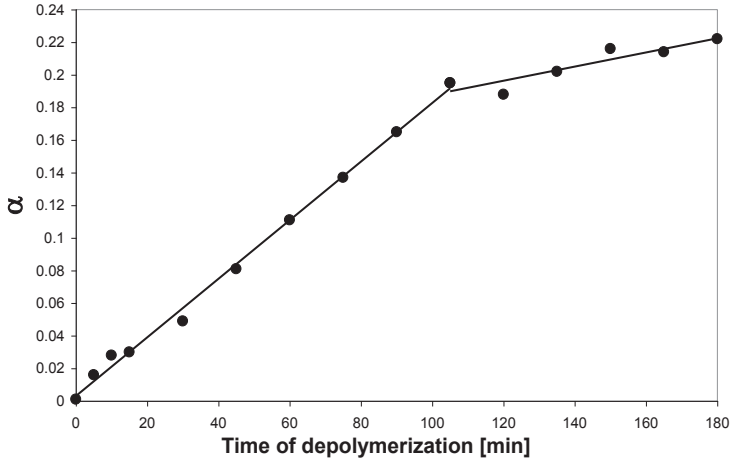


Figure 5. Size-distribution of oligomers after extended hydrolysis of various chitosans with ChiB from *Serratia marcescens*. The pictures show chromatograms revealing the size-distribution of oligomers obtained upon extended hydrolysis of chitosans with F_A of 0.65, 0.50, 0.32 and 0.13 to α-values (corresponding DP_n-values in brackets) of 0.37 (2.7), 0.34 (2.9), 0.22 (4.5) and 0.11 (9.5), respectively. Figure from Sørbotten *et al*. [64]. Reproduced with permission from Wiley-Blackwell.

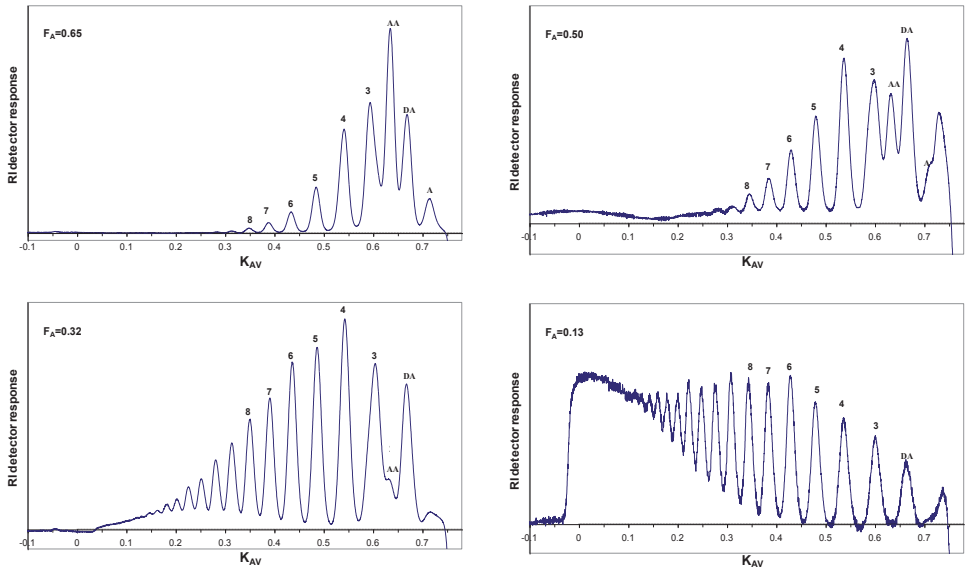
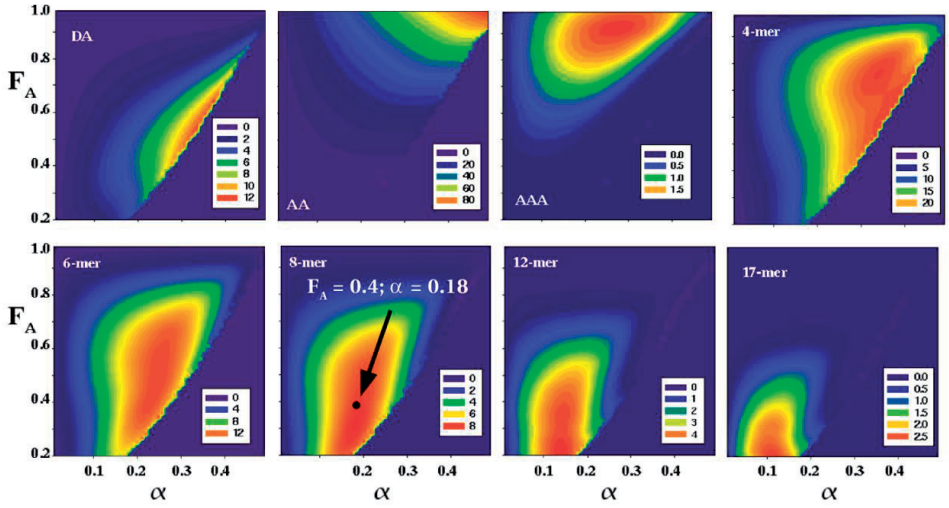


Figure 5 shows how the outcome in terms of the length distribution of products can be manipulated by varying the F_A of the chitosan. Obviously, since we are working with chitinases with clear preferences for GlcNAcs at certain positions in the substrate, the products get longer as the F_A goes down. It is quite remarkable that a chitinase such as ChiB works well on chitosans with F_A close to only 10%.

Sikorski *et al.* [114,118] have produced a model for the degradation of different chitosans with ChiB, which is capable of accurately predicting the outcome of hydrolysis reactions in terms of the length distributions of the products at varying α. This model can be used to predict how reactions need to be set up in order to maximize the production of CHOS of certain lengths (Figure 6).

Figure 6. 2D profiles showing the predicted outcome of chitosan hydrolysis with ChiB from *Serratia marcescens*. The X-axis shows the degree of scission, α, and the Y-axis shows the F_A of the starting chitosan. The predicted amount of a particular product at specific α - F_A combinations is indicated by color (the amounts of oligomers are expressed as % of the total mass of the polymer in the hydrolysis reaction and color coded as defined in the inserts). These profiles allow for selection of optimal reaction and substrate parameters for efficient production of oligomers with desired lengths. For example, high yields of octamer could be obtained if chitosan with F_A 0.4 is hydrolyzed to α = 0.18 (the arrow indicates the maximum level of octamers). For example, for the octamer, at maximum yield conditions, approximately 8% of the polymer is expected to be converted to octamers. Figure taken from Sikorski *et al.* [114], and reproduced with permission from Wiley-Blackwell.



4.2. Degradation of chitosan by family 19 chitinases

ChiG, a bacterial family 19 chitinase from *Streptomyces coelicolor* A3(2), produces quite different CHOS as compared to the family 18 chitinases, reflecting the very different binding preferences in the active sites of the enzymes [115]. Since ChiG, which operates according to a non-processive

endo-mode of action (Figure 7), uses the inverting mechanism, there is no absolute requirement for GlcNAc in subsite -1. This means that the reducing ends of the oligomeric products could be both *N*-acetylated and deacetylated, as was indeed observed. The non-reducing ends of the oligomers were found to be exclusively *N*-acetylated, and the sugar units in the neighboring position to reducing ends were also found to be exclusively *N*-acetylated. Thus, ChiG has an absolute preference for a GlcNAc in subsites -2 and +1. The enzyme also has considerable preference for GlcNAc in -1 and kinetics were clearly biphasic: In the first fast phase cleavage occurred in **A-A$^{\downarrow}$A** sequences, whereas **A-D$^{\downarrow}$A** sequences were cleaved in the slower second phase [115]. So, while the chromatograms of Figure 7 may look somewhat similar to the chromatogram for the family 18 enzyme ChiC in Figure 3, the sequences of the produced oligomers show considerable differences, which may affect bioactivity. More generally, it is clear that ChiG will yield oligomers of different P_A as compared to those obtained by ChiA, ChiB and ChiC. For example, degrading a chitosan with F_A = 0.65 with ChiB and ChiG gives the trimers **DAA** and **AAD**, respectively [64,115].

Figure 7. Size-distribution of oligomers emerging during hydrolysis of chitosan with F_A 0.64 by ChiG from *Streptomyces coelicolor* A3(2). See legend to Figure 3 for further explanation. Figure from Heggset *et al*. [115]. Reproduced with permission from American Chemical Society.

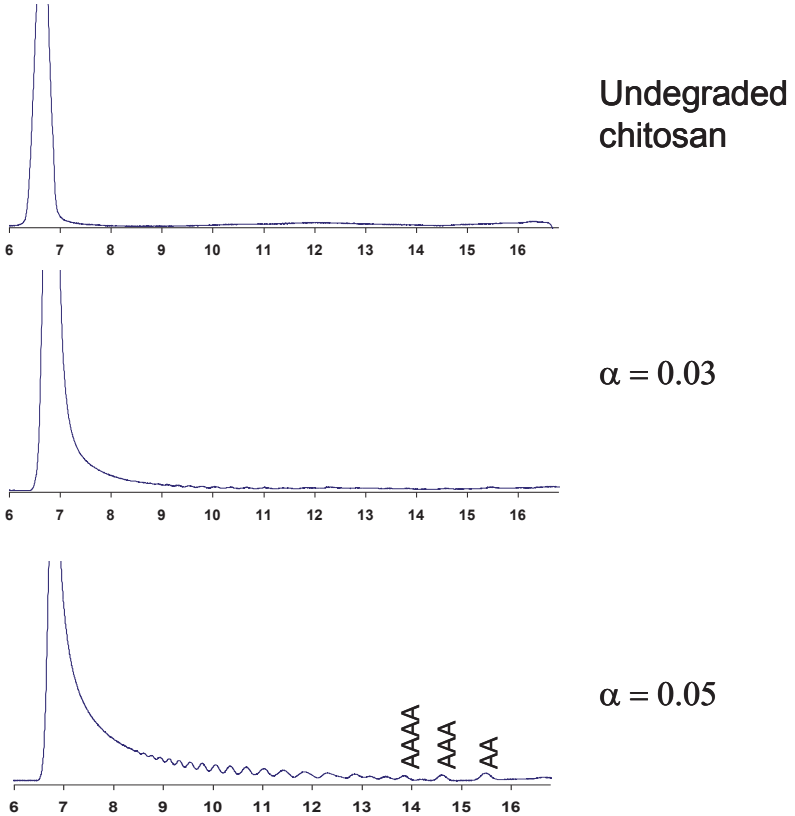


Figure 7. *Cont.*

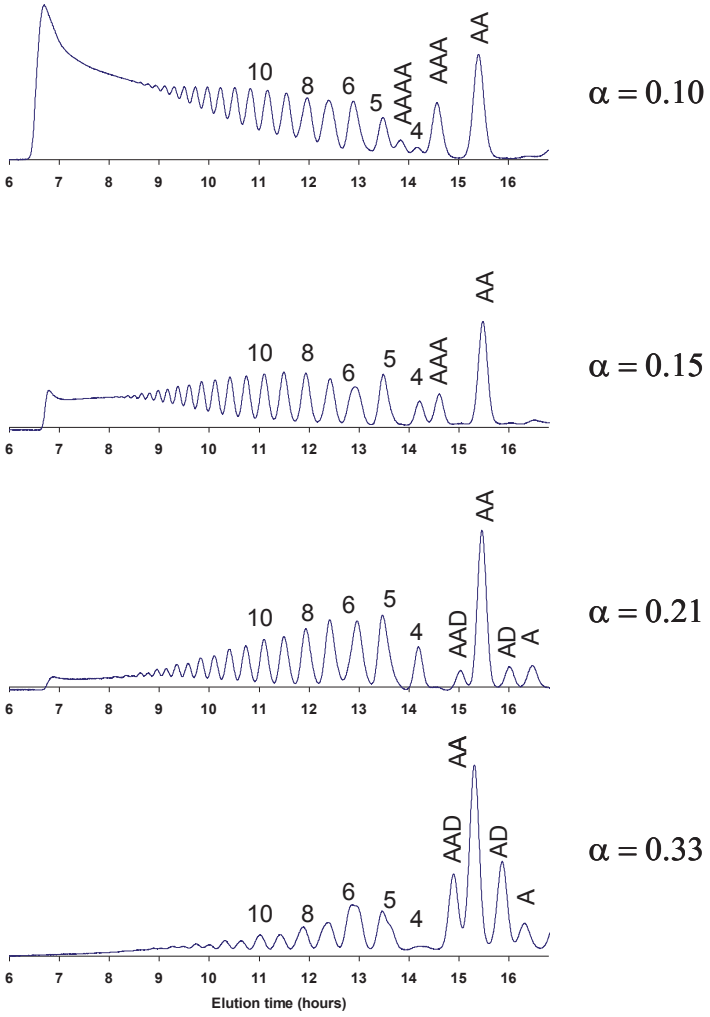
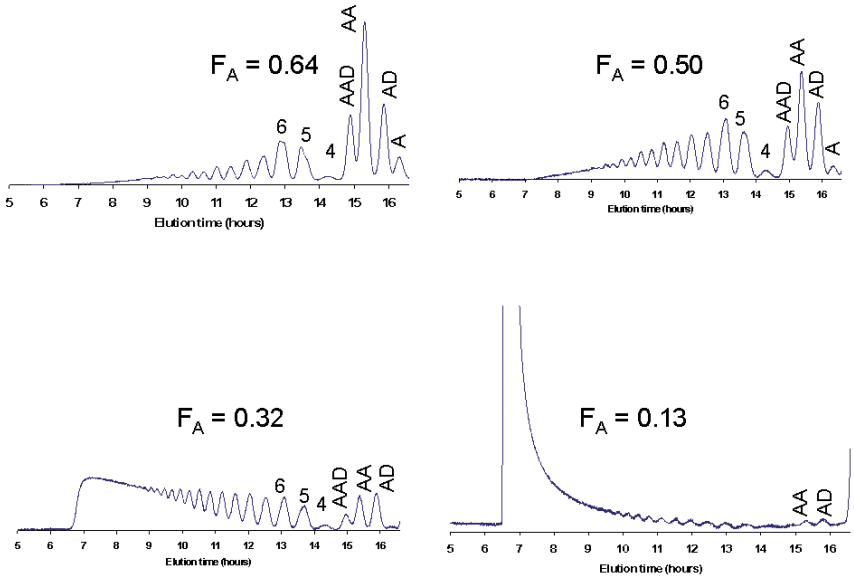


Figure 8 shows that varying the F_A of the chitosan had a major effect on the size distribution of the products and the extent of cleavage. Interestingly, ChiG efficiency is much more sensitive for deacetylation than the efficiency of ChiB (compare Figure 8 with Figure 5).

Other studies on the degradation of chitosan with family 19 chitinases confirm the findings for ChiG. In one study, the family 19 chitinase ChiC from *Streptomyces griseus* HUT 6037 was used to (extensively) degrade a chitosan with a degree of *N*-acetylation of 47% and the products were isolated and characterized [116]. The identities of the isolated CHOS were **AD**, **AAD**, **ADAD**, **ADDAA** and **AADDAA**, and it was concluded that this enzyme has an absolute specificity for *N*-acetylated units in the -2 and +1 subsites, in agreement with the results for ChiG from *Streptomyces coelicolor*. A family 19 chitinase from rice [117] has been found to operate according to a non-processive endo-mode of

action, with strong preferences for *N*-acetylated units in subsites -2 and +1, and with less strong preference for *N*-acetylated units in the -1 subsite.

Figure 8. Size-distribution of oligomers after extended hydrolysis of various chitosans with ChiG from *Streptomyces coelicolor* A3(2). The chitosans with F_A of 0.13, 0.32, 0.50, and 0.64 were degraded to maximum α-values of 0.04, 0.12, 0.23, and 0.33, respectively. Peaks are labeled as in Figure 3. Figure from Heggset *et al*. [115]. Reproduced with permission from American Chemical Society.



4.3. Degradation of chitosan by family 46 chitosanases

The GH 46 family of chitosanases comprises enzymes classified as subclass I (cleaving **A-D** and **D-D** linkages) [60] as well as subclass III (cleaving **D-A** and **D-D** linkages) [120].

In an early study, a chitosan with F_A = 0.25–0.35 was extensively degraded with CsnN174 from *Streptomyces* sp. N174 and the oligomeric products were isolated and characterized. In addition to the fully deacetylated oligomers **D**, **DD** and **DDD**, several hetero-oligomers were identified (the dimer **DA**, the trimers **DDA** and **DAA**, the tetramers **DDAA** and **DAAA** and the pentamer **DDAAA**) [60]. These results suggest that CsnN174 has a high specificity for a GlcN in the +1 subsite (new non-reducing ends), whereas both GlcNAc and GlcN seem to be acceptable in the -1 subsite (new reducing ends). Further insight into the properties of CsnN174 would require studies of oligomer production over time, as well as studies using chitosans of varying F_A.

Recent work in our own laboratories on a family 46 chitosanase, Csn88, from *Streptomyces coelicolor* has shown that this enzyme is capable of degrading chitosans with varying F_A, producing oligomers with both *N*-acetylated and deacetylated reducing ends [121]. ^{1}H NMR spectroscopy analysis showed that when the new reducing ends were *N*-acetylated, the sugar binding in the neighbouring position (*i.e.*, binding in subsite -2) always was deacetylated. However, in oligomers

with deacetylated sugars at the new reducing end, both *N*-acetylated and deacetylated sugar occurred in the neighbouring position (revealed by mass spectrometry). The identity of the new non-reducing ends was studied using ^{13}C NMR spectroscopy and this analysis showed that initial products exclusively had deacetylated non-reducing ends, whereas *N*-acetylated non-reducing ends appeared later during the reaction. All in all, these preliminary data indicate that Csn88 can cleave **D-A**, **A-D** and **D-D** linkages. As expected maximum α values were high, for example 0.59 for a highly deacetylated chitosan (F_A 0.008) and 0.44 for a F_A 0.32 chitosan.

4.4. Degradation of chitosan by unspecific enzymes

Several authors have employed unspecific enzymes such as papaine and cellulases to degrade chitosans (e.g., [122–124]). Since the enzyme preparations used tend to be rather crude and derived from sources (fungi, plants) that are known to produce chitinolytic enzymes, there remains some doubt concerning which enzymes actually catalyze the hydrolysis reactions. However, for the practical purpose of producing CHOS, the use of (cheap) unspecific enzymes may be quite useful.

5. CHOS Production—Chemical Methods

5.1. Acid hydrolysis of chitosan

Of chemical methods for hydrolysis of chitosan [32–35], acid hydrolysis is probably the best known. Early studies on acid hydrolysis of chitosans had shown that it is possible to convert fully deacetylated chitosan to CHOS in concentrated hydrochloric acid [32]. In later studies [33], using a variety of chitosans, the acid-catalyzed degradation rates of chitosans were shown to depend on F_A, and the initial degradation rate constant was found to increase in direct proportion to F_A. Acid hydrolysis was found to be highly specific to cleavage of **A-A** and **A-D** glycosidic linkages, which were hydrolyzed with two to three orders of magnitude higher rates than the **D-D** and **D-A** linkages. This preference is probably due to a combination of two factors: (1) the presence of a positively charged amino group (as in GlcN) close to the glycosidic linkage is inhibitory, and (2) the presence of an acetamidogroup (as in GlcNAc) close to the glycosidic linkage may yield some degree of substrate-assistance to the hydrolytic mechanism. In the same study it was shown that the rate of deacetylation was less than one-tenth of the rate of depolymerization in concentrated acid, whereas the two rates were found to be equal in dilute acid. It was suggested that this is due to these two processes having different reaction mechanisms with different rate-limiting steps [33].

5.2. Chemical synthesis of CHOS

Chemical synthesis of CHOS requires multiple protection and deprotection steps, and is today not a routine procedure. Chemical synthesis of CHOS gives rise to pure compounds, but most methods existing today are time consuming and require extensive use of organic solvents and/or are not capable of producing anything else than homo-oligomers. There are in fact only a few examples of chemically synthesized CHOS in the literature. Kuyama *et al*. [125] performed synthesis of fully deacetylated chitosan dodecamers starting with glucosamine monomers using phthalimido as the amino protective group. Aly *et al*. [126] reported a method for synthesis of fully *N*-acetylated CHOS from GlcN

monomers using dimethylmaleoyl as an amino protective group for synthesis of chitotetraose and chitohexaose. Removal of the amino protective group and *N*-acetylation was performed in a one-pot reaction to give the desired products [127]. In principle it would be possible to combine the use of these two described protection methods to synthesize partly deacetylated CHOS, but this has so far not been reported (to our knowledge).

Trombotto *et al*. [128] have reported a method for chemical preparation of partly deacetylated CHOS from fully deacetylated high molecular weight chitosan. The starting chitosan was partially depolymerized using HCl to produce fully deacetylated oligomers that were fractionated by selective precipitation and ultrafiltration to yield a mixture of DP 2–DP 12. The oligomers were then partly *N*-acetylated using stochiometric amounts of acetic anhydride to reach the decided F_A. In this way, CHOS fractions of DP between 2 and 12 were successfully prepared. The drawback of this method, as for the enzymatic preparation of CHOS, is the heterogeneity of the prepared CHOS.

In an early study, Letzel *et al.* [103] used a similar approach: chitosan with F_A 0.02 was hydrolyzed with HCl, oligomers were separated by DP using gel permeation chromatography and the resulting CHOS fractions were *N*-acetylated using substoichiometric amounts of acetic anhydride to control the F_A. Interestingly, some of the fractions produced in this study inhibited the family 18 chitinase ChiB from *Serratia marcescens*.

In principle, chemoenzymatic synthesis provides opportunities to produce pure CHOS of defined DP, F_A and P_A without the use of extensive protection of the functional groups at the sugar unit. So far this has been done by allowing an oxazoline, imitating the intermediate of chitin hydrolysis, to act as a glycosyl donor in an enzyme-catalyzed reaction where another GlcN/GlcNAc unit acts as a glycosyl acceptor [129]. By using oxazoline oligomeric building blocks of specific DP, F_A, and P_A, longer specific CHOS can be made using this approach. The main disadvantage using the chemo-enzymatic approach is that the product is necessarily also a substrate for the enzyme, which can result in poor yields. To avoid this problem, modified enzymes with reduced hydrolytic activity may be employed. The enzyme modifications would need to be aimed at reducing hydrolytic power, while increasing binding strength for the glycosyl donor in the glycon subsites [130].

6. Purification and Characterization of CHOS

CHOS produced enzymatically or chemically normally consist of a mixture of oligomers differing in DP, F_A and P_A. Several techniques for separation and purification of CHOS have been reported, like gelfiltration [64], ultrafiltration [131], and ion exchange [132] and metal affinity [133] chromatography. Often, such techniques need to be applied in combination to obtain homogeneous CHOS fractions. Despite some successful studies, the production of pure CHOS fractions is generally a time consuming and challenging task.

Preparative separation of CHOS is most commonly based on size, through size exclusion chromatography (SEC). Recently, good methods for the separation of oligomers up to DP 40 (individual oligomers up to DP 20) have been described [64], as illustrated by Figure 3, 5, 7 and 8. The SEC system used for producing the data displayed in this review is based on Superdex^{TM} 30 (GE Healthcare) columns that are coupled in series. The oligomers are detected using an online refractive

index detector. This system allows separation of CHOS with similar DP values ranging from approximately DP 2 to DP 20, independently of F_A and P_A [64].

Further separation of CHOS can be achieved using cation-exchange chromatography, because protonated amino groups on the deacetylated sugars interact with the ion-exchange material. With this method CHOS of identical DP will be separated based on the number of deacetylated units [132]. A further partial separation of isobaric CHOS (identical F_A, different P_A) may be achieved using strong cation exchange chromatography. Although, the latter separations are promising and useful, baseline separation of isobaric CHOS has so far not been achieved [132]. In an alternative strategy, metal affinity chromatography has been successfully used for separation of shorter CHOS. CHOS have a strong affinity for Cu^{2+}, and using copper as a chelating agent gives separation up to 90% of fully deacetylated CHOS of DP 3 and higher [133]. This has not been reported for *N*-acetylated mixtures of CHOS.

In order to characterize CHOS in terms of DP, F_A and P_A, several techniques have been applied, primarily nuclear magnetic resonance (NMR) and mass spectrometry. Using NMR, it is possible to determine F_A in a chitosan or CHOS sample and to (partially) identify the P_A in shorter CHOS depending on the complexity of the oligomer mixture. Resonances detected using NMR reveal that the H-1 resonance of a reducing unit is sensitive to its nearest neighbor, making it possible to (partially) determine the P_A of an oligomer [64]. In addition, the P_A of dimers and trimers can be determined using NMR. The identity of the non-reducing end unit of an oligomer can be determined using ^{13}C NMR, which in some instances also may reveal the identity of its nearest neighbor [108].

Modern mass spectrometry provides excellent tools for the identification of the DP and F_A of CHOS [104,134]. In 1997 Okafo *et al.* [135] reported a reductive amination of CHOS using 2-aminoacridone (AMAC), which is useful for tagging of the reducing end. Building on this labeling technique, Bahrke *et al.* [134] developed a method for sequencing of CHOS up to DP 12 using reducing end derivatization with AMAC. Starting with CHOS fractions of homogeneous DP they used matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) postsource decay (PSD) mass spectrometry (MS) for sequence determination. Reducing end derivatization of CHOS using AMAC favors formation of Y-type ions, meaning that sugars are only cleaved after the oxygen in the $\beta(1\rightarrow4)$ linkage from the reducing end. Consequently, interpretation of the resulting mass spectra is quite straightforward. It should be noted though that the method has limitations when applied to mixtures.

In a later study from the same group [132], a second method for reductive amination, using 3-(acetylamino)-6-aminoacridine, was adopted. Combined detection and fragmentation of isobaric CHOS using MALDI iontrap MS^n was reported. The described technique makes it possible to simultaneously determine sequence and quantity of CHOS of identical DP but different F_A and P_A in an isobaric CHOS mixture.

7. Applications of CHOS

Literature contains numerous papers reporting a remarkably wide range of biological activities of CHOS. As discussed above, the molecular mechanisms behind these bioactivities are mostly unknown and so is the exact nature of the bioactive component. Many activities have been reported only once or twice, providing insufficient basis to make general conclusions about the applicability of CHOS.

Below, we discuss a selection of studies that report such bioactivities, with focus on studies that contain some discussion of the molecular mechanisms involved.

7.1. Tumor growth inhibition

It has been known since the 1970s that CHOS have anti-tumor effects [18], and there is also evidence for positive effects of CHOS in reducing metastasis from tumors [19,20]. It was first suggested that the anti-tumor activity was due to the cationic properties of CHOS [18]. Later, M_W also was proposed to play a major role [136]. Maeda and Kimura [137] found that CHOS enhanced the natural killer activity in intestinal intraepitelial lymphocytes at the same time as they reduced tumor growth in mice, and suggested that this CHOS-activation of intestinal immune functions could be useful in treating tumors.

There are indications that apoptosis is involved in the tumor-reducing effects of CHOS. Harish Prashanth and Tharanathan [138] discovered that DNA from Ehrlich ascites tumor cells was fragmented after incubation with CHOS, an indication of apoptosis. CHOS have also been shown to induce apoptosis of human hepatocellular carcinoma cell via upregulation of the pro-apoptotic protein Bax [139].

In recent years, the hypothesis that the anti-tumor effects of CHOS are related to their inhibitory effects on angiogenesis has received some attention [138,140,141]. Angiogenesis is the formation of new capillary blood vessels from already existing blood vessels. This process is important for tumor formation, since tumor growth and metastasis require angiogenesis when the tumor reaches a certain size. Xiong *et al.* [142] compared effects of dimers to hexamers of fully deacetylated CHOS on angiogenesis and found that the hexamers were the most effective inhibitors, whereas Wang *et al.* [140] showed that *N*-acetylated CHOS were more effective in preventing angiogenesis than fully deacetylated CHOS, both *in vitro* and *in vivo*.

7.2. Asthma

AMCase is induced during T_H2 inflammation through an interleukin (IL)-13 dependent mechanism and has been demonstrated to be heavily over-expressed in human asthmatic tissue [10,12]. Inhibition of the AMCase with the well known chitinase inhibitor allosamidin reduced the inflammation [12]. The fact that chitinases are a factor in host antiparasite responses and in asthmatic T_H2 inflammation led to the hypothesis that asthma may be a parasite-independent antiparasite response [10], which again suggests that inhibition of AMCase is a potential target for asthma therapy [9–12]. It has been shown that partially deacetylated CHOS can function as inhibitors of family 18 chitinases [103–105]. There is therefore a great potential for CHOS as an anti-inflammatory drug in patients with asthma. For a more detailed description of this and related topics, see the review by Muzzarelli in this special issue of Marine Drugs [143].

7.3. Increased bone strength

Mesenchymal stem cells from the bone marrow are able to differentiate into chondrocytes (cartilage), adipocytes (fat) and osteoblasts (bone). Osteoblasts produce osteoid; which is further

mineralized to produce the bone matrix. Bone-tissue is mainly composed of bone matrix and osteoblasts. Chitosan and CHOS are known to increase the differentiation of mesenchymal stem cells to osteoblasts and to consequently facilitate the formation of bone-tissue [21,22].

The mineralization process and bone strength are dependent on Ca^{2+}, which helps to support the structure. There is evidence that CHOS increase calcium-deposition in bone [22,144,145]. Jung *et al.* [144] found that CHOS could efficiently inhibit the formation of insoluble calcium-phosphate salts and consequently increase Ca^{2+} bioavailability and bone strength. They also found that CHOS (<5 kDa) gave increased calcium retention and decreased bone turnover in a rat osteoporosis model. This indicates that CHOS may have beneficial effects as a calcium fortifier in conditions of Ca^{2+} deficiency, such as in osteoporosis.

7.4. CHOS in gene therapy

Chitosans have been successfully used as vectors for delivery of genes (gene therapy) since the first report about 15 years ago [146,147]. Chitosan forms stable complexes with plasmid DNA and can be used as a vector for the administration of genes to mucosal tissues such as the lungs [148] and intestinal epithelium [149,150]. There are, however, certain drawbacks connected to the use of high molecular weight chitosans because of the low solubility at physiological pH, the high viscosity and the fact that the chitosan complexes often tend to form aggregates. By using CHOS instead of chitosan these drawbacks may be overcome [16]. Köping-Höggård *et al.* [16] showed that fully deacetylated CHOS (DP 24) formed stable complexes with plasmid DNA, and *in vitro* and *in vivo* experiments proved that these CHOS were effective vectors for delivery of genes [16,17]. It has been speculated that a delicate balance between the stability of the CHOS-DNA-complexes at lower pH-values (around pH 6) and their instability at higher pH-values (above pH 7) could be the reason for their efficiency [151]. This has recently been confirmed in detailed studies of how chitosan chemistry can be used to create an optimal balance between the stability of the complexes and their unpacking [152].

7.5. Prevention of bacterial adhesion to human cells

Some pathogens can initiate disease in humans by using carbohydrate binding proteins (lectins) to attach to complementary membrane-bound oligosaccharides on host cells [153,154]. Observed antibacterial and anti-infective effects of CHOS [13] are thought to be due to their ability to bind to the lectins on human pathogens and, consequently, prevent adhesion to human cells. *A priori*, one would expect the sequence of GlcNAc and GlcN units in CHOS to be important for binding affinity, and for ensuring selectivity for pathogens (*i.e.*, the CHOS should preferably not bind to lectins of the normal bacterial flora). In a recent study, it was shown that a mixture of 97% deacetylated tetramers significantly inhibited adhesion of certain enteropathogenic *Escherichia coli* strains to human colon adenocarcinoma epithelial (HT29) cells in tissue culture, whereas the binding of other *E. coli* strains was not inhibited [13]. Since pathogens vary in terms of their lectins, identification of both the target-lectins and the possible complementary CHOS are important when pursuing this application route of CHOS.

7.6. CHOS as a chitinase-inhibitor for preventing malaria

Malaria is caused by several species of the parasite *Plasmodium*. *P. falciparum* causes the most serious forms of malaria in humans, whereas *P. vivax, P. ovale* and *P. malarie* give a milder disease that is not generally fatal [155]. Each year, 350–500 million cases of malaria occur worldwide, and over one million people die, most of them young children in sub-Saharan Africa. There is no vaccine available, and the identification of molecular targets for vaccine development is of great importance. *Anopheles* mosquitoes transmit the malaria-parasite from one infected person to another and the most important control strategy for malaria today is to interfere with different stages in the life cycle of the *Plasmodium* parasite.

During its life cycle, the *Plasmodium* parasite must be capable of penetrating the chitin-containing periotrophic matrix surrounding the mosquito midgut, to make the mosquito infective. To do so, *Plasmodium* species secrete family 18 chitinases capable of degrading the periotrophic matrix [23,156–160]. The *P. falciparum* chitinase (PfCHT1), the *P. vivax* chitinase (PvCHT1) and the *P. gallinaceum* chitinase (PgCHT1) have been characterized [161–163]. *P. gallinaceum* is the only malaria parasite species where more than one chitinase gene has been identified, and PgCHT1 and PgCHT2 are both family 18 chitinases. One approach to vector control might be inhibition of the secreted *Plasmodium* chitinases by chitinase inhibitors that are taken up by the mosquito via the blood meal [23,156–160]. Several studies have convincingly shown that inhibition of *Plasmodium* chitinases indeed reduces infectivity [23,163,164]. CHOS may perhaps be developed as nontoxic, inexpensive small-molecule inhibitors of these chitinases.

7.7. Applications of chitosan/CHOS in wound-dressings

The use of chitosan in wound dressings has been explored to a certain extent and positive effects have been documented in several studies [14,165–169]. Similar positive effects have been documented for CHOS, which were shown to accelerate the wound healing process [14,15]. It is quite likely that chitosan is converted to CHOS by naturally occurring enzymes and that the activity observed for chitosan might in fact be caused by CHOS. It may thus be advantageous to use CHOS in wound dressings to get a more immediate effect.

CHOS are thought to accelerate wound healing by enhancing the functions of inflammatory and repairing cells [170–172]. For example, it has been shown that subcutaneous injection of hexamers of CHOS enhanced migration of polymorphonuclear cells in dogs [171]. Hexamers of *N*-acetylated and fully deacetylated CHOS were shown to induce persistent release of IL-8, a potent activator and chemoattractant of polymorphonuclear cells, from fibroblasts from rats *in vitro* [170]. It must be noted that most authors ascribe the beneficial effect of longer CHOS and (polymeric) chitosan on wound healing to the ability of these compounds to form biocompatible ordered tissue-like structures (see [167] and references therein).

Hemostatic effects may also contribute to the beneficial effects of chitosan/CHOS in wound dressings. Chitosan enhances platelet adhesion and aggregation [165,173] and increases the release of the platelet derived growth factor-AB (PDGF-AB) and the transforming growth factor-β1 (TGF-β1) from platelets in canine blood [173]. These two factors retract inflammatory cells which are thought to

be important in wound healing. Chitosan also has the ability of causing erythrocytes to aggregate [169].

Minagawa *et al.* [166] compared wound break strength and the increase in collagenase activity in wounds in rats after exposure to monomers, oligomers and polymers of the chitin-group (GlcNAc/*N*-acetylated CHOS/chitin) and the chitosan-group (GlcN/fully deacetylated CHOS/chitosan). They found that all six compounds increased both the wound break strength and the collagenase activity. Overall, the non-acetylated compounds were most effective compared to the corresponding *N*-acetylated compounds. The oligomers of fully deacetylated CHOS were most effective for wound break strength and GlcN gave the highest activity of collagenase. The enzyme collagenase is produced mainly by fibroblasts and inflammatory cells and its activity is related to remodeling in wound healing [174].

7.8. Antifungal effects

The antifungal activity of chitosan was discovered already in 1979 [175], and has been utilized to inhibit fungal growth in crops [176]. The antifungal potential of CHOS has to a lesser extent been investigated. In 1984 Kendra and Hadwiger [177] tested the antifungal activity of monomers–heptamers of deacetylated CHOS on *Fusarium solani*, which is infectious to pea crops, and found that the heptamer was most effective.

Subsequent research has revealed that longer oligomers of CHOS (also called low molecular weight chitosan, LMWC) are more effective. LMWC (4.6 kDa, average DP of 23) shows antifungal activity against *Candida krusei* and inhibits spore germination in *Fusarium oxysporum* [178]. Seyfarth *et al.* [26] found antifungal effects of LMWC on different *Candida* species. Both DP and F_A of the chitosan/CHOS are of great importance for the antifungal potential and LMWC with low F_A so far seems to be the most promising type of compound [179].

The anti-fungal effect of LMWC seems to be caused by its interaction with lipids in the plasma membrane, leading to morphological changes and cell surface disruptions [180,181]. The composition of the fungal plasma-membrane seems to be important for the sensitivity against chitosan, and a higher content of polyunsaturated fatty acids makes the fungi more sensitive [182].

From literature studies, as well as from own unpublished work on non-medical use of LMWC as anti-fungals, it is clear that LMWC indeed have a considerable potential in this area. This is a good reason for giving the application of LMWC, to combat fungal infections in humans, more research attention than it has received so far.

8. Concluding Remarks and Future Perspectives

Despite major progress in the past decade, the production of pure CHOS with defined DP, F_A and P_A is still a challenge. However, it is now fully possible to carry out controlled and reasonably well understood enzymatic production processes that yield CHOS preparations that are enriched for certain known compounds. The outcome of such processes can be controlled by controlling the enzyme, the starting chitosan (primarily F_A), and the extent to which the degradation reaction is allowed to develop. Further refinement of the production step may be achieved by using engineered enzymes with changed

binding preferences in one or more of their subsites and by carrying out specific deacetylation steps with chitin deacetylases.

Techniques for further purification of CHOS as well as for sequence determination are now available, but are still quite challenging to exploit. Scaling up purification methods at an economically acceptable cost is another challenge, meaning that, from an economical point of view, it is probably cheaper to produce CHOS mixtures that are enriched for a bioactivity, rather than producing truly pure compounds. It is conceivable that the further development of CHOS as bioactive molecules may include further functionalization by chemical methods, for example by coupling groups to the reducing end.

These improved methods for producing (almost) pure, well-characterized CHOS will help to create a knowledge base for understanding how CHOS exert bioactivities. For example, it may soon be possible to determine the crystal structures of chitinases in complex with a CHOS acting as an inhibitor. Likewise, the interaction of CHOS with AMCase [183] or HCgp39 [184] may be assessed by structural studies.

CHOS have a remarkably wide spectrum of possible bioactivities. While highly promising, there is no doubt that these bioactivities need to be substantiated by further studies with well-defined CHOS preparations, as well as by fundamental research on the molecular mechanism behind the activity. Only then the great promise of converting an abundant bioresource, chitin, to CHOS-based medicines can be met.

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Paper II

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Paper III

Human Chitotriosidase Catalyzed Hydrolysis of Chitosan

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RUNNING TITLE: Processivity and exo-endo mode of the human chitotriosidase

[1]ABBREVIATIONS: ChiA, chitinase A from *Serratia marcescens*; ChiB, chitinase B from *Serratia marcescens*; ChiC, chitinase C from *Serratia marcescens*; DP, degree of polymerization; GlcN, glucosamine; GlcNAc, *N*-acetylated glucosamine; HCHT, human chitotriosidase; HPLC, high pressure liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PDB, protein data bank; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ABSTRACT: Chitotriosidase (HCHT) is one of two family 18 chitinases produced by humans, the other being acidic mammalian chitinase (AMCase). The enzyme is thought to be part of the human defense mechanism against fungal parasites, but its precise role and the details of its enzymatic properties have not yet been fully unraveled. We have studied properties of HCHT by analyzing how the enzyme acts on high molecular-weight chitosans, soluble co-polymers of β-1,4-linked *N*-acetylglucosamine (GlcNAc, A) and glucosamine (GlcN, D). Using methods for in-depth studies of the chitinolytic machinery of bacterial family 18 enzymes, we show that HCHT degrades chitosan primarily via an endo-processive mechanism, as would be expected on the structural features of its substrate-binding cleft. The preferences of HCHT subsites for acetylated versus non-acetylated sugars were assessed by sequence analysis of obtained oligomeric products showing a very strong, absolute, and a relative weak preference for an acetylated unit in the –2, –1, +1 subsite, respectively. The latter information is important for the design of inhibitors that are specific for the human chitinases and also provide insight into what kind of products may be formed *in vivo* upon administration of chitosan-containing medicines or food products.

KEYWORDS: Human chitinase; chitosan; chitin; processivity; chitotriosidase.

Chitin, an insoluble linear polysaccharide consisting of repeated units of β-1,4-*N*-linked acetylglucosamine [$(GlcNAc)_n$], is common as a structural polymer in crustaceans, arthropods, fungi, and parasitic nematodes. The metabolism of chitin in nature is controlled by enzymatic systems that produce and break down chitin, primarily chitin synthases and chitinases, respectively. Chitinases are thought to play important roles in anti-parasite responses in several life forms, including humans (*1-4*). Even though chitin and chitin synthases have not been found in humans, we produce two active chitinases that are categorized as family 18 chitinases based on sequence-based classification of glycoside hydrolases (*5*). These two enzymes are called acidic mammalian chitinase (AMCase) (*6*) and human chitotriosidase (HCHT) (*7*) and both are believed to play roles in anti-parasite responses (*8, 9*). While AMCase is found in the stomach (*6*), in tears (*10*), sinus mucosa (*11*), and lungs (*12*), HCHT is primarily expressed in activated human macrophages (*13*).

HCHT is up-regulated in a series of diseases and medical conditions such as Gaucher's disease (*13*), sarcoidosis (*14, 15*), cardiovascular risk (*16*), coronary artery disease (*17*), primary prostate cancer and benign prostatic hyperplasia (*18*), nonalcoholic steatohepatitis (*19*), and Niemann-Pick disease (*20*). The only currently known physiological implications of the elevated HCHT levels are a better defense against chitin-containing pathogens (*4*) and the triggering of human macrophage activation by HCHT-mediated chitin and chitosan degradation (*21*).

HCHT is synthesized and secreted as a 50-kDa protein in human macrophages. A considerable portion of produced enzyme is routed to lysosomes and processed into a 39-kDa isoform lacking the C-terminal chitin binding domain (*22*). The 39 kDa catalytic domain comprises a $(\beta/\alpha)_8$ barrel with a so called α/β insertion domain that contributes to endorsing the enzyme with a deep catalytic cleft (*23*) (Figure 1B). The catalytic acid, Glu-140, is located at the end of the conserved DxxDxDxE motif that includes strand β4 of the $(\beta/\alpha)_8$ barrel. The

91 substrate-binding cleft of HCHT extends over one face of the enzyme and is lined with
92 solvent exposed aromatic residues (Fig 1B.) (*23*). Whereas some chitinases with such deep
93 clefts have long loops that form a "roof" over the substrate-binding cleft (*24, 25*), such a
94 "roof" is absent in HCHT (Figure 1).

95 Family 18 chitinases employ a substrate-assisted catalytic mechanism in which the *N*-
96 acetyl group of the sugar bound in the –1 subsite (*24, 26-28*). Because of this, family 18
97 chitinases have an absolute preference for acetylated units in the –1 subsite. This may be
98 exploited in the design of inhibitors based on partially acetylated chito-oligosaccharides
99 (CHOS). CHOS whose preferred binding mode places a deacetylated unit in subsite –1 will
100 bind non-productively, and hence serve as an inhibitor (*29*). CHOS bear great promise as
101 building blocks for chitinase inhibitors, because they are natural products and potentially
102 highly selective (*30*).

103 While family 18 chitinases share this special catalytic mechanism, family members
104 may differ in many other aspects. One variable concerns their tendency to cleave the
105 polymeric substrate at chain ends (exo-action) or at random positions (endo-action). Both
106 modes of action may occur in combination with processivity, which implies that the enzyme
107 remains attached to the substrate in between subsequent hydrolytic reactions (*31*). Another
108 variable within the family 18 chitinases concerns the binding affinities and selectivity of their
109 individual subsites. To analyze these characteristics, studies on the degradation of chitosan,
110 the water soluble partially deacetylated polymeric chitin analogue, have shown to be useful
111 (*32-35*).

112 Being a part of the innate immune system and associated with so many diseases,
113 detailed knowledge of the mechanistic properties of HCHT is of great interest. Several studies
114 of the properties of HCHT have appeared in the literature (*4, 21, 36*), but issues related to the

mode of action and subsite-binding preferences have so far received limited attention. Insight in subsite-binding preferences is particularly important because inhibition of human chitinases is of medical interest. Inhibition of AMCase has been suggested as a therapeutic strategy against asthma (*12*), while there is no evidence that inhibition of HCHT will be beneficial. In fact, due to the beneficial fungistatic effect of HCHT, inhibition of this enzyme could be unfavorable. Thus, there is a need to develop inhibitors that are selective for AMCase, and to do so, insight in the binding preferences of both AMCase and HCHT is required. Here, we describe novel insights into the enzymatic properties of HCHT derived from an in-depth analysis of HCHT action on chitosan.

EXPERIMENTAL PROCEDURES

Materials. Chitin was isolated from shrimp shells as described and milled in a hammer mill to pass through a 0.1 mm sieve (*37*). Chitosans with different fractions of *N*-acetylated units (F_A) were prepared by homogenous de-*N*-acetylation of chitin (*38*). The characteristics of the chitosans used in this study are given in Table 1. Chitinase B (ChiB) from *Serratia marcescens* was purified as described (*39*).

HCHT Expression and Purification. *Pichia pastoris* cells expressing the 39 kDa form of HCHT were grown in 100 mL buffered glycerol-complex (BMGY) medium at 28 ºC for 24 hours and 10 mL of this culture was used to inoculate 500 mL fresh BMGY. After incubation for 48 hours at 30 ºC and 200 rpm, cells were harvested through centrifugation at 3500 rpm for 30 min at 20 ºC. Subsequently, pellets were re-suspended in 500 mL fresh BMGY and incubated for additional 120 hours at 30 ºC and 200 rpm. Every 24 hour 5 mL of high quality methanol were added to the culture. After 4 additions of methanol, cells were

139 harvested through centrifugation for 30 minutes at 3500 rpm and 20 ºC. HCHT is secreted
140 into the culture medium and is present in the supernatant after centrifugation. The supernatant
141 was filtered through a 0.22 µm filter and concentrated using a Vivaflow 200 PES, 10 000
142 MWCO, until a total volume of 30-50 mL. Concentrated supernatant was dialyzed against 50
143 mM sodium acetate pH 4.2 at 4 ºC for 72 hours in order to get rid of components from the
144 medium. HCHT was then purified using ion exchange chromatography with a HiTrap CM FF
145 5 mL column (GE Healthcare), using 50 mM sodium acetate pH 4.2 as running buffer and a
146 flow of 5 mL/min. The protein was eluted from the column by applying a linear gradient to
147 100% 50 mM sodium acetate pH 6.5 over 20 column volumes, and detected using a UV-
148 detector. The contents of the collected fractions were analyzed using SDS-PAGE. Fractions
149 containing HCHT were pooled and concentrated to approximately 2 mg/mL by centrifugation
150 at 4000 rpm for approximately 20 minutes in Amicon centrifuge tubes 10 000 MWCO.
151 Enzyme purity was analyzed by SDS-PAGE and found to be over 95% in all cases (Figure
152 S1). Protein concentrations were determined by using the Quant-It protein assay kit and a
153 Qubit fluorometer from Invitrogen (CA, USA).

154 **Degradation of High Molecular Mass Chitosan with F_A = 0.62, F_A = 0.49, F_A =**
155 **0.35 and F_A = 0.18**. Chitosan was dissolved in 80 mM sodium acetate buffer pH 5.5 to a final
156 concentration of 10 mg/mL (*35*). Chitosan with F_A = 0.62 was depolymerized by adding
157 0.075 µg HCHT pr mg chitosan. Samples were taken at various time points between 2.5 min.
158 and 9 days after starting the reaction and enzyme activity was stopped by adjusting the pH to
159 2.5 with 5 M HCl followed by boiling for 2 minutes. Chitosans with F_A = 0.49, 0.35 or 0.18
160 were depolymerized (as described above) to a maximum degree of scission (α). The degree of
161 scission was determined by NMR (see below) and was considered maximal after it had been
162 established that addition of fresh enzyme to the reaction mixtures did not yield a further
163 increase in the degree of scission.

Analytical Instrumentation. Oligomers produced from the enzymatic depolymerization of chitosan were separated on three columns packed with Superdex^{TM} 30 from GE Healthcare, coupled in series (overall dimensions 2.60 x 180 cm) (*35*). Fractions of 4 mL were collected for further analyses of the depolymerization products. Using this method, oligomers are separated by DP only, except for oligomers with the lower DPs (< DP = 5), where there also is some separation according to sugar composition; see results section.

In order to determine the sequence of chitosan oligomers, the oligosaccharides were derivatized by reductive amination of the reducing end with 2-aminoacridone (AMAC) (*29, 40*). Sequencing of chitosan oligomers was performed using MALDI-TOF/TOF mass spectrometry.

Samples from enzymatically depolymerized chitosan were lyophilized and dissolved in D_2O, after which the pD was adjusted to 4.2 using DCl for NMR experiments. The ^{1}H-NMR spectra were obtained at 85 ºC at 300 MHz (Oxford NMR^{300}, Varian) (*41, 42*). The deuterium resonance was used as a field-frequency lock, and the chemical shifts were referenced to internal sodium 3-(trimethylsilyl)propionate-d4 (0.00 p.p.m.). The ^{1}H-NMR spectra were used to determine the degree of scission, α through the anomer (H-1) resonance as follows: DP_n = [area of H-1 resonances (internal and reducing end]/(area of reducing end resonances) (*35*). The degree of scission was calculated as $\alpha = 1/DP_n$.

For determination of relative viscosity and reducing ends of solutions, chitosan with an F_A of 0.62 was dissolved to a final concentration of 1 mg/mL in 40 mM acetate buffer pH 5.4 containing 0.1 M NaCl. HCHT was added to a final concentration of 10 ng/mL. Determination of the relative viscosity of the polymer solution and determination of the total number of reducing ends using the MBTH method (*43*) were performed as described by Sikorski et al. (*34*).

MS spectra were acquired using an UltraflexTM TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with gridless ion optics under control of Flexcontrol 4.1. For sample preparation, 1 µL of the reaction products was mixed with 1 µL 10% 2,5-dihydroxybenzoic acid (DHB) in 30% ethanol and spotted onto a MALDI target plate. The MS experiments were conducted using an accelerating potential of 20 kV in the reflectron mode.

RESULTS AND DISCUSSION

Degradation of High Molecular Mass Chitosans with HCHT; Subsite-Preferences. High molecular chitosan (M_r = 140 000) with F_A 0.62 was degraded with HCHT to different degrees of scission (α), which is the fraction of glycosidic linkages that has been cleaved by the enzyme. The degree of scission at any time point of the reaction was determined by monitoring the increase in reducing end resonances relative to resonances from internal protons in a ^{1}H-NMR spectrum of the reaction mixture (*35*). Figure 2 shows the time course for the reaction where the observed maximum α-value was found to be 0.33. The initial phase of hydrolysis (Figure 2 insert) suggested biphasic kinetics. The experimental data were fitted to double-exponential equations (Equation 1) where A_1 and A_2 represent the amplitudes of the biphasic time course, r_1 and r_2 stand for the corresponding rates, and B represents the end point of the hydrolysis reaction (maximum α).

$$\text{Fraction reacted} = -A_1 e^{(-r_1 t)} - A_2 e^{(-r_2 t)} + B \qquad (1)$$

The fit yielded apparent rate constants (k_{cat}^{app}) of 102 s^{-1} and 14 s^{-1} with amplitudes of 0.17 and 0.14, respectively.

Figure 3 shows chromatograms for SEC of the reaction mixtures obtained after HCHT degradation of chitosan with F_A of 0.62 to α = 0.03, 0.08, and 0.13; Figure 4 shows a chromatogram for α = 0.33. High molecular chitosan (DP > 40) is eluted in the void peak at approximately 550 minutes, while chitosan oligomers are eluted in separate peaks from 700-1200 minutes. Generally, oligomers are separated by DP only, but at low DP some separation according to sugar composition (acetylated, A, versus deacetylated, D) is observed as indicated in Figure 3. The DP3 to DP6 fractions were subjected to sequence analysis and the results are shown in Table 2. The reducing ends of the observed products reflect binding preferences in the negative subsites, whereas the non-reducing ends of the products reflect binding preferences in the positive subsites. The combining the data of Figure 3 with the sequence data of Table 2 shows that early on in the reaction, cleavage almost exclusively occurs in the sequence **AA-A** bound to subsites –2 to +1. Almost all products have **AA** on their reducing ends in all phases of hydrolysis indicating that there is a strong preference for an acetylated unit in the –2 subsite. Products ending at **–DA** were observed in the dimer and trimer fractions, at the very end of the reaction only (Figure 4, Table 2). Significant amounts of products with a **D** at the non-reducing end appear earlier in the reaction indicating that the preference for an acetylated unit in the +1 subsite is not as strong as in the –2 subsite. These preferences may help to explain the kinetic behavior described above (Figure 2). As the hydrolysis reaction progresses, the reaction will slow down because optimal cleavage sites, containing the **AA-A** stretch as well as perhaps adjacent sequence features that cannot be resolved from the present data, will decrease.

Three other high molecular mass chitosans with F_A of 0.49, 0.35, and 0.18 were also incubated with HCHT and extensively depolymerized to maximum α. As expected based on the clear preferences for acetylated units discussed above, the size distribution of the product mixtures shifted towards higher oligomer lengths and the maximum α became lower for

237 substrates with lower F_A values (Figure 4). It has previously been shown that chitinases that
238 use aromatic side chains to interact with their substrate are more "tolerant" for deacetylation
239 than chitinases that primarily bind the substrate through specific hydrogen bonds involving
240 polar side chains (*44*). This is due to the fact that aromatic residues stack with the
241 hydrophobic faces of the sugars, an interaction type that is less specific than hydrogen bonds
242 that may involve the *N*-acetyl groups. Clearly, both the structural data shown in Figure 1 and
243 the observations displayed in Figure 4 show that HCHT belongs to the former category. The
244 ability of HCHT to degrade chitosans with low F_A should be noted, since such chitosans have
245 several (potential) applications in human food (*45*).

246 **Determination of Endo/exo Mode**. By studying the relative viscosity of the chitosan
247 solution during chitinase-catalyzed hydrolysis, it is possible to determine whether the
248 enzymes act in an endo- or an exo-fashion. Endo-acting enzymes will reduce viscosity much
249 faster than exo-acting enzymes (see Sikorski et al. (*34*) for a detailed discussion). Acid
250 hydrolysis of chitosan is used as a model for the endo-mode because this process introduces
251 random cleavages along the polymer chain. Another control for endo-activity is ChiB from *S.*
252 *marcescens* for which highly detailed studies have shown that endo-type of action is
253 predominant when acting on chitosan. Figure 5 displays relative viscosity over time for a
254 chitosan solution (F_A = 0.62) hydrolyzed by acid, ChiB, and HCHT. In all three cases relative
255 viscosity was quickly reduced, indicating that HCHT acts in the endo-mode when hydrolyzing
256 chitosan.

257 Figure 1 shows that HCHT, chitinase A (ChiA) from *S. marcescens*, and ChiB have
258 relatively deep substrate-binding clefts, a property that is often considered to be indicative of
259 exo-activity and/or processivity (*46*). Nevertheless all three enzymes were found to
260 predominantly act in an endo-mode when hydrolyzing chitosan (Figure 5 and Sikorski et al.
261 (*34*)). It should be noted that the enzymes may behave differently when acting on crystalline

chitin. For example, there are indications that solid β-chitin fibrils are degraded from the reducing end by ChiA and the non-reducing end by ChiB (*47*). Studies with ChiA have shown that substrate association is the rate determining step in the hydrolysis of chitin, whereas product release is rate determining when the substrate is soluble chitosan (*48*). This implies that association to a soluble substrate is much less energetically demanding than association to an insoluble substrate. In the crystalline substrate, the ends of the polysaccharide chains are the most accessible, and are thus likely to be highly preferred by the enzymes. Soluble substrates have much better accessibility, and the number of potential "internal" binding sites heavily outnumbers the number of chain ends. Thus, endo-activity is likely to become dominant, even for enzymes that have an intrinsic tendency to act in an exo-mode. So far, it is not known whether HCHT acts in an exo-mode on chitin. For comparison, enzymes of the ChiC-type (Figure 1C) have much more open and shallow substrate-binding clefts than HCHT and are considered true endo-acting enzymes.

Processivity. ChiA and ChiB (Figure 1A and 1D) are both processive enzymes that degrade chitin chains in opposite directions, while cleaving off GlcNAc dimers (*32, 47, 49*). For ChiB, mutational studies have shown that Trp^{97} and Trp^{220} in the +1 and +2 subsites, respectively, are important for the enzyme's processive action on chitosan (*32*). ChiA also has aromatic residues at these positions (Trp^{275} & Phe^{396}), but their mutation had only a limited affect on processivity. Instead, processivity in ChiA depends heavily on the presence of Trp^{167} in the –3 subsite (*49*). HCHT has an aromatic residue (Trp) in all these three positions and also contains Trp^{71} and Tyr^{34} in subsites –6 and –5, respectively, which are Phe^{232} and Tyr^{170}, respectively, in ChiA. Thus, in terms of the "aromatic signature" of the substrate-binding cleft, HCHT resembles ChiA. HCHT is expected to be processive and it might seem that the enzyme degrades chains from their reducing ends, as does ChiA. It should also be noted that ChiA and ChiB has a chitin binding domain containing three and four,

287 respectively, solvent exposed aromatic amino acids (Figure 1A and 1D), which the tested
288 isoform of HCHT does not have, that may also contribute to the degree of processivity.

289 The degree of processivity of HCHT was assessed by plotting the relative viscosity of
290 the polymer solution from which the α of the polymer fraction, α_{pol}, may be calculated, versus
291 the total number of reducing ends (α_{tot}) (Figure 6). The inverse of the slopes of the lines
292 shown in Figure 6 represent the number of cuts (N_{cuts}) per formation of an enzyme-substrate
293 complex (*34*). The observed number of cuts is expressed as relative number, where N_{cuts}
294 observed for acid hydrolysis is set to 1. The results indicate that HCHT is processive with an
295 average of 2.5 cuts per formation of enzyme-substrate complex during hydrolysis of a
296 chitosan with F_A of 0.62. The same numbers are 9.1 and 3.4 cuts per formation of enzyme-
297 substrate complex for ChiA and ChiB, respectively (*34*). As a control, the value for ChiB was
298 also determined and found to be 3.6 (Figure 6) in good accordance with the work of Sikorski
299 *et al*.

300 Processivity in family 18 chitinases leads to a diagnostic product profile dominated by
301 even-numbered products early on in the reaction with chitosan (*21, 35, 50*). HCHT showed
302 this clear dominance of even-numbered only very early in the reaction (insert in Figure 3).
303 The ratio between the size of an even-numbered peak and an odd-number peak may serve for
304 a relative quantification of processivity; in this study, the DP6 and DP7 peaks were used. At α
305 below 0.01 the DP6/DP7 ratio was about 3, but it rapidly decreased via 1.5 at $\alpha = 0.03$ to
306 about 1.3 at $\alpha = 0.08$ (Figure 3). For ChiA and ChiB the DP6/DP7 ratios at $\alpha \approx 0.08$ are
307 approximately 4 and 3, respectively (*33*). The initial dominance of even numbered products
308 for HCHT has also been detected by Gorzelanny et al. (*21*) using a different approach based
309 on the use of electrophoresis and MS. Another characteristic feature of endo-acting processive
310 enzymes is the slow disappearance of the polymer peak. This is indeed the case for HCHT,
311 where disappearance of this peak is much slower (at $\alpha > 0.13$, Figure 3) than for non-

312 processive endo-acting family 18 chitinases such as ChiC from *S. marcescens* where the
313 polymer peak disappears at $\alpha \approx 0.05$ (*33*). For ChiA and ChiB, the polymer peak disappears at
314 $\alpha \approx 0.20$. The combination of slow disappearance of the polymer peak (Figure 3) and a clear
315 endo-activity (Figure 5) coupled with an initial dominance of even-numbered products and an
316 estimated 2.5 cuts per formation of enzyme-substrate suggest that HCHT is processive, albeit
317 possibly to a lesser degree than ChiA and ChiB.

318 It is conceivable that the analysis of processivity in HCHT to some extent is disturbed
319 by transglycosylation. HCHT is known to have relatively high transglycosylation activity and
320 recent mutational work on ChiA has shown that the introduction of a Trp at position +2
321 drastically increase transglycosylation activity (*51*). This Trp is naturally present in HCHT,
322 whose active site is highly similar to that of the engineered hypertransglycosylating ChiA
323 mutant. Perhaps, the rapid disappearance of the dominance of even-numbered products during
324 the course of the reaction is somehow linked to increased occurrence of transglycosylation.
325 Furthermore, the isoform of HCHT tested in this work does not contain the C-terminal chitin
326 binding domain that contains eight aromatic amino acids (*52*) (unknown if these are solvent
327 exposed due to lack of crystal structure for this isoform), and it is conceivable that the
328 presence of this chitin binding domain may increase the degree of processivity.

329 In summary, we provide insight into how HCHT acts on chitosan, which is useful to
330 understand enzyme properties such as endo- vs. exo-action, processivity, and substrate
331 binding preferences. This information is important for further work on understanding the roles
332 of human chitinases, the faith of chitosan-containing food products or medicines, and the
333 development of inhibitors that are specific for certain chitinases. HCHT acts on fungal cell
334 walls (*4*) and it is conceivable that its processive mechanism contributes to its fungistatic
335 effect. Association to the insoluble polymer is the rate-determining step in chitin hydrolysis
336 (*48*) and a processive mode allows for more hydrolytic events to take place upon each

association compared to a non-processive mechanism. The ability to bind in an endo-mode may also promote substrate-binding since the enzyme may not be dependent of finding chain ends.

HCHT is called chitotriosidase most likely because in the original studies it was found to release 4-methylumberriuferyl from the artificial substrate 4-methylumbelliferyl-β-D-*N*-*N*'-*N*''-triacetylchitotriose. In retrospect, it is clear that chitinase action of artificial substrates is not a good way to determine the mode of action of these enzymes (*53, 54*). Also, naming an enzyme chitotriosidase when chitobiose is *produced* is somewhat strange. Regardless of formal considerations, it must be noted that the present data clearly show that the main hydrolysis product is chitobiose, i.e. the A-A- dimer (Figure 4, $F_A = 0.62$ experiment). This is fully consistent with HCHT acting as a "normal" processive enzyme. Formally, in analogy with nomenclature used in the cellulose field, it would probably be better to refer to the enzyme as chitobiohydrolase.

SUPPORTING INFORMATION. Figure S1. SDS-PAGE gel of collected fractions after ion exchange chromatography. This material is available free of charge via the Internet at http://pubs.acs.org.

ACKNOWLEDGMENTS

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FIGURE CAPTIONS

Figure 1. Crystal structures of: A ChiA from *S. marcescens* ((*55*); pdb code 1ctn), B: the 39 kDa from of HCHT ((*23*); pdb code 1guv), C: the catalytic domain of ChiC from *L. lactis* (pdb code 3ian; this domain has 67 % sequence identity with ChiC from *S. marcescens*), and D: ChiB from *S. marcescens* ((*25*); pdb code 1e15). The structures have been aligned by the position of their (conserved) catalytic centers, meaning that the substrate-binding clefts are shown in the same view. ChiA, ChiB, and HCHT contain the α/β insertion domain (a darker grey) and have deep substrate binding clefts, while ChiC has a shallower and open substrate-binding cleft. The side chains of solvent exposed aromatic amino acids in equivalent structural positions are shown in blue. HCHT has all six of these: it has an aromatic motif the in –6 to –3 subsites similar to the aromatic motif in ChiA (W71, Y34, W31), the same Trp-Trp motif in the +1 and +2 subsites as ChiB (W99, W218) and a Trp (W321) at the bottom of the –1 subsite that is fully conserved in all family 18 chitinases (labeled W539 in ChiA, W403 in ChiB, and W321 in ChiC, respectively). Aromatic amino acids in the substrate-binding clefts are known to be important for substrate-binding (*56*) and for a processive mode of action (*32, 49*). Note the "roof" over that active site cleft in ChiB (indicated by an arrow). Both ChiA and ChiB have chitin-binding domains (indicated by "CBM") with opposite orientations relative to the catalytic domain that contains solvent exposed aromatic amino acids.

Figure 2. Time course for degradation of chitosan F_A 0.62 with HCHT. The graph shows the degree of scission (α = the fraction of cleaved glycosidic bonds) as a function of time. The initial first 600 minutes of the degradation are shown as an insert.

Figure 3. Size exclusion chromatograms of oligomers obtained after degradation of chitosan (F_A = 0.62) to different degrees of scission (α) by HCHT. Peaks are labeled with DP-values or, in case of mono-component peaks with known content) with the sequence of the oligomer; the large top to the left represents the void top, containing material with a DP larger than approximately 40 (see Sørbotten et al. (*35*) for a detailed description of how the chromatograms are interpreted). The insert for SEC chromatogram for α = 0.03 is resulting oligomers at α below 0.01. A picture for maximally degraded chitosan (α = 0.33) is provided in Figure 4.

Figure 4. Degradation of chitosans with varying F_A to maximum degree of scission. To ensure reaching maximum α, samples were collected after it had been established that addition of enzyme to the reaction mixtures did not yield further increase in α.

Figure 5. Changes in the DP_n^{pol} as a function of the reaction extent α.

Figure 6. Degree of scission of the polymer fraction (α_{Pol}) as a function of the total degree of scission (α_{tot}).

TABLES.

Table 1. Characterization of Chitosans[a]

chitosan (F_a)	[η] (mL/g)	MW
0.18	800	257 000
0.35	730	233 000
0.49	746	238 000
0.62	865	280 000

[a] Fraction of acetylated units (F_A), intrinsic viscosities ([η]), and average molecular weight (MW) of the chitosans. The molecular weights were calculated from the intrinsic viscosity vs. molecular weight relationship (*57*).

Table 2. Sequences of the isolated oligomers of different length obtained after hydrolysis of high molecular chitosan, $F_A = 0.62$, at different degrees of scission.[a]

DP_n	Species	**α = 0.03**	**α = 0.08**	**α = 0.13**	**α = 0.33**
DP3	A3	AAA	AAA	AAA	
	A2D	DAA	DAA	DAA	DAA
					ADA
	AD2				ADD
DP4	A4	AAAA	AAAA	AAAA	
	A3D	DAAA	DAAA	DAAA	
				ADAA	ADAA
	D2A2				DDAA
DP5	A4D	AADAA	ADAAA	ADAAA	
		ADAAA		AADAA	
	A3D2		DADAA	DADAA	ADDAA
			DDAAA	DDAAA	
	A2D3				DDDAA
DP6	A5D	AAADAA	AAADAA	AADAAA	
		AADAAA	AAADAA	ADAAAA	
	A4D2	ADADAA	DADAAA	DAADAA	
		ADDAAA	ADADAA	ADADAA	
	A3D3		DDDAAA	DADDAA	ADDDAA
			DADDAA	DDDAAA	DDADAA
				DDADAA	
	A2D4				DDDDAA

[a] Note that the sequencing method is based on labeling of the reducing end and that sequences therefore are determined "from the reducing end" (*40*). When two different sugars appear in a certain position, ambiguities are introduced for the "remaining" sequence towards the non-reducing end. The sequences shown are those that are compatible with the mass spectra and not all shown sequences may actually occur. For example, the pentamer fraction at alpha = 0.13 only contains products ending at -ADAA and –DAAA but it is not certain that all four given pentamer sequences actually occur. For the hexamer fraction ambiguities of course are even larger.

FIGURES

Figure 1

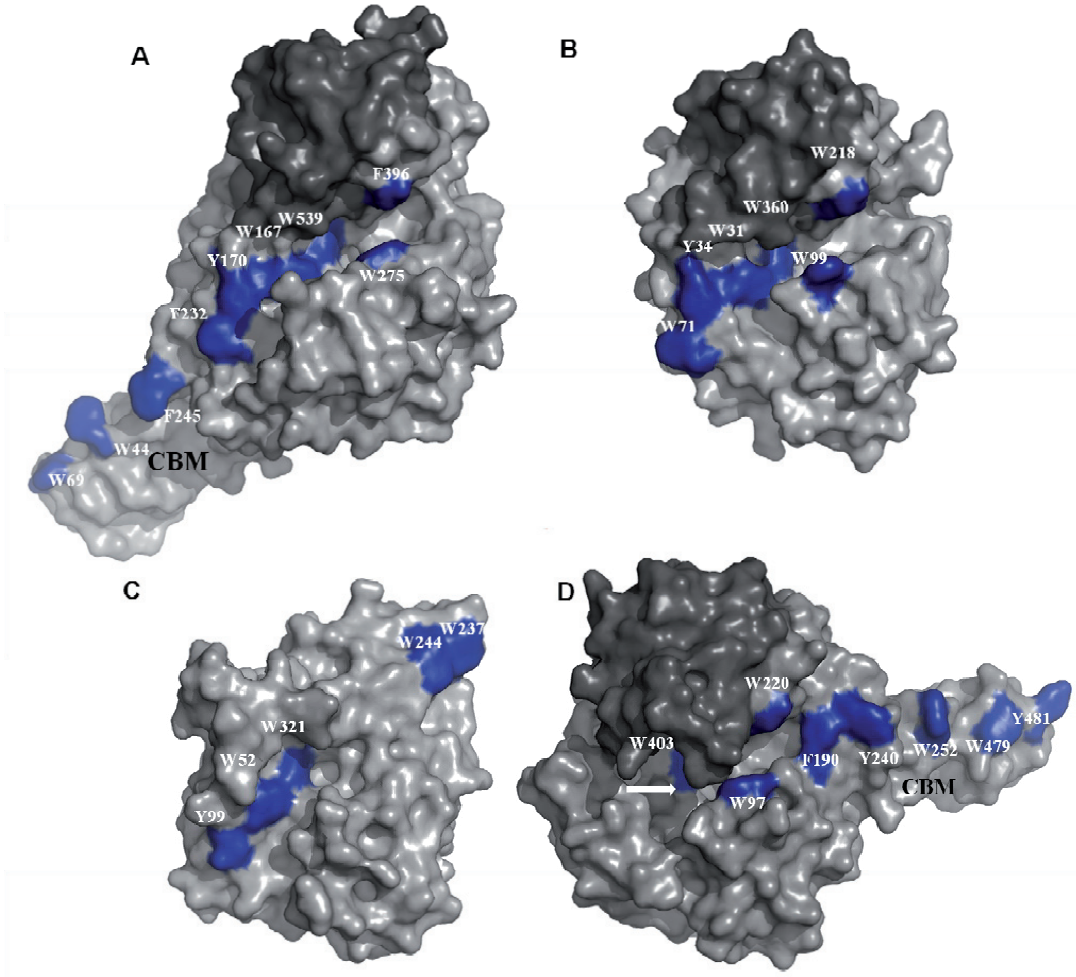

Figure 2

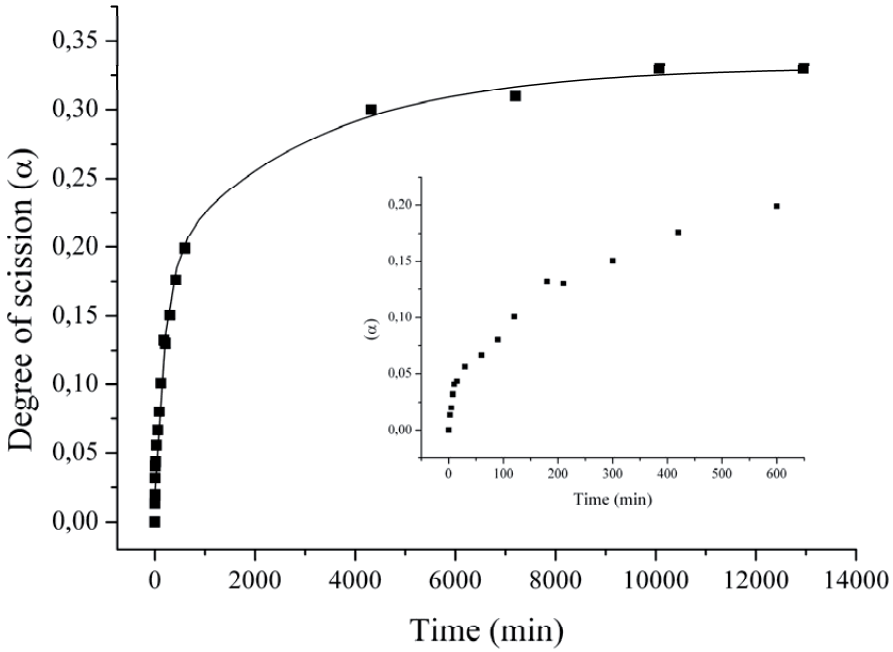
Degree of scission (α)
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
0
2000
4000
6000
8000
10000
12000
14000
Time (min)
(α)
0,20
0,15
0,10
0,05
0,00
0
100
200
300
400
500
600
Time (min)

Figure 3

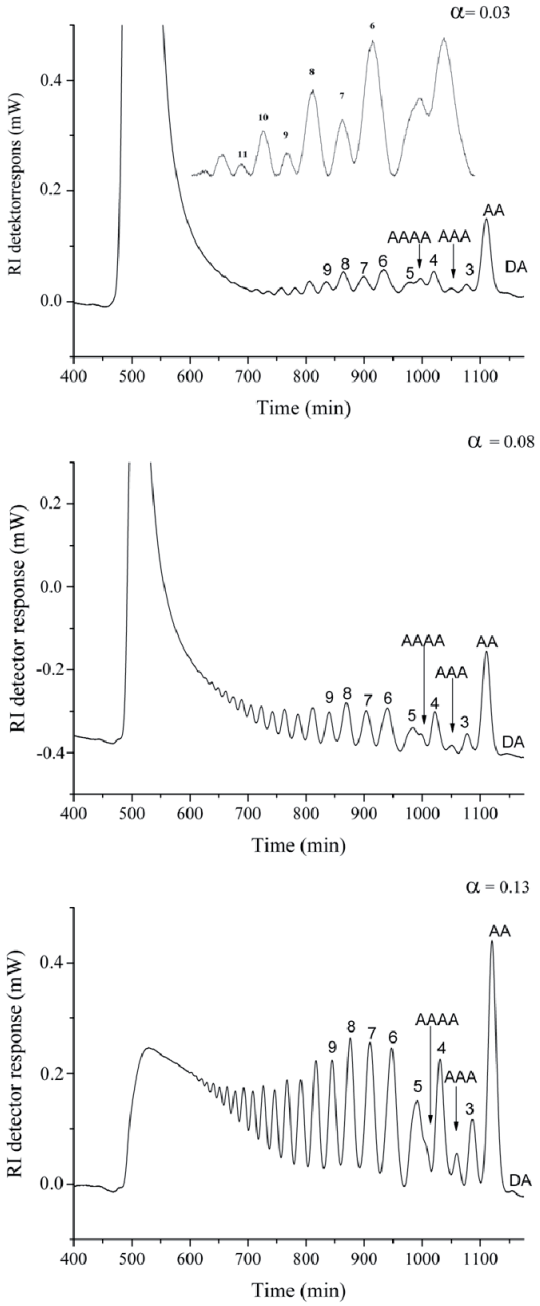
α= 0.03
RI detektorrespons (mW)
Time (min)
α = 0.08
RI detector response (mW)
Time (min)
α = 0.13
RI detector response (mW)
Time (min)
AA
AAAA
AAA
DA

Figure 4

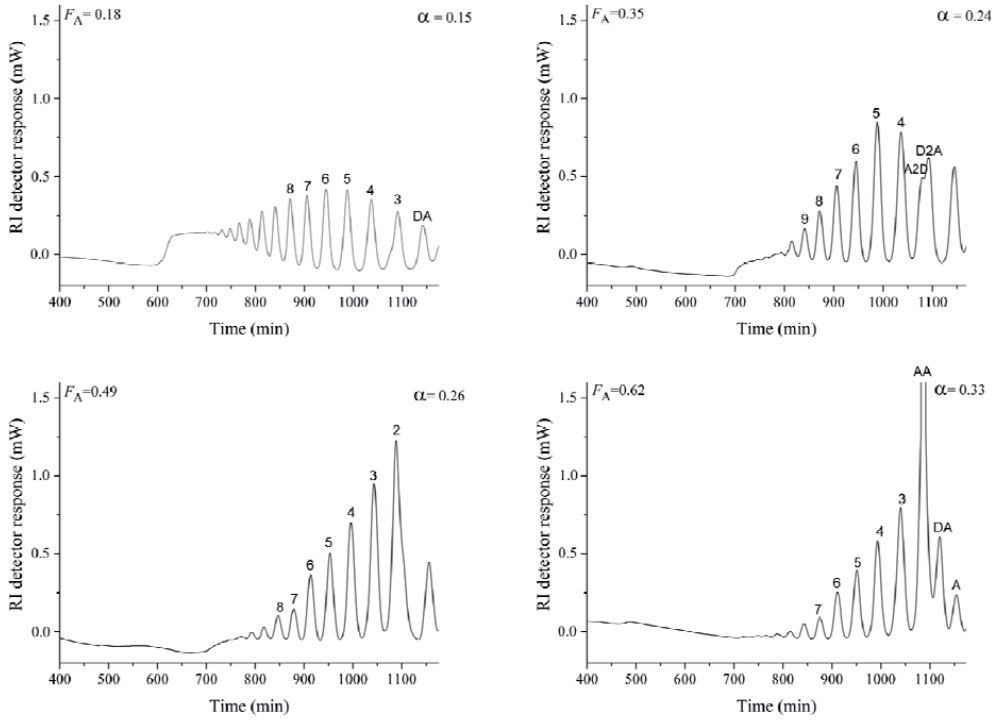
F_A= 0.18
α = 0.15
8
7
6
5
4
3
DA
F_A=0.35
α = 0.24
9
8
7
6
5
4
D2A
A2D
F_A=0.49
α= 0.26
8
7
6
5
4
3
2
F_A=0.62
α= 0.33
AA
7
6
5
4
3
DA
A
RI detector response (mW)
Time (min)
0.0
0.5
1.0
1.5
400
500
600
700
800
900
1000
1100

Figure 5

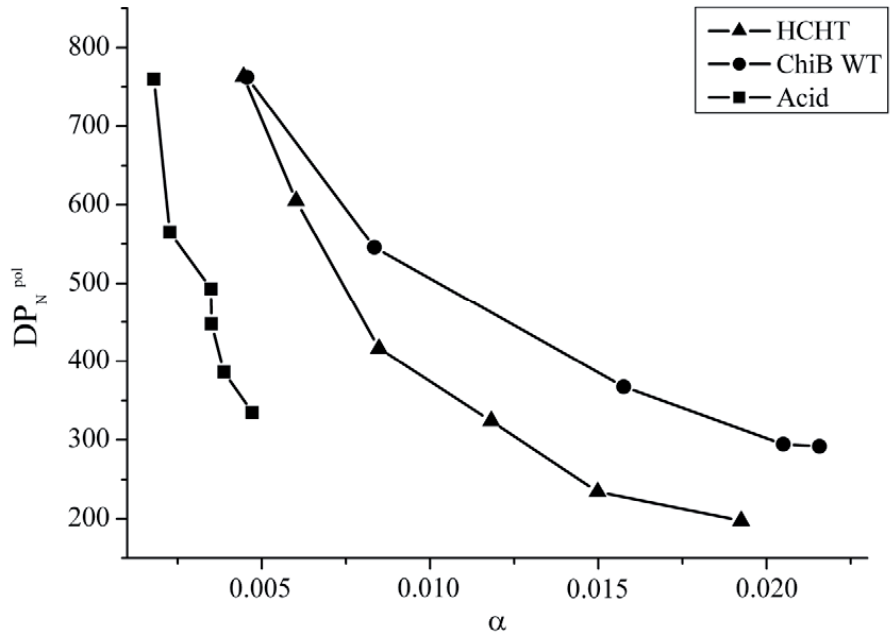
HCHT
ChiB WT
Acid
800
700
600
500
400
300
200
DP_N^{pol}
0.005
0.010
0.015
0.020
α

Figure 6

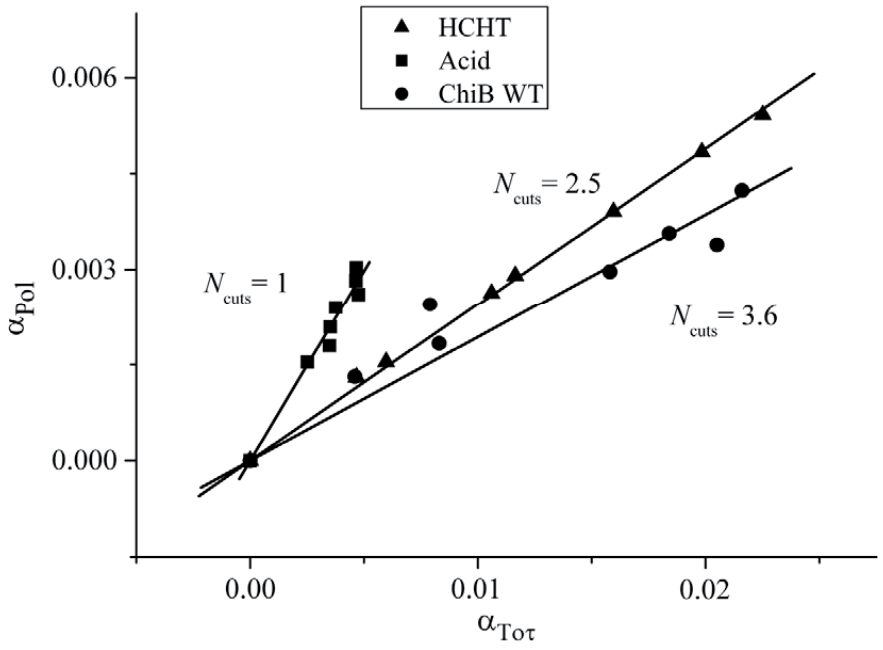
HCHT
Acid
ChiB WT
N_{cuts} = 2.5
N_{cuts} = 1
N_{cuts} = 3.6
0.006
0.003
0.000
α_{Pol}
0.00
0.01
0.02
α_{Tot}

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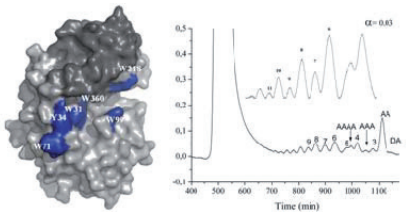
Table of Contents Graphic

Human Chitotriosidase Catalyzed Hydrolysis of Chitosan

Kristine Bistrup Eide, Anne Line Norberg, Ellinor Bævre Heggset, Anne Rita Lindbom, Kjell Morten Vårum, Vincent G.H. Eijsink, and Morten Sørlie



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Paper IV

Degradation of Chitosans with Chitinase G from *Streptomyces coelicolor* A3(2): Production of Chito-oligosaccharides and Insight into Subsite Specificities

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We have studied the degradation of soluble heteropolymeric chitosans with a bacterial family 19 chitinase, ChiG from *Streptomyces coelicolor* A3(2), to obtain insight into the mode of action of ChiG, to determine subsite preferences for acetylated and deacetylated sugar units, and to evaluate the potential of ChiG for production of chito-oligosaccharides. Degradation of chitosans with varying degrees of acetylation was followed using NMR for the identity (acetylated/deacetylated) of new reducing and nonreducing ends as well as their nearest neighbors and using gel filtration to analyze the size distribution of the oligomeric products. Degradation of a 64% acetylated chitosan yielded a continuum of oligomers, showing that ChiG operates according to a nonprocessive, endo mode of action. The kinetics of the degradation showed an initial rapid phase dominated by cleavage of three consecutive acetylated units (**A**; occupying subsites −2, −1, and +1), and a slower kinetic phase reflecting the cleavage of the glycosidic linkage between a deacetylated unit (**D**, occupying subsite −1) and an **A** (occupying subsite +1). Characterization of isolated oligomer fractions obtained at the end of the initial rapid phase and at the end of the slower kinetic phase confirmed the preference for **A** binding in subsites −2, −1, and +1 and showed that oligomers with a deacetylated reducing end appeared only during the second kinetic phase. After maximum conversion of the chitosan, the dimers AD/AA and the trimer AAD were the dominating products. Degradation of chitosans with varying degrees of acetylation to maximum degree of scission produced a wide variety of oligomer mixtures, differing in chain length and composition of acetylated/deacetylated units. These results provide insight into the properties of bacterial family 19 chitinases and show how these enzymes may be used to convert chitosans to several types of chito-oligosaccharide mixtures.

Introduction

Chitin is the most abundant biopolymer in the animal kingdom, occurring mainly as a structural polysaccharide in the exoskeleton of crustaceans and insects.[1] Chitin is insoluble in aqueous solutions and chemically composed of (1→4)-linked units of 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc, **A**-unit) that may be de-*N*-acetylated to various extents.[2] Chitosans are linear, cationic polysaccharides composed of (1→4)-linked units of 2-amino-2-deoxy-β-D-glucopyranose (GlcN, **D**-unit) and **A**-units, which are soluble in acidic solutions.[3] Chitosans with widely varying degrees of *N*-acetylation and chain length can be prepared from chitin. Variation in the chemical composition and chain length of chitosans affects properties such as solubility,[4] binding to lysozyme,[5] susceptibility to degradation by lysozyme,[6,7] as well as functional properties in drug delivery[8,9] and gene delivery systems.[10−13] Previous studies have shown that chitosans obtained by homogeneous de-*N*-acetylation of chitin, using methods such as those used in the present study, have a random distribution of *N*-acetyl groups.[14−16]

Chitinases and chitosanases are glycoside hydrolases that convert chitin and chitosans to low molecular weight products (chito-oligosaccharides, CHOS) by hydrolyzing the β(1→4) glycosidic linkages between the sugar units. Glycoside hydrolases can be divided into different families based on primary sequence, three-dimensional structure, and catalytic mechanism.[17,18] There are several families of chitinases (primarily glycoside hydrolase families 18 and 19) and chitosanases (primarily glycoside hydrolase families 5, 8, 46, 75, and 80).[19] Family 18 and 19 chitinases have very different three-dimensional structures and use different catalytic mechanisms. While family 18 chitinases use a substrate-assisted double-displacement reaction with retention of the configuration of the anomeric carbon,[20] family 19 chitinases use a single-displacement reaction with inversion of the configuration of the anomeric carbon.[18,21] Because of the involvement of the *N*-acetyl group in catalysis,[20,22] productive substrate-binding of chitosans to family 18 chitinases requires that the −1 subsite is occupied with an acetylated sugar (**A**).[23]

Water-soluble chitosans provide unique opportunities to study enzyme properties such as processivity that are difficult to study with the insoluble crystalline chitin substrate.[24] We have recently undertaken a detailed study of the mode of action of the family 18 chitinases ChiA, ChiB, and ChiC from *Serratia marcescens*,[23,25] using chitosans as substrates, and found that ChiA and ChiB are processive enzymes that tend to bind the substrate in an endofashion, while ChiC is an nonprocessive endochitinase.[26] These studies also revealed preferences for **A** versus **D** in subsites −2 and +1, showing that the enzymes had no preference in +1, whereas there was a preference for **A** in +2,

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which in the case of ChiC was almost absolute. In addition to providing important insight into enzyme properties and how these enzymes function together, these studies showed how different mixtures of CHOS could be produced by varying the enzyme concentration, the extent of cleavage and the degree of acetylation of the substrate.[23,25,27] Controlled production of CHOS is of interest because they have several potential applications in agriculture[28−32] and medicine.[33−37]

While family 18 chitinases are well studied, less is known about the properties of family 19 chitinases. These enzymes are commonly found in plants and several of them have been characterized to some extent. Bacterial family 19 chitinases were only recently discovered[38] and are primarily found in *Streptomyces* spp., the main decomposers of chitin in the soil. The genome sequence of *S. coelicolor* A3(2) revealed 11 putative family 18 chitinases and two putative family 19 chitinases, ChiF and ChiG.[39−41] ChiF is a two-domain enzyme similar to ChiC from *S. griseus* HUT6037,[38] consisting of a catalytic domain and an N-terminal chitin-binding domain. ChiG consists of a catalytic domain only.[42] The recently determined crystal structures of ChiC[43] and ChiG[44] revealed clear differences between the catalytic domains of these bacterial enzymes and those of plant enzymes.[44]

Here, we have analyzed the degradation of well-characterized, fully water-soluble, high-molecular weight chitosans by ChiG, to gain insight into the mode of action and subsite binding preferences of the enzyme and to explore the possibilities of producing new CHOS mixtures. We have used chromatographic methods for oligomer separation and previously established NMR-based methods for analyses of the reaction progress and the chemical composition of purified oligomer fractions. The results show that ChiG acts as an endochitinase with clear binding preferences in subsites −2, −1, and +1, quite different from family 18 chitinases. This is reflected in the formation of oligomers with widely varying size distributions and chemical compositions that differ from those obtained with family 18 chitinases.

Experimental Section

Chitosans. Chitin was isolated from shrimp shells as described by Hackman,[45] and milled in a hammer mill to pass through a 1.0 mm sieve. Chitosans with various degrees of *N*-acetylation of 64, 50, 32, and 13% (i.e., fraction of acetylated units (F_A) = 0.64, 0.50, 0.32, and 0.13 and intrinsic viscosities of 740, 850, 820, and 910 mL/g, respectively) were prepared by homogeneous de-*N*-acetylation of chitin.[46] The detailed characterization of these chitosans have previously been reported,[23] showing that the chitosans have a random distribution of **A**- and **D**-units, that is, according to a Bernoullian distribution.[15,16]

Chitinase G. ChiG was overexpressed in *Escherichia coli* BL21Star (DE3) and purified as described earlier.[44] The purified enzyme was dialyzed against 20 mM Tris-HCl, pH 8.0, and stored at 4 °C under sterile conditions. Protein concentration measurements were done according to Bradford[47] using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, U.S.A.), with bovine serum albumin (BSA) as a standard.

Enzymatic Degradation of the Chitosans. Chitosan (as chitosan hydrochloride) was dissolved in water to 20 mg/mL and incubated with shaking in room temperature, overnight. Subsequently, an equivalent volume of 0.08 M sodium acetate buffer, pH 5.5 (containing 0.2 M NaCl), and 0.2 mg BSA was added. The chitosan solutions (final chitosan concentration, 10 mg/mL) were incubated in a shaking water bath at 37 °C, and degradation reactions were started by adding 0.05 μg ChiG per mg chitosan, using a highly concentrated ChiG stock solution. Samples were taken from the reactions at regular time intervals from 2 to 23200 min after the initiation of the reaction, and the reaction was stopped by boiling these samples for 5 min, followed by a centrifugation to remove potential precipitates. Insolubility of the oligomer products was not observed with ChiG. A negative control where chitosan (F_A = 0.64) was solubilized and incubated (for 24 h) without adding ChiG was included (see Figure 4, top chromatogram). This chromatogram shows that the chitosan elute in the void volume of the column without detectable amounts of oligomers.

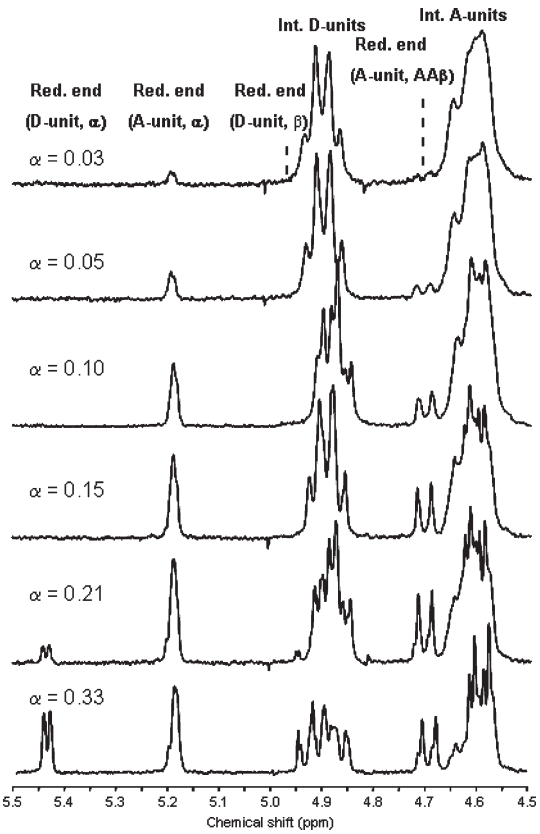

Figure 1. ^{1}H NMR spectra showing the anomer region of chitosan (F_A = 0.64) after hydrolysis with ChiG to α-values between 0.03 and 0.33. Spectra were acquired at 300 MHz at 90 °C and pD 4.2, and resonances were assigned according to previously published ^{1}H NMR spectra of degraded chitosan.[15,23,57] The α-anomer of a **D** resonates at 5.43 ppm and the β-anomer at 4.92 ppm. The α-anomer of the **A**-unit resonates at 5.19 ppm, whereas the β-anomer resonates at 4.705 ppm, if the neighboring residue is an **A** (visible in the spectrum) or at 4.742 ppm if the neighboring residue is a **D** (not visible in the spectrum). The internal **D**-units resonate at 4.8−4.95 ppm, while the internal **A**-units resonate at 4.55−4.68 ppm. The β-resonance for a **D** is only partially resolved from the resonance for the internal **D**-units.[15,58]

For maximum degradation of chitosans, reactions were performed by adding 2 μg ChiG per mg chitosan, and the incubation times were 10080 min (one week). The samples were stored at −20 °C until further analysis.

Control experiments were performed to check whether ChiG was inactivated during the (one week) incubation with substrate. Degradation reactions were allowed to proceed for one week (as described above), and the size-distribution of oligomers determined by SEC (see below). More enzyme were added to the degraded chitosan, and the reaction were allowed to proceed for one additional week. The size-distribution of oligomers determined by SEC (see below), without any detectable change in size-distribution, showing that ChiG is not significantly

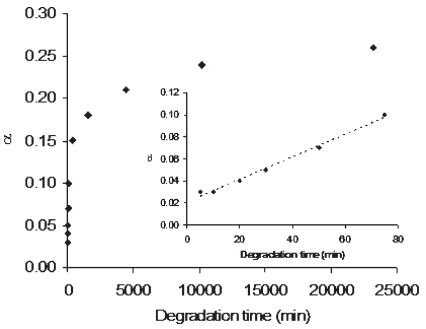

Figure 2. Degradation of a $F_A = 0.64$ chitosan with ChiG. The degree of scission, α, as determined from ^{1}H NMR spectra, as a function of the incubation time.

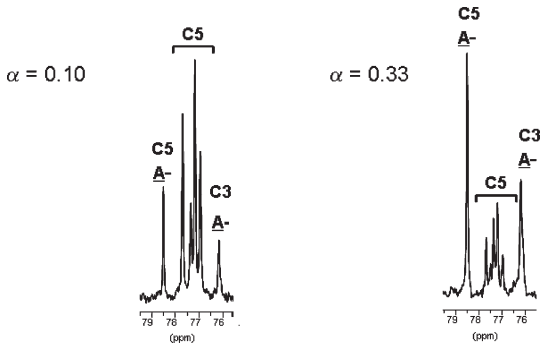

Figure 3. Part of the ^{13}C NMR spectra (between 79.5−75.5 ppm, C5 and C3 region) of partially and extensively degraded $F_A = 0.64$ chitosan (α = 0.10 and 0.33, respectively). Spectra were acquired at 400 MHz, 90 °C and pD 4.2. Acetylated nonreducing ends of carbon 5 (C5) resonate at 78.5 ppm. Deacetylated nonreducing ends of C5 resonate at 79.1 ppm[7] and are absent in the spectra. Acetylated nonreducing ends of carbon 3 (C3) with an **A** in the neighboring position (AA-) resonate at 76.2 ppm, while **A** nonreducing ends with a **D** in the neighboring position (AD-) resonate at 76.1 ppm.

inactivated after one week. Similar experiments were performed to test product inhibition where more substrate was added, without any detectable change in the size-distribution. These results show that neither enzyme inactivation nor product inhibition was important at the current experimental conditions.

Calculation of the Specific Activity. The change in the degree of scission of the degradation was calculated from the initial (linear) part of the line in Figure 2. Specific activity was calculated by converting the change in α to mole of cleaved bonds per second and dividing the result by the molar ChiG concentration. To compare the specific activity of ChiG with that of ChiB from *S. marcescens*, we used the data presented in Figure 3 in Sørbotten et al. (2005),[23] and similar calculations as described for ChiG.

Size Exclusion Chromatography (SEC). Chitosan and CHOS were separated on three XK 26 columns, coupled in series, packed with Superdex 30 from Pharmacia Biotech (Uppsala, Sweden), with an overall dimension of 2.60 × 180 cm. The mobile phase used was 0.15 M ammonium acetate, pH 4.5, and a LC-10ADvp pump (Shimadzu GmbH, Duisburg, Germany) delivered the mobile phase at a flow rate of 0.8 mL/min. The columns were coupled to a refractive index (RI) detector (Shodex RI-101, Shodex Denko GmbH, Dusseldorf, Germany), and the data were logged with a CR 510 Basic Data logger (Campbell Scientific Inc., Logan, UT, U.S.A.). It has previously been shown that there is a linear relationship between peak areas and the amount (mass) of injected oligomer, irrespective of DP and degree of *N*-acetylation.[23] Fractions of 3.2 mL were collected using a fraction collector, for characterization of the isolated oligomers. For quantitative studies of degradation, the sample size was typically 10 mg degraded chitosan. When oligomers were collected for NMR characterization, degraded chitosan samples (50−200 mg) were to be injected.

NMR Spectroscopy. Samples for ^{1}H NMR and ^{13}C NMR were dissolved in D_2O, and the pD was adjusted to 4.2 with DCl/NaOD. The deuterium resonance was used as a field-frequency lock, and the chemical shifts were referenced to internal sodium 3-(trimethylsilyl-)propionate-d_4 (0.00 ppm). The spectra was obtained at 300 or 400 MHz (^{1}H NMR) at 90 °C, as previously described.[7,15,16] To further resolve the sequence dependent shift of the C3 nonreducing end, that is, +2 subsite preferences (see Figure 3, α = 0.10), the FID was reprocessed (using an exponentially decaying window-function) with a line broadening factor of 1 Hz instead of 3 Hz.

As earlier described in Sørbotten et al., 2005,[23] ^{1}H NMR was used to determine the sequence of shorter oligomers and to calculate the number average degree of polymerization, DP_n, of the enzyme-degraded chitosans. The degree of scission, α, which gives the fraction of cleaved glycosidic linkages is determined as $\alpha = 1/DP_n$.

Results

Degradation of a Highly Acetylated Chitosan with $F_A = 0.64$. *Binding Preferences in Subsites −1 and −2 and Time Course of the Reaction.* Progress of the chitosan degradation reaction after incubation with ChiG was followed by determining the increase in reducing end resonances relative to internal H-1 protons using ^{1}H NMR spectroscopy. Because the NMR spectrum provides information about the identity of the new reducing ends (**A** or **D**), it reveals the binding preference of the −1 subsite. Also, because the resonance of the anomeric proton is influenced by the identity of the neighboring sugar, the NMR spectrum yields information about the identity of the sugar unit bound productively to the −2 subsite. The degree of scission, α, is the fraction of glycosidic linkages that has been cleaved. Figure 1 shows ^{1}H NMR spectra of the anomer region at α-values between 0.03 and 0.33.

At low α-values (from 0.03 to 0.10), the new reducing ends are exclusively acetylated, as can be seen from the signals at 5.19 ppm for −**A**(α) and at 4.705 ppm for −AA(β). The β-anomer signal for a reducing end **A** occurs at different chemical shift values depending on whether the preceding sugar is an **A** or a **D**, that is, at 4.705 and 4.742 ppm, respectively. For ChiG, we could not identify, not even after extended degradation, any resonances at 4.742 ppm, showing that ChiG exclusively binds **A** productively in the −2 subsite. After more extensive degradation, at α-values above 0.15, resonances for deacetylated reducing ends became apparent, at 5.43 ppm (−**D**(α)) and 4.92 ppm (−**D**(β)). It is not possible to discriminate between the identity of the neighboring unit to a reducing end **D** (see legend to Figure 1) but the observations for the acetylated reducing ends described above and the sequence analysis of products described below, do strongly suggest that ChiG exclusively binds **A** productively in the −2 subsite with a **D** in the −1 subsite.

The kinetics of the degradation of the high molecular weight chitosan substrate ($F_A = 0.64$) reveal a rapid initial degradation phase at α-values below 0.10 (Figure 2). This faster and linear part of the reaction correlates with the phase with productive binding of **A** in subsites −1 and −2 both. Apparently, binding of sequences that position a **D** in subsite −1 slows down the reaction rate. Extended incubations, with high enzyme concentrations showed that the maximum value of α was 0.33, meaning that about 1 in 3 of the glycosidic bonds had been cleaved.

+1 and +2 Subsite Specificities. ^{13}C NMR spectroscopy can reveal the identity of the new nonreducing ends in degraded

chitosans[7] and can thus be used to obtain information concerning the specificity of the +1 subsite. Figure 3 shows part of two ^{13}C NMR spectra (from 79.5 to 75.5 ppm) of partially and extensively degraded chitosan ($F_A = 0.64$), with the resonances of carbon 5 (C5) and the nonreducing end of carbon 3 (C3). The spectra show an almost absolute dominance of acetylated units at the new nonreducing ends. Further work, described below, confirmed that all oligomers produced have an **A** at the nonreducing end.

It has previously been found that **A/D** preferences in subsite +2 can be determined as the nonreducing **A** residue of C3 shows a sequential shift depending on the neighboring unit.[7] The resonance of the nonreducing **A** of C3 reveals a splitting which corresponds to AA- and AD- (76.2 and 76.1 ppm, respectively). The ratio between the resonances was 80:20 (results not shown), showing a low, but significant preference toward **A** in subsite +2, because the F_A is increased in the +2 subsite as compared to the F_A of the substrate chitosan (0.64).

Size-Distribution of Oligomers as a Function of α. Figure 4 shows the size-distribution of oligomers obtained from hydrolysis of chitosan ($F_A = 0.64$) at degrees of scission (α) between 0.03 and 0.33. Identification of the DP of the peaks was performed by comparing these chromatograms with chromatograms of standard-samples of fully acetylated and fully deacetylated oligomers (DP 1→6). At DP > 4, CHOS with varying **A/D** compositions tend to elute as one peak, whereas, at lower DP fully acetylated dimers/trimers are separated from partially acetylated dimers/trimers (see Figure 4 and ref 23 for further details). The chromatograms show that the shorter oligomers produced during the initial, faster phase of the reaction are fully acetylated, confirming that ChiG has a clear preference for cleaving consecutive sequences of **A**. As the reaction proceeds the void peak (DP > 50) gradually disappears and short partially *N*-acetylated oligomers become apparent, for example, the new tetramer peak at α = 0.10 and the trimer AAD and the dimer AD at α = 0.21. While species such as AAD and AD emerge due to increased cleavage after a **D** (see Figure 1), shorter fully acetylated products such as AAAA and AAA are further degraded and disappear. Conversion of these fully acetylated oligomers is also demonstrated by the appearance of the acetylated monomer (A) in the chromatograms at α-values of 0.21 and higher. After extensive degradation with ChiG (α = 0.33), the product spectrum is strongly dominated by AAD, AA, and AD, and the vast majority of oligomers have a DP less than 10. An intriguing observation is the almost complete absence of tetramers after extensive degradation of the chitosan substrate.

Chemical Composition of Oligomers after Degradation to α = *0.10 and 0.33.* Individual oligomer fractions obtained after degradation of the $F_A = 0.64$ chitosan to low α (α = 0.10) and high α (α = 0.33) were collected (see Figure 4) and characterized by ^1H NMR spectroscopy.

α = *0.10.* The dimer and trimer fractions were confirmed to consist exclusively of AA and AAA (DP and F_A were determined as 2.1 and 1.0 and 3.0 and 1.0, respectively), as indicated in the chromatogram (Figure 4). The two tetramer fractions (labeled "AAAA" and "4" in the chromatogram) yielded a DP of 4.1 and a F_A of 0.77 for "4" and a DP of 3.9 and a F_A equal to 1.0 for the peak labeled with AAAA. Considering the observation from NMR (Figure 1) that the reaction mixture at α = 0.10 did only contain oligomers with −AA at the reducing ends and **A** at the nonreducing end, the tetramers in the peak labeled with "4" must be ADAA.

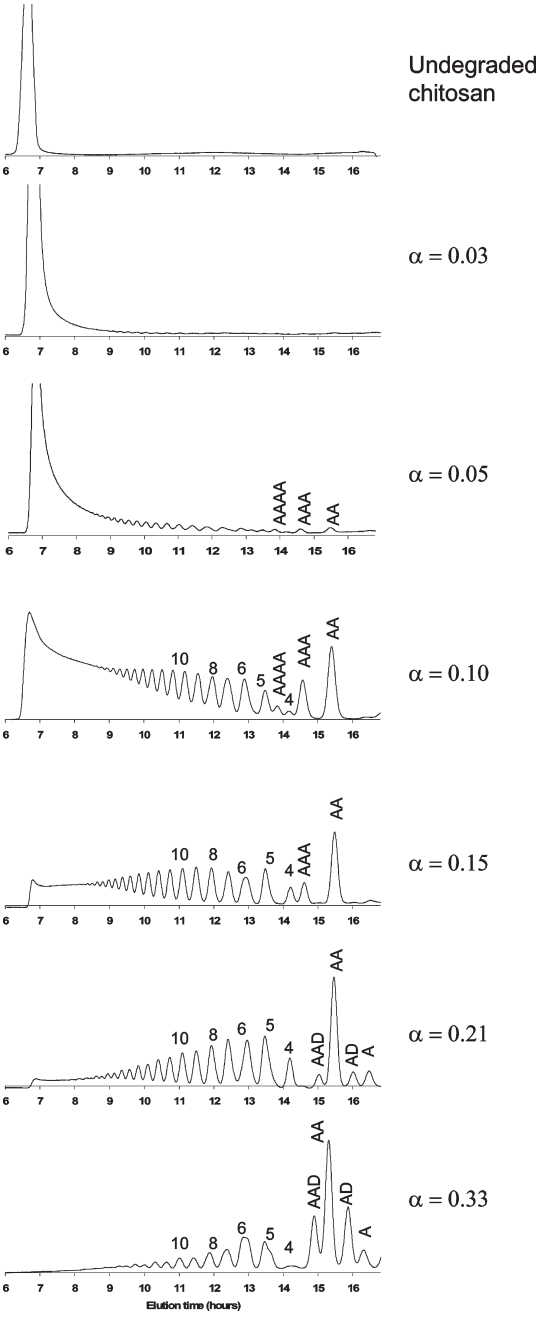

Figure 4. SEC chromatograms showing the size-distribution of oligomers obtained after degradation of chitosan ($F_A = 0.64$) with ChiG. The degree of scission, α, varies from essentially 0 (undegraded chitosan) to 0.33. Peaks are labeled with DP-values or with the oligomer sequence.

Figure 5 shows the ^1H NMR spectrum of the isolated pentamer fraction. The spectrum indicates a DP of 5.0 and an F_A of 0.79, suggesting a predominant A_4D_1 composition. Considering the information about the reducing and nonreducing ends, the sequence of the pentamer must be ADAAA and/or AADAA.

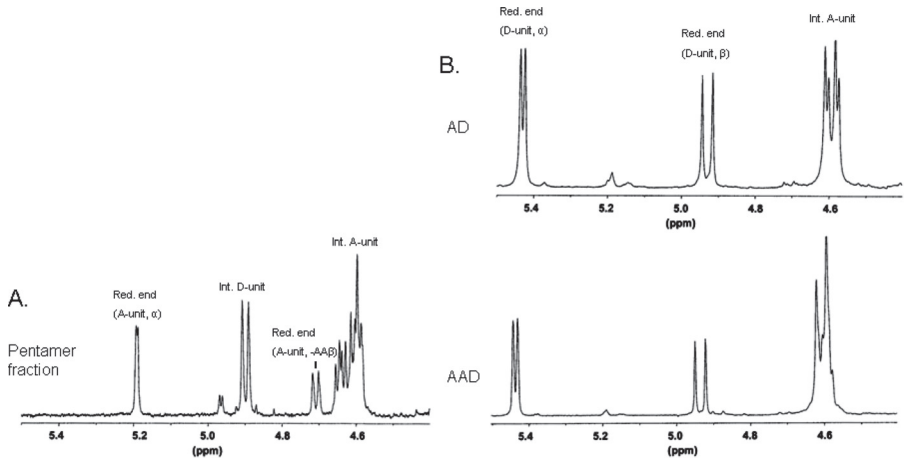

Figure 5. ^{1}H NMR spectra (anomer region) of selected oligomer fractions. (A) Pentamer fraction obtained after degradation to a degree of scission of 0.10. The minor resonance at 4.98 ppm is tentatively assigned to a Schiff-base proton (-CH=N-) formed between the reducing end aldehyde group and an amino group from a **D**. (B) Dimer and trimer fractions obtained after extensive degradation ($\alpha = 0.33$).

For higher oligomers, the NMR spectra became too complicated to derive sequence information. The spectra did show, however, that all oligomers had −AA at their reducing ends. The spectra also showed that the F_A-value decreased with increasing chain length. This was to be expected since the clear preferences of ChiG for binding **A** in several of its subsites (see above) makes oligomers with lower F_A-values less favorable substrates.

$\alpha = 0.33$. ^{1}H NMR analysis of the fraction eluting last from the column showed a DP- and a F_A-value equal to 1.0, showing that the fraction consists of the monomer A. The de-*N*-acetylated monomer (GlcN) could not be detected. The following two fractions (elution time between 15 and 16 h) had both a DP-value close to 2 (2.1 and 2.0, respectively) and F_A-values of 0.55 and 1.0, confirming that they contain AD (eluting at the highest elution time) and AA, respectively. For illustration, the ^{1}H NMR spectrum of the AD fraction is shown in Figure 5, revealing deacetylated reducing ends and acetylated nonreducing end in equal amounts (the integrated area of the two resonances labeled reducing end -**D**α and -**D**β equals the integrated area of the resonance labeled internal **A**-units). ^{1}H NMR analysis of the next fraction gave a DP of 3.1 and a F_A of 0.68 and the spectrum (Figure 5) showed almost exclusively deacetylated units at the reducing end. Thus the sequence of this oligomer is AAD. These two partially *N*-acetylated oligomers could be obtained in relatively high yields of 11% (AD) and 9% (AAD) by weight, making this enzyme/substrate/degradation extent combination an attractive way of producing the oligomers.

The next fraction, containing tetramers, was too small for further analysis. The ^{1}H NMR spectrum of the pentamer fraction revealed a DP of 5.0 and a F_A-value of 0.53. The relative amounts of **A**- and **D**-units at the reducing end are 68 and 32%, respectively. Considering the fact the absolute preference for **A** is at the position next to the reducing end and at the nonreducing end, the dominant pentamer sequences must be ADDAA and ADDAD.

Degradation of Chitosans with Varying F_A. Chitosans with varying degrees of acetylation ($F_A = 0.13$, 0.32, and 0.50) were degraded to maximum α-values of 0.04, 0.12, and 0.23, respectively. Chromatographic analysis of the products (Figure 6) showed that the DP of the oligomers increased as the degree of acetylation decreased. Degradation of a highly deacetylated chitosan ($F_A = 0$; results not shown) did not yield any oligomers with DP < 50, showing that acetylated units are required in the active site for productive binding. For the chitosan with $F_A = 0.13$ oligomers with DP < 50 were observed, but the major part of the degraded chitosan eluted in the void volume, showing DP-values above 50. When comparing the oligomers with DP from 6 and below for the three chitosans with F_A of 0.64, 0.50, and 0.32, their relative amounts are quite similar, and the main difference is related to the increasing amount of oligomers with DP > 6 with decreasing F_A.

The chemical composition of the oligomers produced after degradation of the $F_A = 0.32$ chitosan was analyzed by ^{1}H NMR spectroscopy (results not shown). The trimer fraction had a DP_n of 3.3 and a F_A of 0.65, and the ^{1}H NMR spectrum showed that the reducing end was exclusively a **D**. Thus, this fraction is dominated by the trimer AAD. The tetramer fraction was too small for further analysis. The ^{1}H NMR spectrum of the pentamer fraction showed a DP of 5.0 and a F_A of 0.45, and the reducing end had 45% **A** and 55% **D**. This suggests that the pentamer fraction primarily contains ADDAD and ADDAA. The ^{1}H NMR spectra of the hexamer and the heptamer fraction showed a DP of 6.2 and a F_A of 0.43 and a DP of 8.2 and a F_A of 0.37, respectively. Thus, it would seem that the hexamer and the heptamer fractions mainly consist of $A(D)_xAD$ and $A(D)_x$-AA, where x is equal to 3 for the hexamer fraction and 4 for the heptamer fraction. Oligomers with stretches of consecutive **D**-units will not be cleaved by ChiG, because of the specificity for **A** in subsite −2 and +1.

The spectra for the longer oligomers were too complex in composition, to be interpreted with respect to the composition and sequence of the oligomers. All spectra showed the resonance at 4.705 ppm showing the occurrence of −AA at the reducing end, while the nonreducing ends were exclusively acetylated units.

Discussion

ChiG degradation of a water-soluble chitosan ($F_A = 0.64$) reveals that the enzyme operates according to a nonprocessive endo mode of action (Figure 4). We have found that ChiG can hydrolyze chitosans of varying degrees of acetylation although to very different extents (Figure 6). The subsite specificities of

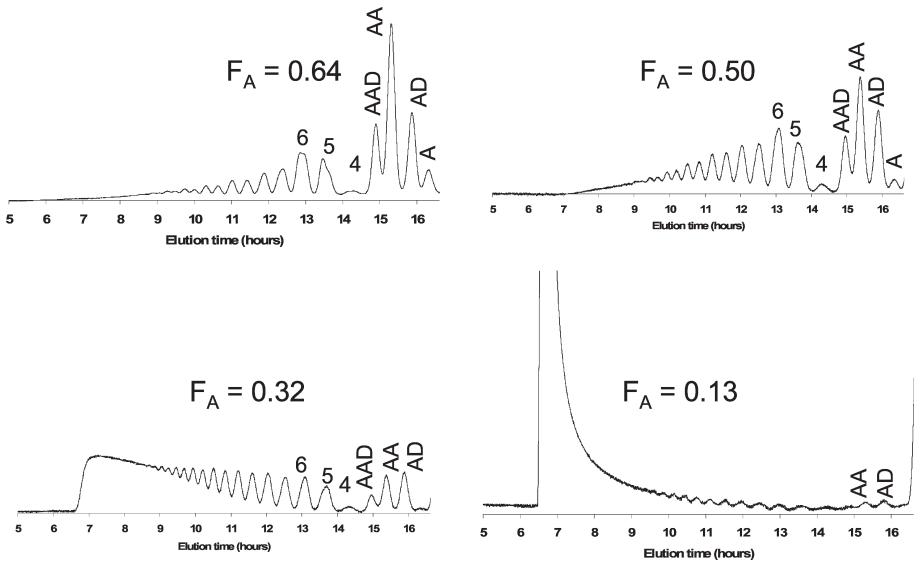

Figure 6. SEC chromatograms showing the size-distribution of oligomers obtained after maximum degradation of chitosans with various F_A. The chitosans with F_A of 0.13, 0.32, 0.50, and 0.64 were degraded to maximum α-values of 0.04, 0.12, 0.23, and 0.33, respectively. Peaks are labeled with DP-values or with the oligomer sequence.

ChiG show that this family 19 chitinase has a strong preference for acetylated units in subsite −2 and +1, in addition to a clear preference for **A** in subsite −1 and a less clear preference in subsite +2, as revealed from the sequential information in the NMR-spectra (Figures 1 and 3). The subsite preferences of ChiG are summarized schematically in Figure 7, illustrating that only 2 of the 16 different chitosan tetrade sequences occupying subsites −2 to +2 are cleaved with a high rate while two additional sequences are cleaved with a lower rate.

The oligomers that are produced using the family 19 chitinase ChiC from *S. griseus* HUT 6037 upon extensive degradation of a chitosan with a degree of acetylation of 47% have been isolated and characterized.[48] From the structure of isolated oligomers, this chitinase is found to be absolute specific for **A** in −2 and +1 subsite, in agreement with our results from *S. coelicolor*. A family 19 chitinase from rice[49] has been found to operate according to a nonprocessive endo mode of action and with strong preferences for acetylated units in subsites −2 and +1 and with preference for acetylated units in the −1 subsite, showing a similar mode of action and subsite preferences as ChiG. Huet et al. (2008) reports that a family 19 chitinase from *Carica papaya* preferentially binds two GlcNAc monomers in the subsites −2 and +1,[50] in agreement with the ChiG preferences for **A** in the same subsites when degrading chitosan.

This is the first in-depth study of a family 19 chitinase degrading fully water-soluble chitosan substrates of varying chemical composition and degrees of scission, which provide insight into the properties of a bacterial family 19 chitinase, and how these enzymes can be used to convert chitosans to several types of chito-oligosaccharide mixtures. Our study allows a detailed comparison of chitosan degradation of a family 19 chitinase with similar studies of family 18 chitinases from *S. marcescens*.[23,25,51] The chain length distribution of oligomers produced after hydrolysis of a highly acetylated chitosan with ChiG at widely different α-values (Figure 4) shows a continuum of oligomers with no preference for even-numbered oligomers, indicating that ChiG operates according to an endo mode of

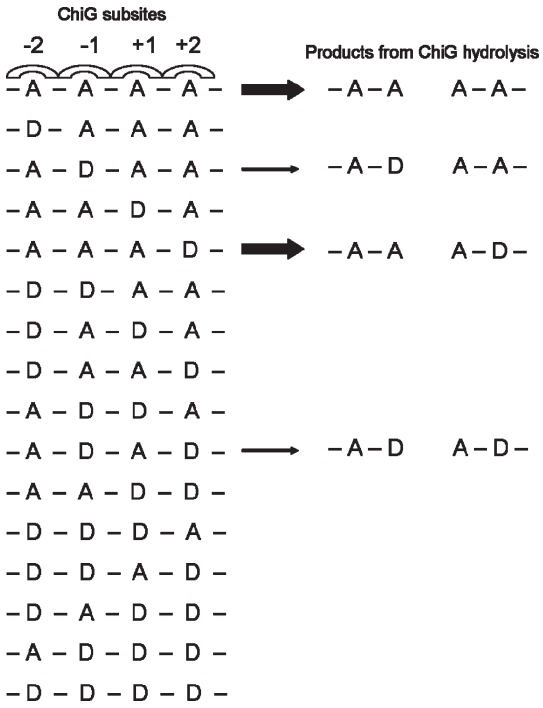

Figure 7. Schematic illustration of the subsite specificities of ChiG toward the 16 possible tetrade sequences in the chitosan substrate. Cleavage occur between sugars bound to −1 and +1.[59] Thick arrow indicates a faster degradation rate as compared to a thin arrow.

action, with no indication of processivity as observed previously for ChiA and ChiB from *S. marcescens*.[23,25] This mode of action is consistent with the open active site of ChiG that was revealed from the crystal structure.[44] The results for ChiG, however,

resemble the results for the degradation of chitosan with ChiC from *S. marcescens*, another nonprocessive chitinase.[25] While the three family 18 chitinases ChiA, ChiB, and ChiC from *S. marcescens* show a preference for an **A** in −2 subsite, an absolute requirement of an **A** bound to subsite −1 and no preference for **D** or **A** in subsite +1,[25] ChiG shows quite different binding preferences. This is reflected in the very different composition of the oligomers produced by ChiG, showing preferences for **A**, **A/D**, and **A** in subsite −2, −1, and +1, respectively.

The experiments with chitosans with lower degrees of acetylation reveal additional differences between ChiB and ChiG. While ChiB is quite capable of degrading such chitosans, yielding degrees of scissions of 0.34, 0.22, and 0.11 for F_A values of 0.50, 0.32, and 0.13, respectively,[23] the efficiency of ChiG decreases dramatically at lower F_A (Figure 6). Thus, ChiG is more dependent on the presence of acetylated sugars than ChiB. Interestingly, enzyme−substrate interactions in ChiB[52] involve several aromatic residues that stack with the hydrophobic faces of the sugars. Such contacts, which do not necessarily depend on the presence of the *N*-acetyl group are absent in the interaction between family 19 chitinases and their substrates. In the catalytic clefts of family 19 enzymes, substrate binding is dominated by specific hydrogen bonds that often involve the *N*-acetyl groups.[50] This provides an explanation for the low efficiency of ChiG toward more highly deacetylated chitosans.

While ChiG is not particularly effective against chitosan with low F_A, it degrades $F_A = 0.64$ chitosan about 2.4 times faster than ChiB with specific activities of ChiG and ChiB of 2280 and 970 min^{-1}, respectively. This is remarkable because ChiB is much more efficient in degrading β-chitin than ChiG.[53] Related family 19 enzymes such as the *Streptomyces* enzymes ChiC and ChiF[42,54] also have low activity against crystalline chitin. This difference between the family 19 enzymes and ChiB further strengthens the idea that these enzymes have different roles in chitin conversion. It has previously been shown that the presence of aromatic residues in the substrate-binding cleft of ChiB increases efficiency toward chitin while reducing efficiency toward chitosan.[51]

Remarkably, extensively degraded chitosan was almost devoid of the tetramers (Figures 4 and 6). This can be explained by the subsite specificities of the enzyme. For an endoenzyme such as ChiG, tetramers are the result of two independent degradation events. The reducing end sugar and its nearest unit must have been productively bound to the subsite −1 and −2, respectively, meaning that the reducing end may be an **A** or **D**, whereas the next sugar must be an **A**. The nonreducing end and its nearest neighbor must have been productively bound to subsite +1 and +2, meaning that the nonreducing end sugar must be an **A**, whereas the sugar next to it can be **A** or **D**. Thus, all tetramers formed must have the sequence AXAX (X being **A** or **D**), and these tetramers can be degraded further by ChiG (albeit probably with varying efficiencies). In contrast, the trimers accumulated at the end of the degradation reaction (AAD, Figure 4) can not bind productively. The accumulated pentamers, with their most probable sequences ADDAA and ADDAD, can not be degraded either because they can not bind with an **A**, simultaneously occupying the −2 and +1 subsites (see Figure 7).

The present study provides in-depth insight into how bacterial family 19 chitinases degrade chitosan and how they can be used to convert various chitosans into mixtures of chito-oligosaccharides. Whereas our results suggest that plant and bacterial family 19 chitinases have rather similar substrate-specificities, the differences between family 18 and family 19 enzymes are remarkable. These differences are not only relevant from an applied point of view, but may also have biological implications. Whereas family 18 chitinases, at least the processive variants among them, can degrade heavily deacetylated chitin quite well, family 19 chitinases need higher degrees of acetylation to be effective. Differences in domain structure within and in between the two chitinase families, as well as differences in enzyme mode of action (endo versus exo, processive or not[25,51]) will effect enzyme efficiency in a substrate-dependent manner, conferring different functional roles to the many different chitinases that may be found within one organism.[54−56] It is conceivable that variation in the dependency of enzyme efficiency on the degree of acetylation of the substrate provides the basis for additional functional differentiation of chitinases.

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Paper V

Degradation of Chitosans with a Family 46 Chitosanase from *Streptomyces coelicolor* A3(2)

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We have studied the degradation of well-characterized soluble heteropolymeric chitosans by a novel family 46 chitosanase, ScCsn46A from *Streptomyces coelicolor* A3(2), to obtain insight into the enzyme's mode of action and to determine its potential for production of different chitooligosaccharides. The degradation of both a fully deacetylated chitosan and a 32% acetylated chitosan showed a continuum of oligomeric products and a rapid disappearance of the polymeric fraction, which is diagnostic for a nonprocessive endomode of action. The kinetics of the degradation of the 32% acetylated chitosan demonstrated an initial rapid phase and a slower second phase, in addition to a third and even slower kinetic phase. The first phase reflects the cleavage of the glycosidic linkage between two deacetylated units (D−D), the primary products being fully deacetylated dimers, trimers, and tetramers, as well as longer oligomers with increasing degrees of acetylation. In the subsequent slower kinetic phases, oligomers with a higher degree of acetylated units (**A**) appear, including oligomers with **A**'s at the reducing or nonreducing end, which indicate that there are no absolute preferences for **D** in subsites −1 and +1. After maximum degradation of the chitosan, the dimers DA and DD were the dominant products. The degradation of chitosans with varying degrees of acetylation to a maximum degree of scission showed that ScCsn46A could degrade all chitosan substrates extensively, although to decreasing degrees of scission with increasing F_A. The potential use of ScCsn46A to prepare fully deacetylated oligomers and more highly acetylated oligomers from chitosan substrates with varying degrees of acetylation is discussed.

Introduction

Chitin is an essential structural component in the exoskeleton of crustaceans and insects, and is also found in the cell walls of certain fungi and in algae.[1] This insoluble polymer is composed of (1−4)-linked units of 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; **A**-unit). Chitosans are a family of water-soluble linear binary heteropolysaccharides composed of (1→4)-linked **A**-units and 2-amino-2-deoxy-β-D-glucopyranose (GlcN, **D**-unit), which can be prepared from chitin with varying extents of deacetylation. Variation in the chemical composition and chain length of chitosans have been shown to affect their properties and functionalities.[1] From chitosans with a defined chemical composition, well-defined mixtures of chitooligosaccharides can be prepared, and we have recently reviewed some of their most promising applications.[2] Chitosans prepared by homogeneous de-*N*-acetylation of chitin, such as those used in the present study, have a random distribution of **A**- and **D**-units.[3−5]

Chitinases and chitosanases are glycoside hydrolases that are capable of converting chitin and chitosans to low molecular weight products (chitooligosaccharides, CHOS) by hydrolyzing the glycosidic linkages between the sugar units.[2] Chitosanases (EC 3.2.1.132) are enzymes that can hydrolyze glycosidic linkages in chitosans and can be divided into subclasses, depending on their specificity toward **A**- or **D**-units bound to subsites −1 and +1.[6] Chitosanases are produced by various organisms such as fungi and bacteria and occur in six different families of the glycoside hydrolases (GHs), that is, families 5, 7, 8, 46, 75, and 80.[7] Families 5, 7, and 8 primarily contain nonchitosanases but include a few members for which chitosan-hydrolyzing activity has been detected. The other three families, GH46, GH75, and GH80, exclusively contain chitosanases. Chitinases occur in families GH18 and GH19. The genome of the Gram-positive bacterium *Streptomyces coelicolor* A3(2)[8] contains 13 chitinase genes (11 GH18 and 2 GH19[9]), two genes putatively encoding GH46 chitosanases and one gene putatively encoding a chitosanase belonging to family GH75.

Generally, glycoside hydrolases can either degrade polymeric substrates from the chain end (exo attack) or from a random point along the polymer chain (endo attack), and each of these mechanisms can occur in combination with a processive mode of action. The enzymes employ either a retaining "double-displacement" mechanism or an inverting "single displacement" mechanism.[10−12] There are several types of remote structural similarities between GH families. For example, family 46 chitosanases, family 19 chitinases, and lysozymes belonging to GH families 22−24 share a structural core consisting of two α-helixes and a three-stranded β-sheet and are said to form "the lysozyme superfamily".[13,14]

We have previously performed in-depth studies of several chitinases, including the GH18 enzymes ChiA, ChiB, and ChiC from *Serratia marcescens*[15−20] and a family 19 chitinase, ChiG, from *Streptomyces coelicolor* A3(2),[21] using chitosans as substrates. These studies have provided protocols for the production of specific chitooligosaccharides as well

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Table 1. Characterization of Chitosans[a]

chitosan (Fa)	[η] (mL/g)	M_W (g/mol)
0.008	590	185000
0.32	730	233000
0.46	746	238000
0.63	865	280000

[a] Fraction of acetylated units (F_A), intrinsic viscosities ([η]), and molecular weight (M_W) of the chitosans. The molecular weight were calculated from the intrinsic viscosity vs molecular weight relationship.[38]

as in-depth insight into enzyme function. First, a compositional analysis of the products yields information on subsite specificities (for acetylated, **A**, or deacetylated, **D**, sugars) in the active site of the enzyme. Second, insight into processivity may be obtained.[15,17,20] Such in-depth studies have only been performed to a limited extent for chitosanases. The chitosan oligomer products formed upon degradation of a chitosan with chitosanase GH46 from *Streptomyces* sp. (CsnN174) have been studied and the structure of this enzyme is known, and site-directed mutagenesis and biochemical studies have revealed the roles of specific residues in substrate-binding and catalysis.[22−25]

In the present study we have expressed one of the two putative GH46 chitosanases from *Streptomyces coelicolor* A3(2) annotated as Q9RJ88. According to current CAZY nomenclature, the resulting enzyme has been named ScCsn46A. We have used the purified enzyme to carry out a detailed study of the degradation of fully water-soluble and well-characterized chitosans, providing insight into subsite binding preferences and modes of action, as well as in the suitability of the enzyme for the production of oligosaccharides. The results show that ScCsn46A operates according to a nonprocessive endomode of action with an initial preference for cleavage of the linkage between two deacetylated units. Quite remarkably, however, extended incubations revealed that also the linkage between two acetylated units may be hydrolyzed.

Experimental Section

Chitosans. Chitin was isolated from shrimp shells as previously described by Hackman[26] and milled in a hammer mill to pass through a 1.0 mm sieve. Chitosans with a fraction of *N*-acetylated units (F_A) of 0.008, 0.32, 0.46, and 0.63 were prepared by homogeneous de-*N*-acetylation of chitin.[27] The characterization of the chitosans is given in Table 1.

ScCsn46A. *DNA Techniques.* The *q9rj88* gene was amplified from genomic DNA (ATCC BAA-471D) from *Streptomyces coelicolor* A3(2) with primer *q9rj88*-F: 5′GCAT**CGTCTC**ACATGGCGTCCGCGC3′(*Bsm*BI restriction site is in bold face) and primer *q9rj88*-R: 5′GCAT**AAGCTT**TCAGCCGATGTGGTAGGC3′ (*Hind*III restriction site is in bold face). PCR reactions were conducted with *Phusion* DNA polymerase (Finnzymes, Espoo, Finland) in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). The amplification protocol consisted of an initial denaturation cycle of 30 s at 98 °C, followed by 35 cycles of 10 s at 98 °C, 10 s at 67 °C and 30 s at 72 °C, followed by a final step of 10 min at 72 °C. The 749 bp amplified fragment was ligated into vector pCR4Blunt-TOPOZero Blunt TOPO (Invitrogen, Carlsbad, CA, USA). The gene fragment was excised from the TOPO vector using *Bsm*BI and *Hind*III for cloning into *NcoI*-*Hind*III digested pETM11 vector (Günter Stier, EMBL Heidelberg, Germany). The pETM11 vector is constructed so that the cloned gene is preceded by a sequence encoding an N-terminal His_6-tag followed by a TEV-protease cleavage site. The final constructs were transformed into *Escherichia coli* BL21Star (DE) (Invitrogen). DNA sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer/Applied Biosystems).

Production and Purification of Recombinant Protein. A total of 300 mL of *E. coli* BL21Star (DE3) transformants containing the pETM11-*q9rj88* construct were grown at 37 °C in LB-medium, with 100 μg mL^{-1} kanamycin at 225 rpm, to a cell density of 0.6 at 600 nm. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM, and the cells were further incubated for 4 h at 37 °C, followed by harvesting by centrifugation (9829 × g, 10 min at 4 °C).

The cell pellet was resuspended in 16 mL of 20 mM Tris-HCl, pH 8.0. To weaken the cell walls, 0.1 mg/mL lysozyme and 1 μL/mL DNaseI were added, and the mixture was incubated at room temperature for 20 min with occasional stirring. The cells were split into two 15 mL tubes and lysed by sonication at 25% amplitude for 60 × 5 s pulses (with a 5 s delay between pulses) on ice, with a Vibra cell Ultrasonic Processor, converter model CV33, equipped with a 3 mm probe (Sonics, Newtown, CT). After sonication, the cell-lysate was centrifuged (7741 × g, 10 min at 4 °C), and the supernatant was pressed through a 0.20 μm sterile filter, supplied with 20 μL of 50 mM PMSF per 10 mL extract, and stored at 4 °C.

ScCsn46A was purified on a Ni-NTA column (Qiagen, Venlo, The Netherlands) using a flow rate of 2 mL/min. The column was equilibrated in 100 mM Tris-HCl buffer, pH 8.0, containing 20 mM imidazole. After loading the protein sample, the column was washed with the starting buffer. The His-tagged protein was then eluted with 100 mM Tris-HCl buffer, pH 8.0, containing 100 mM imidazole. The purified protein was dialyzed against 20 mM Tris-HCl, pH 8.0, and stored at 4 °C.

Removal of the $(His)_6$-tag was performed by mixing 0.1 mg $(His)_6$-ScCsn46A with 75 μL 10× TEV protease buffer (0.5 M Tris-HCl, pH 8.0 and 5 mM EDTA), 1 mM DTT, 0.005 mg TEV protease, and dH_2O up to 750 μL. This mixture was incubated at 37 °C for 3 h. After incubation, the mixture was dialyzed against 100 mM Tris-HCl, pH 8.0, and 20 mM imidazole overnight. The dialyzed mixture was then applied onto a Ni-NTA column, as described above. The flow-through fraction, now containing the ScCsn46A-protein with no $(His)_6$-tag, was dialyzed against 20 mM Tris-HCl, pH 8.0, upconcentrated and stored at 4 °C. Protein concentration measurements were done according to Bradford[28] using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA), with bovine serum albumin (BSA) as a standard. The protein produced via this procedure contains a three-residue N-terminal extension (Gly-Ala-Met) compared to the mature wild-type enzyme.

Enzymatic Degradation of the Chitosans. Chitosan (as chitosan hydrochloride) was dissolved in water to 20 mg/mL and incubated with shaking at room temperature overnight. An equivalent volume of 0.08 M sodium acetate buffer, pH 5.5 (containing 0.2 M NaCl), and 0.2 mg BSA was added. Chitosan solutions (final concentration of 10 mg/mL) were incubated in a shaking water bath at 37 °C, and the degradation reactions were started by adding 0.5 μg ScCsn46A per mg chitosan (for degradation reactions of chitosan F_A 0.008, only 0.05 μg ScCsn46A per mg chitosan was added). Samples were taken from the reactions at regular time intervals between 15 and 20160 min after the initiation of the reaction. The samples were immediately immersed in boiling water for 5 min, followed by centrifugation to remove potential precipitates. The samples were stored at −20 °C until further analysis. To produce negative controls, chitosans with F_A 0.008 or 0.32 were solubilized and incubated for 72 h without adding ScCsn46A. All chromatograms for these negative controls showed that the chitosans elute in the void volume during size-exclusion chromatography (SEC; see below) and that there are no detectable amounts of oligomers present (see below).

Reactions aimed at obtaining the maximum degradation of chitosans with various degrees of *N*-acetylation (F_A = 0.008, 0.32, 0.43, and 0.63) were performed using 2.0 μg ScCsn46A per mg chitosan. After 1 week (10080 min), more enzyme was added (6.0 μg per mg chitosan F_A = 0.008 or 2.0 μg for chitosans F_A = 0.32, 0.43, and 0.63), and the degradation reaction was incubated for one more week.

For all chitosans, control experiments were performed to check whether ScCsn46A was inhibited by the oligomer products produced

during the degradation process. Degradation reactions (2.0 μg ScCsn46A per mg chitosan) were allowed to proceed for 1 week, and the quantity and size distribution of the hydrolysis products were determined by SEC (see below). More substrate was then added (doubling the total amount of added substrate), and the reaction was incubated for one additional week. Subsequent SEC analyses revealed that the size distributions were the same as those obtained after 1 week, whereas the amounts per product almost doubled, demonstrating that product inhibition was not important. These observations also show that the enzyme was stable during the incubation. This was further tested by analyzing the effect of adding additional enzymes to reactions that were considered to have been run to completion. Adding additional enzymes had no effect on the product profile.

Size Exclusion Chromatography (SEC). Chitosan and CHOS were separated on three XK 26 columns connected in series and packed with Superdex 30 (Pharmacia Biotech, Uppsala, Sweden), with an overall dimension of 2.60 × 180 cm. The elution buffer used was 0.15 M ammonium acetate, pH 4.5. The elution buffer was pumped through the system using an LC-10ADvp pump (Shimadzu GmbH, Duisburg, Germany), delivering the elution buffer at a flow rate of 0.8 mL/min. The relative amounts of oligomers were monitored with a refractive index (RI) detector (Shodex RI-101, Shodex Denko GmbH, Dusseldorf, Germany) coupled to a CR 510 Basic Data logger (Campbell Scientific Inc., Logan, UT). For the characterization of isolated chitooligosaccharides, fractions of 3.2 mL were collected using a fraction collector. For quantitative studies of degradation, typically 10 mg degraded chitosan was injected. When oligomers were collected for NMR and MS characterization, samples of 50−200 mg degraded chitosan were injected.

NMR Spectroscopy. Samples for ^{1}H and ^{13}C NMR were dissolved in D_2O and 0.1 M DCl/NaOD was used to adjust the pD to 4.2 and 4.6, respectively. The deuterium resonance was used as a field-frequency lock, and the chemical shifts were referenced to internal sodium 3-(trimethylsilyl-)propionate-d_4 (0.00 ppm). ^{1}H NMR spectra was obtained at 300 or 400 MHz at 90 °C, and ^{13}C NMR spectra were obtained overnight at 400 MHz at a temperature of 90 °C, as previously described.[4,5,29]

To determine the sequence of shorter oligomers and to calculate the number average degree of polymerization, DP_n, of the enzyme-degraded chitosans, ^{1}H NMR spectroscopy was used, as described earlier in Sørbotten et al., 2005.[20] The degree of scission, α, which gives the fraction of cleaved glycosidic linkages, was calculated as the inverse value of DP_n.

MS Experiments of Chitooligosaccharides. Underivatized heterochitooligosaccharides fragment almost exclusively by cleavage of the glycosidic bond, generating Y- and Z-type ions if the charge is retained on the reducing end or B- and C-type ions if the charge is retained on the nonreducing end, respectively. Due to overlapping *m*/*z* values of ions of identical monosaccharide composition, distinguishing between fragmentations of chitooligosaccharides from the reducing or nonreducing end cannot be done. For the sake of sequencing, the introduction of tag at the reducing end of the molecule is essential.[30] In general, the fragmentation of chitooligosaccharides yields predominantly B-type ions from the nonreducing end and Y-type ions from the reducing end. Because the B-type ion fragmentation can occur at random within the sugar chain, they are of little diagnostic value.[31]

Matrix-Assisted Laser Desorption Ionization with Orthogonal Time-of-Flight Mass Spectrometry (MALDI TOF/TOF MS). A reductive amination of chitooligosaccharides with 2-aminoacridone (AMAC) was performed as previously described.[30,32] MS^1 and MS^2 spectra were acquired using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) under the control of Flexcontrol 3.0. For sample preparation, 1 μL of purified and isolated AMAC−CHOS and 2 μL of DHB (2.5- dihydroxybenzoic acid) matrix solution (15 mg/mL in 30% aqueous ethanol) were mixed. An aliquot of 1 μL of the mixed solution was spotted onto the target plate and the spotted samples were air-dried at room temperature.[32] The MS experiments were conducted in the reflector mode using external calibration and an accelerating potential of 25.1 kV. The MS^1 and MS^2 signals were recorded over a range of *m*/*z* 100−4000 with a resolution set to 2.00 Gs/s. In the TOF/TOF-MS^2 analysis, precursor ions were accelerated at 13 kV, and fragment ions generated from these precursors were subsequently accelerated to 26 kV. Due to the AMAC derivatization, oligosaccharides carrying the reducing end will show a mass increment of 194 Da, allowing for identification of Y-type ions and permitting a straightforward sequence determination of chitooligosaccharides.[30,32]

Enzymatic Degradation of the Fully Acetylated Hexamer $(GlcNAc)_6$. The degradation of $(GlcNAc)_6$ (concentration: 300 μM) was carried out in a 50 mM sodium acetate buffer, pH 5.5, with 0.1 mg BSA added. The degradation reaction was started by adding 60 μg ScCsn46A per mg $(GlcNAc)_6$, and the reaction mixture was incubated at 37 °C for several hours. Samples were taken from the reaction at regular time intervals between 0 and 96 h after the initiation of the reaction. To stop the reaction, aliquotes of the reaction mixture were transferred to new tubes, and the pH was adjusted to 2.5 using HCl and boiled for 2 min. The samples were stored at −20 °C until further analysis by HPLC. Prior to HPLC analysis, all samples were diluted with 1 volume of acetonitrile. All reactions were analyzed in triplicate.

An HPLC analysis of 20 μL portions of the stored degradation reaction mixtures was performed at room temperature on a Gilson HPLC system (Gilson, Inc., Middleton, WI) equipped with a Tosoh TSK-Gel amide-80 column (0.46 × 25 cm; Tosoh Bioscience, Montgomeryville, PA) and a 234 autoinjector (Gilson). The elution buffer consisted of 70% acetonitrile, and the elution buffer was delivered at a flow rate of 0.7 mL/min. Oligosaccharides were detected by recording absorption at 210 nm.

Results

Degradation of a Chitosan, $F_A = 0.008$. Progress in chitosan degradation was determined as the degree of scission (α), that is, the fraction of glycosidic linkages in the chitosan cleaved by the enzyme, by monitoring the increase in reducing end resonances relative to resonances from the internal anomer protons using ^{1}H NMR spectroscopy. A fully deacetylated chitosan ($F_A = 0.008$) was degraded with ScCsn46A to different degrees of scission. The time course of the reaction (Figure 1) demonstrates that the reaction rate gradually decreases as the substrate becomes degraded. Figure 2 shows the size distribution of oligomers after degradation of the chitosan to α-values of 0.06, 0.20, and 0.59. The void peak (elution time from 6−7.5 h; DP (degree of polymerization) > 50) gradually disappears as the reaction proceeds and is completely absent in the chromatogram of the $\alpha = 0.20$ sample. As the void peak disappears, a continuum of shorter oligomers appears with no preference for even-numbered oligomers, thereby indicating that ScCsn46A operates according to an endomode of action with no processivity.[17] Identification of the peaks was performed by comparing the chromatograms with chromatograms of standard samples of fully de-*N*-acetylated oligomers, as well as from ^{1}H NMR spectra of isolated oligomers (spectra not shown). At maximum degree of scission ($\alpha = 0.59$), obtained after prolonged incubation and repetitive addition of the enzyme, the most dominant product is the dimer DD, with considerable amounts of monomer present. The peaks eluting before the dimer DD (elution time from 14.5−15.8 h) are artifacts due to Schiff bases.

Degradation of a Chitosan, $F_A = 0.32$. *Subsite Specificity in Subsite −1 and −2.* The proton NMR spectrum yields information about the identity of the new reducing ends (**A** or **D**), which reveals information about the productive binding preference in the −1 subsite of the enzyme. In addition, information about the neighboring unit next to the new reducing

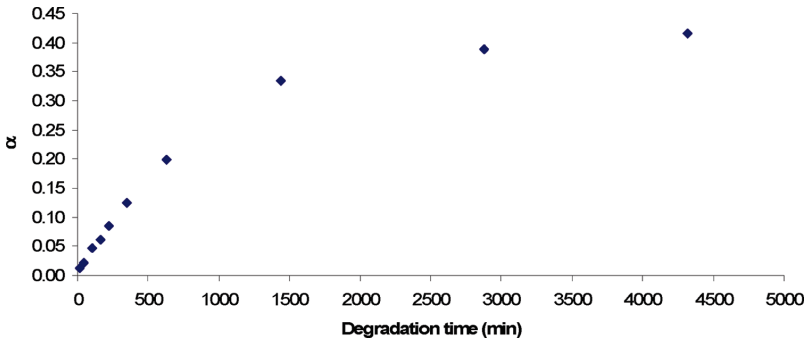

Figure 1. Time course for chitosan ($F_A = 0.008$) degradation by ScCsn46A. The graph shows the degree of scission, α (from ^{1}H NMR) of a $F_A = 0.008$ chitosan, as a function of the time of degradation. The maximum α-value obtained after extended hydrolysis with a large amount of enzyme (see Experimental Section) was 0.59. This α-value is not shown in the graph because an increased amount of enzyme was added and the α-value cannot be compared directly with the other α-values.

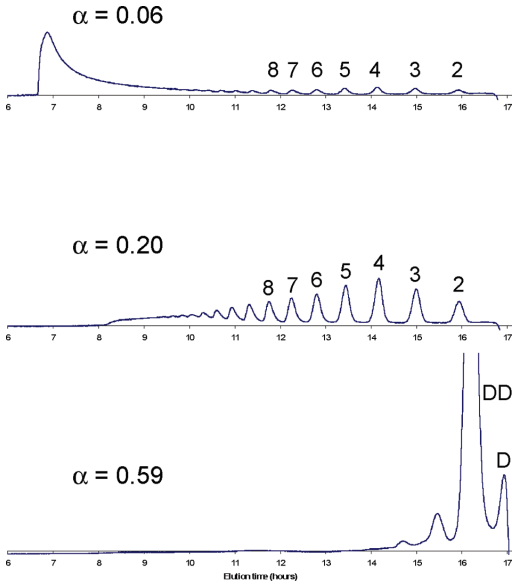

Figure 2. SEC chromatograms showing the size distribution of oligomers obtained after ScCsn46A degradation of a highly deacetylated chitosan ($F_A = 0.008$) to different degrees of scission, α. Peaks are labeled with the DP or the sequence of the oligomer.

end can be obtained (the sugar unit which binds in subsite −2) because the resonance of the acetylated reducing end proton is influenced by the neighboring sugar.[20]

Figure 3 shows the anomer region of ^{1}H NMR spectra of chitosan ($F_A = 0.32$) degraded to α-values between 0.06 and 0.44. At α-values below 0.10, the new reducing ends are almost exclusively deacetylated (signals at 5.43 ppm for -**D**(α) and 4.92 ppm for -**D**(*β*)), while new acetylated reducing ends (signals at 5.19 ppm for -**A**(α) and 4.742 ppm for -DA(*β*)) only appear on more extensive degradation (α-values above 0.1). Thus, ScCsn46A has a preference for **D**-units in the −1 subsite, although the enzyme can also hydrolyze the glycosidic linkage following an **A**-unit. The neighboring sugar next to the acetylated reducing end, that is, the sugar unit bound productively in the −2 subsite, is exclusively deacetylated (signal at 4.742 ppm; -DA(*β*)), as no resonance corresponding to an acetylated reducing end with an acetylated neighboring unit can be identified (i.e., -AA(*β*); expected at 4.705 ppm),[20] even on prolonged degradation of the chitosan (α = 0.44). When a deacetylated unit is productively bound in subsite −1, it is not possible from the proton NMR spectrum to discriminate between the identities of the neighboring sugar next to the reducing end **D**.[21]

Time Course of the Reaction. The time course of the degradation of chitosan ($F_A = 0.32$) reveals multiphasic kinetics. After an initial rapid phase lasting until the degree of scission reaches approximately 0.10, a slower second kinetic phase dominates until the degree of scission reaches α = 0.30. Subsequently, the reaction continues at an even slower pace and is followed by a third, even slower, kinetic phase continuing until α = 0.34. Prolonged incubation with more enzyme added resulted in the maximum obtainable α-value of 0.44. The initial fast product formation rate correlates with the exclusive formation of new deacetylated reducing ends (see Figure 3 and above). Compared to the time course of degradation of the fully deacetylated substrate (Figure 1), the time course shown in Figure 4 is more discontinuous, with distinct phases that reflect different rates of degradation for different sequences in the chitosan and intermediate products.

Subsite Specificity in +1 Subsite of ScCsn46A. To analyze the productive binding specificity of subsite +1, oligomer mixtures obtained after degradation of chitosan ($F_A = 0.32$) to α-values of 0.10 and 0.29 were analyzed using ^{13}C NMR, and Figure 5 shows the resonances of carbons 3 and 5 (from 73.5−79.5 ppm). The ^{13}C NMR spectrum of the partially degraded chitosan (α = 0.10, Figure 5A) shows almost exclusively **D**-units at the nonreducing end, revealing a clear preference for **D**-units in the +1 subsite. The spectrum of the more extensively degraded chitosan (α = 0.29, Figure 5B) reveals a small but significant fraction of acetylated nonreducing ends (signal at 76.1 ppm), demonstrating that ScCsn46A has no absolute specificity toward **D**-units in the +1 subsite.

Size Distribution of Oligomers Produced after Degradation of Chitosan ($F_A = 0.32$). The size distributions of oligomers resulting from degradation of an $F_A = 0.32$ chitosan to α-values between 0.06 and 0.44 are shown in Figure 6. The void peak (elution time from 6−7.5 h; DP > 50) gradually disappears as the reaction proceeds, and is completely absent in the chromatogram of the α = 0.29 sample. As the void peak disappears, a continuum of shorter oligomers appears with no preference for even-numbered oligomers, indicating (as with the fully deacetylated substrate) that ScCsn46A operates according to an endomode of action with no processivity. Initially (up to α = 0.21), the dimer fraction only contains the fully deacetylated

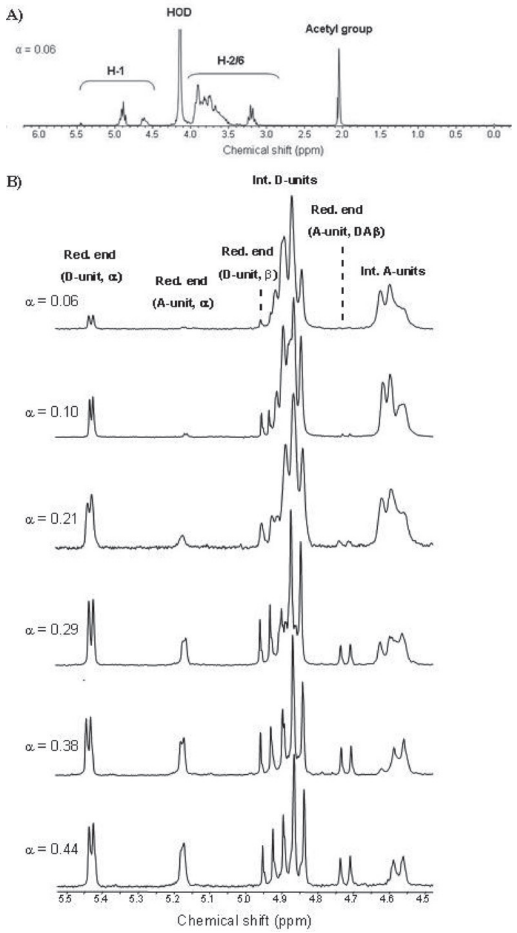

Figure 3. ^{1}H NMR spectra of chitosan (F_A= 0.32) after degradation by ScCsn46A. (A) ^{1}H NMR spectrum of chitosan (F_A= 0.32) degraded with ScCsn46A to $\alpha = 0.06$. The spectra were acquired at 300 MHz at 90 °C and pD 4.2. The resonances were assigned according to previously published ^{1}H NMR spectra of degraded chitosan.[5] The resonances of H-1 of both **D**- and **A**-units (anomer region) occur from 5.5 to 4.5 (see further information below). The H-2 of a **D**-unit resonates at 3.2 ppm, while the resonance of H-2 of an **A**-unit and H-3−6 of both **A**- and **D**-units occurs between 3.5−4.0 ppm. The resonance at 2.05 ppm is from protons of the acetyl group. (B) ^{1}H NMR spectra showing the anomer region of chitosan ($F_A = 0.32$) after hydrolysis with ScCsn46A to α-values between 0.06 and 0.44. The spectra were acquired at 300 or 400 MHz at 90 °C and pD 4.2. The resonances were assigned according to previously published ^{1}H NMR spectra of degraded chitosan.[5,20,21,37] The α-anomer of a **D** resonates at 5.43 ppm and the β-anomer at 4.92 ppm. The α-anomer of the **A**-unit resonates at 5.19 ppm, whereas the β-anomer resonates at 4.705 ppm if the neighboring residue is an **A** (not visible in the spectrum) or at 4.742 ppm if the neighboring residue is a **D** (visible in the spectrum). The internal **D**-units resonate at 4.8−4.95 ppm, while the internal **A**-units resonate at 4.55−4.68 ppm. The β-resonance for a terminal **D** is only partially resolved from the resonance for the internal **D**-units.[5,39]

dimer, DD, while the chromatograms for $\alpha = 0.29$ and higher show that the dimer fraction becomes separated into two peaks, identified as DA and DD. The dimers, AD and AA, were not detected. As the chitosan is further degraded, an increasing amount of oligomers with lower DP-values appears, and dimers

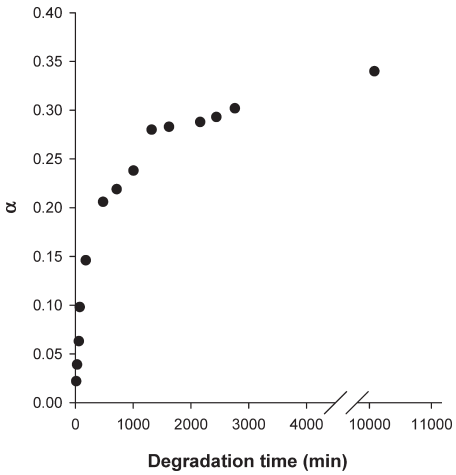

Figure 4. Time course for degradation of an $F_A = 0.32$ chitosan with ScCsn46A. The graph shows the degree of scission, α (as determined from ^{1}H NMR spectra), as a function of the time of degradation. The maximum α-value obtained after extended hydrolysis with a large amount of enzyme (see Experimental Section) was 0.44 (this α-value is not comparable to the other α-values due to the increased amount of added enzyme and are therefore not shown in the graph).

appear as the dominant products. The chromatogram of the $\alpha = 0.38$ sample reveals a small peak eluting after 17 h, which increases upon prolonged degradation to $\alpha = 0.44$. This peak was identified as the deacetylated monomer (D).

Chemical Composition of Oligomeric Products. Individual oligomer fractions obtained after degradation of the chitosan with $F_A = 0.32$ to different α-values ($\alpha = 0.10$, 0.29 and 0.44, see Figure 6) were isolated and further characterized using ^{1}H NMR spectroscopy and mass spectroscopy (MS). The NMR method has limitations, as mixtures of complex oligomers with higher DP values cannot be fully sequenced. Therefore, mass spectroscopy was employed for sequencing the isolated oligosaccharides. In the following, the results from the characterization of the isolated trimers and tetramers are presented.

Figure 7A,B shows the MS^1 spectra of chitosan oligomers (trimer and tetramer) obtained from the $\alpha = 0.10$ and 0.44 samples. The mass spectra show that fully deacetylated oligomers dominate during the initial phase of the degradation ($\alpha = 0.10$), whereas acetylated units appear upon further degradation ($\alpha = 0.44$). This is in agreement with the F_A values determined from NMR (Figure 7C): at $\alpha = 0.10$, the trimer and tetramer fractions had an F_A of 0.05 and 0.089, respectively, while at $\alpha = 0.44$, the F_A of the trimer and tetramer fractions were 0.47 and 0.59, respectively. Figure 7A shows the MS spectra revealing the fully deacetylated isomer (D3) and the trimer isomer containing one acetylated unit (D2A1) in the DP3 fraction at $\alpha = 0.10$. At $\alpha = 0.44$, the fully deacetylated trimer is not present, whereas A2D1 and D2A1 appear. At $\alpha = 0.10$ (Figure 7B), the DP4 fraction contains D4, D3A1, and D2A2, while at $\alpha = 0.44$, the DP4 fraction reveals D1A3 and D2A2 without any D4 or D3A1. All in all, these observations clearly show that **A**-rich sequences are not cleaved to a large extent before **D**-rich sequences are exhausted.

The ^{1}H NMR spectrum of the isolated tetramer at $\alpha = 0.10$ ($F_A = 0.089$, DP = 3.7; Figure 7C) is dominated by resonances of **D**-units, both internally and at the reducing end. The F_A of the reducing end signals was determined to be 0.094, which is not much different from the F_A of the tetramer. This shows little

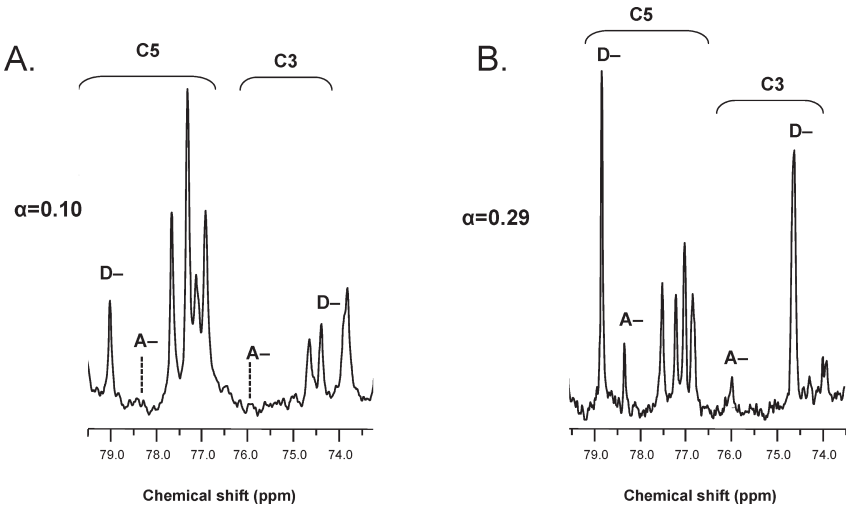

Figure 5. ^{13}C NMR spectrum of chitosan (F_A = 0.32) after degradation by ScCsn46A. Part of the ^{13}C NMR spectra (from 73.5 to 79.5 ppm) of the C5 and C3 region of partially (α = 0.10) and extensively (α = 0.29) degraded chitosan F_A = 0.32. The spectra were acquired at 400 MHz at 90 °C and pD 4.6. Acetylated nonreducing ends of C5 resonate at 78.5 ppm, while the deacetylated nonreducing ends of C5 resonate at 79.1 ppm. The internal **D**- and **A**-units of the C5 resonate between 76.5−78.0 ppm. Acetylated nonreducing ends of C3 resonate at 76.1 ppm and deacetylated nonreducing ends of C3 at 74.4 ppm.[29]

preference for **A**-units at the reducing end and from the MS spectra (Table 2) tetramers (D3A1), with **A**-units in all positions except the nonreducing end being identified. The ^{1}H NMR spectrum of the isolated tetramer at α = 0.44 in (Figure 7C) reveals F_A = 0.59 and DP = 3.9. The F_A of the reducing end signals was determined at 0.58, confirming the results of the nearly equal F_A of the reducing end and the total F_A from the trimer fraction. Using MS, the sequences of all dimers, trimers, and tetramers were determined at α = 0.10 and 0.44, with the results summarized in Table 2. An example of oligomer sequencing from MS spectra is given in Figure 8, where ions are assigned and labeled according to the nomenclature of Domon and Costello.[31] Figure 8A shows the Y-type ions fragmentation of an AADA-AMAC tetramer, and the actual MALDI-TOF/TOF-MS/MS spectrum of the D1A3-AMAC ([M + Na]$^+$ 1005.51 *m/z*) is shown in Figure 8B. The mass spectrum (Figure 8B) shows only one Y_1-type ion (*m/z* 438.03), indicating that all D1A3 tetramers have an acetylated residue (A) at the reducing end. There are two Y_2-type ion peaks appearing at *m/z* 599.15 and 641.18, respectively, corresponding to tetramers with DA and AA at the reducing end. Furthermore, there are Y_3-type ions at *m/z* 802.32 and 844.36, that correspond to tetramers having ADA/DAA and AAA at their reducing end, respectively. Finally, the mass difference between the precursor ion (*m/z* 1005.51) and the Y_3-type ion leaves an **A** or **D** residue as the fourth residue from the reducing end. Thus, as illustrated in Figure 8B, the D1A3 fraction consisted of a maximum of three isomers, one being DAAA and the other one or two being AADA and ADAA, most probably AADA, as the NMR data do not reveal the presence of **A**-units at the reducing end with acetylated neighbors. The different oligomers cannot be quantified because the peak intensities are dependent on the collision energy (extent of fragmentation), as well as on the type of monosaccharide (**D**-unit vs **A**-unit) involved in the glycosidic bond.[33]

Extensive Degradation of Chitosans with Varying F_A. Figure 9 shows the product profiles obtained upon extensive degradation of chitosans with varying F_A values (0.008, 0.32, 0.46, and 0.63) to maximum α-values of 0.59, 0.44, 0.37, and 0.32, respectively. Clearly, the average DP values of the oligomeric products increase as the F_A of the chitosan increases. The increasing presence of A is also seen in the split dimer peak, which shows an increasing fraction of DA (relative to DD) as the F_A value increases.

The chemical composition of the isolated oligomers produced after extensive degradation of the F_A = 0.63 chitosan was analyzed by ^{1}H NMR spectroscopy. As expected, the dimer fraction was found to consist of DD and DA, as shown in the chromatogram. For the other oligomer fractions, the NMR spectra became too complicated to derive sequence information, so the samples were analyzed with MS (both MS^1 and MS^2). The combined NMR and MS results are shown in Table 3. The sequence information from the MS analyses reveals that the oligomers produced are highly acetylated and that the F_A of the oligomers increases with the DP of the oligomer (this was also observed in NMR analyses; results not shown). For the oligomer fractions with DP > 3, the nonreducing end (as seen from the sequence information derived from MS) were almost exclusively acetylated, while the reducing end could be both acetylated and deacetylated.

Discussion

ScCsn46A degradation of chitosan revealed a continuum of oligomers, demonstrating that ScCsn46A degraded the substrate according to a nonprocessive endomode of action (Figures 2 and 6). The degradation of chitosans with varying F_A (F_A = 0.008, 0.32, 0.46, and 0.64) showed that ScCsn46A could degrade all chitosan substrates extensively, although to a decreasing degree of scission as F_A increases (Figure 9).

The active site of ScCsn46A has an open substrate-binding cleft with no aromatic residues, which suggests that ScCsn46A degrades the substrate in a nonprocessive endomode of action.[10,16,34] This is confirmed in the present study by the rapid disappearance of the void peak in addition to the production of a continuum of shorter oligomers (Figures 2 and 6).

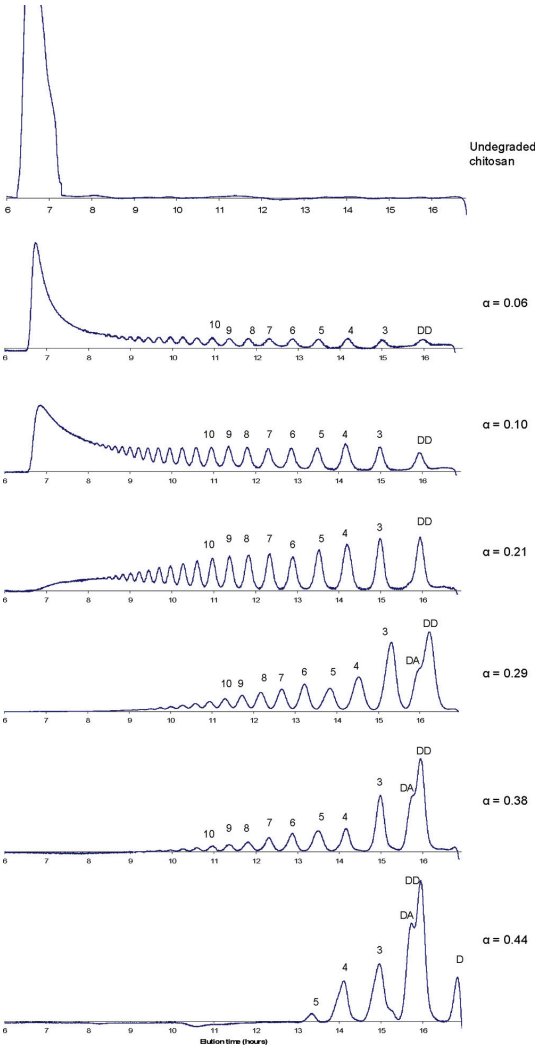

Figure 6. SEC chromatograms showing the size distribution of oligomers obtained after ScCsn46A degradation of a chitosan (F_A = 0.32) as a function of the degree of scission, α (from 0−0.44). Peaks are labeled with DP values or with the oligomer sequence.

Table 2. MALDI TOF/TOF MS^1/MS^2 Analysis of Chitooligosaccharide Fractions[a]

α = 0.10		α = 0.44	
CHOS	sequences	CHOS	sequences
D2	DD	D2	DD
D1A1		D1A1	DA
D3	DDD	D3	
D2A1	DDA	D2A1	DDA
	DAD		DAD
	ADD		ADD
D1A2		D1A2	ADA
			DAA
D2A2	AADD	D2A2	AADD
	ADAD		ADAD
	ADDA		ADDA
	DADA		
	DAAD		
D3A1	DDDA	D3A1	
	DDAD		
	DADD		
D4	DDDD	D4	
D1A3		D1A3	AADA
			ADAA
			DAAA

[a] MALDI TOF/TOF MS^1/MS^2 analysis of chitooligosaccharide fractions (dimer, trimer, tetramer) obtained after degradation of chitosan (F_A = 0.32) with ScCsn46A to α-values of 0.10 and 0.44 and subsequent gel filtration chromatography. The analysis of AMAC-derivatized oligomers was conducted using MALDI TOF/TOF MS^1 and MS^2.

Table 3. Combined NMR and MS Results after Analysis of Oligomer Fractions Obtained after Maximum Degradation of a F_A = 0.63 Chitosan[a]

	NMR		
CHOS	DP	F_A	MS sequence
DP3	3.0	0.55	ADA
			ADD
			DDA
DP4	4.1	0.66	AADA
			ADDA
			AADD
DP5	5.2	0.72	AAADD
			AADDA
			AAADA
			AADAA
DP6	5.9	0.76	AAADDA
			AAAADD
			AAAADA
			AAADAA

[a] ^1H NMR spectroscopy was used to obtain information about the DP and the F_A of the isolated CHOS after maximum degradation of chitosan (F_A = 0.63) with ScCsn46A. MS was employed for sequencing of the CHOS.

The initial specific activities (calculated as previously described[21]) for ScCsn46A against the chitosan substrate with F_A of 0.32 were found to be 325 min^{-1} for the initial degradation phase and 27 and 2 min^{-1} for the following two degradation phases (Figure 4). For degradation of the fully deacetylated chitosan, the initial specific activity was 816 min^{-1}, compared to a specific activity of ChiG and ChiB degrading a chitosan (F_A = 0.64) of 2280 and 970 min^{-1}, respectively.[21] Consequently, ScCsn46A is less effective when degrading chitosans compared to ChiG and ChiB.

ScCsn46A is well-suited for preparing fully deacetylated oligomers with good yields, and the yield of an oligomer with a given length can be optimized by controlling the degree of scission (Figure 2). This is particularly important to degrade a fully deacetylated chitosan, as this chitosan is very resistant to acid hydrolysis, in which the rate of hydrolysis of a glycosidic linkage following a deacetylated unit has been found to be at least 2 orders of magnitude lower in comparison to the rate of a glycosidic linkage following an **A**-unit.[35−37] Moreover, using ScCsn46A is clearly preferable to acid degradation for high yields of oligomers because the enzyme will yield almost no monomer in contrast to acid hydrolysis.

The initial subsite preferences of ScCsn46A are summarized schematically in Figure 10, illustrating that 4 of the 16 possible chitosan tetrade sequences binding in subsites −2 to +2 of the enzyme are cleaved. Thus, ScCsn46A initially cleaves the glycosidic linkage between two deacetylated units, that is, with a **D**-unit in subsite −1 and +1. Nevertheless, extensive degradation of the chitosan substrates showed somewhat surprisingly that the chitosanase could cleave all four glycosidic linkages (D−D, D−A, A−D, and A−A). To verify that the linkage A−A actually was cleaved, ScCsn46A degradation of the fully acetylated chitin hexamer ($(GlcNAc)_6$) was performed,

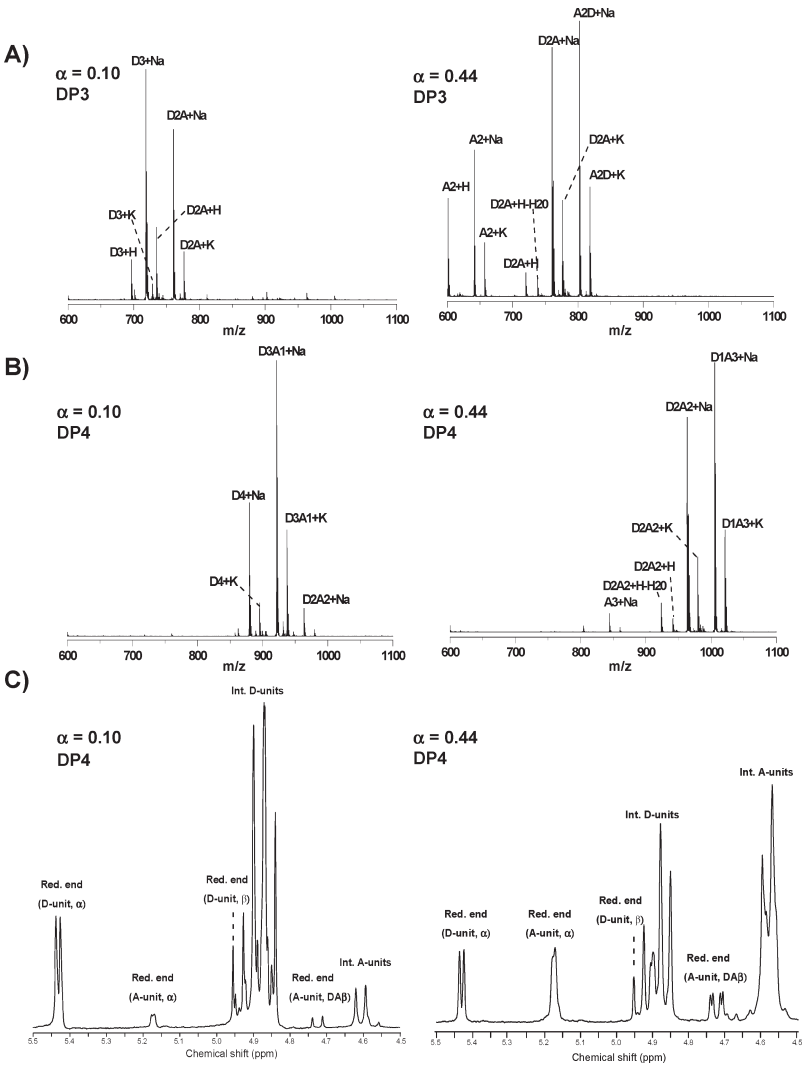

Figure 7. Analysis of chitooligosaccharide fractions obtained after size exclusion chromatography of chitosan ($F_A = 0.32$) hydrolyzed with ScCsn46A to α-values of 0.10 and 0.44. (A, B) MALDI TOF MS spectra of AMAC derivatized chitooligosaccharides in the trimer and tetramer fractions, respectively. Peaks are labeled with the composition of the oligomer (**A** is *N*-acetylglucosamine and **D** is glucosamine). Oligomer sequences were determined using the MS^2 mode, as illustrated in Figure 8; data are reported in Table 2. (C) ^1H NMR spectra (anomer region) of the tetramer fraction at both α-values.

revealing the products $(GlcNAc)_4$, $(GlcNAc)_3$, and $(GlcNAc)_2$, as shown in the HPLC chromatograms of Figure 11. This demonstrates that ScCsn46A is capable of hydrolyzing a glycosidic linkage between two **A**-units. The specific rate of the degradation of $(GlcNAc)_6$ was calculated to be 0.006 min^{-1} (results not shown). Compared with the degradation of the fully deacetylated chitosan (initial specific activity of 816 min^{-1}), the degradation of $(GlcNAc)_6$ is more than 5 orders of magnitude slower. However, this is the first time that a chitosanase has been shown to be able to cleave the A−A linkage.

It is likely that the cleavage of the D−A linkage takes place, as the tetramer ADAD is one of the initial products (Table 2). Because ScCSn46A is an endoenzyme, ADAD must be the result of two independent degradation events: X-ADAD-X (X being **A** or **D**). The cleavage that produces the new nonreducing end of the tetramer could be a cleavage of an A−A or D−A linkage, and it is conceivable that this is the D−A linkage, as the cleavage of an A−A linkage only occurs after prolonged incubation. Still, the dimers AD and AA were not detected as products, even after prolonged incubation (Table 2). The dimers must be the result of two independent degradation events, and the absence of the dimers AD and AA would imply that ScCsn46A either has an absolute specificity for D-units in subsite −2 or that the enzyme does not actually cleave the D−A linkage. As a result, we cannot conclude whether ScCsn46A can cleave the D−A linkage.

Because the ^1H NMR spectra were too complicated to derive sequence information from isolated oligomers with DP > 2, MS was employed for sequencing the chitooligosaccharides, from which more detailed qualitative information of the sequences

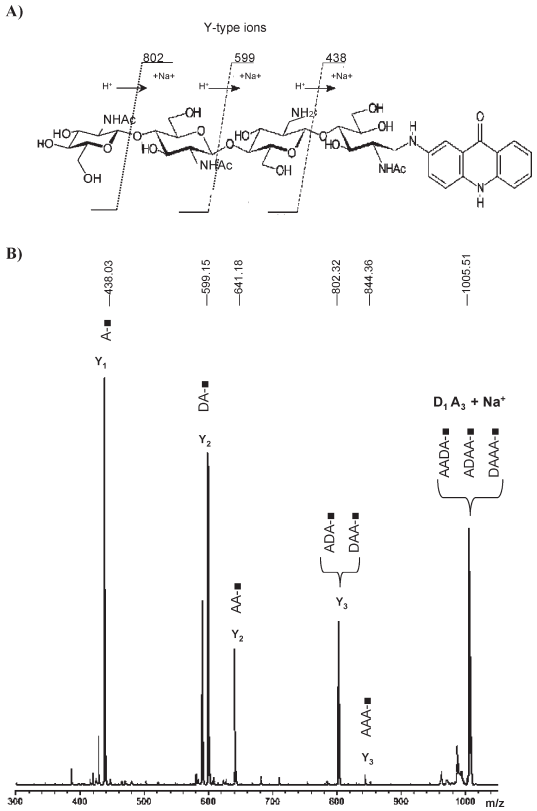

Figure 8. Structure determination of chitooligosaccharides obtained with MS. (A) MALDI-LIFT-TOF/TOF-MS^2 fragmentation scheme of an A-A-D-A-amac tetramer derivative produced after hydrolysis of chitosan ($F_A = 0.32$) with ScCsn46A to α-value of 0.44. (B) Sequence spectra of the AMAC-derivatized D_1A_3 tetramer. The ■ sign stands for AMAC, located at the reducing end of the CHOS. The Y-type ions are labeled. The *m*/*z* for the precursor ion is 1005.51.

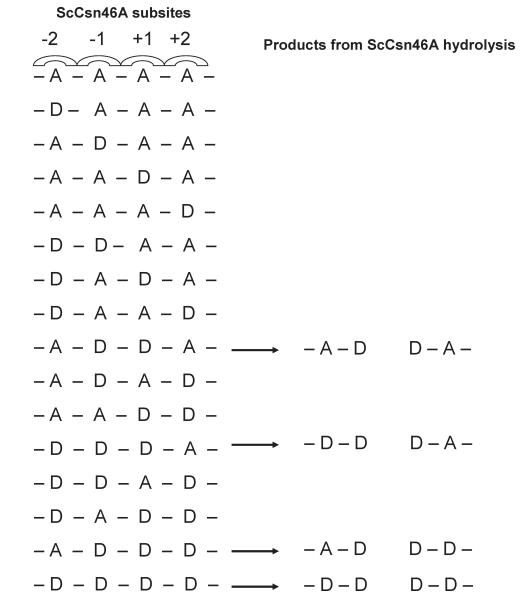

Figure 10. Schematic illustration of the initial subsite specificities of ScCsn46A toward the 16 possible tetrade sequences in the chitosan substrate.

was obtained (Tables 1 and 2). The oligomer products of ScCsn46A degradation of the $F_A = 0.32$ chitosan ($\alpha = 0.10$) are highly deacetylated (see Table 2). Initially, only the fully deacetylated dimer (DD) was produced, but when the chitosan becomes more degraded, the dimer DA appears. Upon further degradation ($\alpha = 0.29$), the fully deacetylated tetramer and pentamer were degraded, which is revealed from the MS results. It also appears that oligomers that are highly deacetylated were degraded (e.g., the tetramers DDDA, DADA, and DADD and the pentamers DADDD, DDADD, DDDDA, DDDAD, and DDA-DA). These oligomers are degraded into dimers and trimers, as can be seen from the increase of the fraction of dimers and trimers in the chromatograms at the higher α-values (Figure 6). Hence, ScCsn46A has a low subsite specificity toward acetylated/deacetylated units. After the rapid initial degradation rate (Figure 4), medium-sized oligomers (DP 5−8) with a very

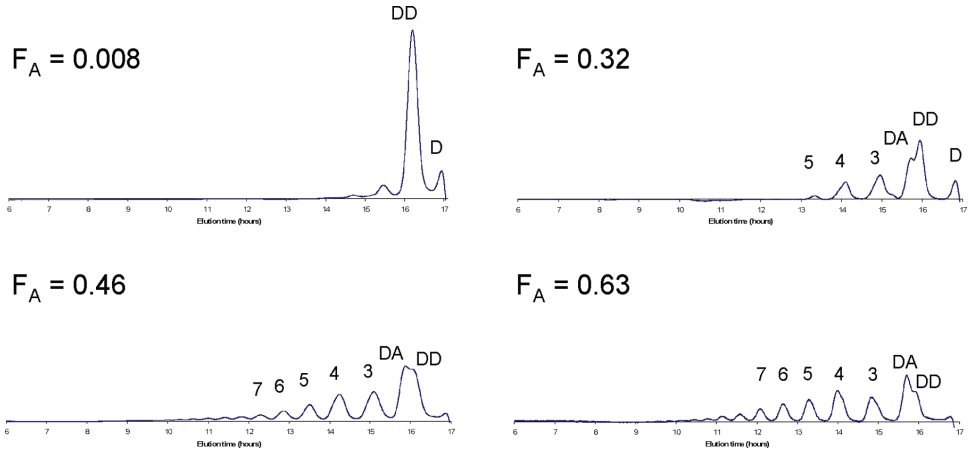

Figure 9. Size-distribution of oligomers obtained after maximum degradation of chitosans with varying F_A. SEC chromatograms showing the size distribution of oligomers obtained after maximum degradation of chitosans with varying F_A. Chitosans with F_A of 0.008, 0.32, 0.46, and 0.64 were degraded to α-values of 0.59, 0.44, 0.37, and 0.32, respectively. Peaks are labeled with DP values or with the sequence of the oligomer.

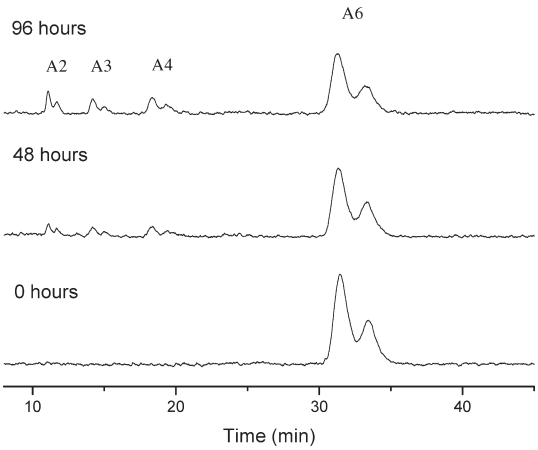

Figure 11. HPLC analysis of the ScCsn46A degraded $(GlcNAc)_6$. A total of 60 μg ScCsn46A/mg $(GlcNAc)_6$ were incubated with 300 μM $(GlcNAc)_6$ for 0, 48, and 96 h. The chromatograms show the CHOS produced after 48 and 96 h of incubation. Peaks are labeled with the identity of the CHOS.

complex composition are formed in high yields (e.g., Figure 6, $\alpha = 0.29$) in contrast to those oligomers that can be produced with ChiA, ChiB, and ChiC from *S. marcescens* or ChiG from *S. coelicolor*.[17,20,21]

For the extensively degraded chitosan ($\alpha = 0.44$) with $F_A = 0.32$, the deacetylated monomer (D) appears in the chromatogram (Figure 6). In the dimer and trimer fractions, the same dimers and trimers are present as for the chitosan degraded to $\alpha = 0.29$, except for the fully deacetylated trimer, which could not be detected in the trimer fraction of the extensively degraded chitosan, meaning that DDD is further degraded to D and DD. This is also clearly seen from the results of the degradation of the fully deacetylated chitosan (Figure 2). The ^{1}H NMR spectra of the pentamer fraction showed almost a complete absence of deacetylated reducing ends (results not shown), which was confirmed by the MS results, demonstrating that pentamers with only acetylated reducing ends were produced upon prolonged degradation (results not shown). The oligomers in the tetramer and pentamer fractions have a much higher F_A of 0.59 (for both) when compared to the initial chitosan ($F_A = 0.32$) and this, together with the initial specificity for the productive binding of deacetylated units, shows that ScCsn46A has a preference for deacetylated units.

Oligomers produced using a family 46 chitosanase from *Streptomyces* sp. N174 upon extensive degradation of a partially *N*-acetylated chitosan ($F_A = 0.25-0.35$) have previously been isolated and characterized.[24] The results suggested that this chitosanase had a high specificity for a deacetylated unit in the +1 subsite, whereas both **A** and **D** were accepted in the −1 subsite, and the oligomers produced were somewhat different from the oligomers produced after extensive degradation of the $F_A = 0.32$ chitosan with ScCsn46A (see Table 2). However, this is the first in-depth study of a family 46 chitosanase degrading fully water-soluble chitosan substrates of varying degrees of acetylation and degrees of scission. In comparison to previously characterized chitinases, ScCsn46A has a much lower subsite specificity resulting in the formation of a complex mixture of chito-oligomers upon degradation of chitosans. Such chito-oligomers are interesting as enzyme inhibitors and as elicitors, and further work is in progress.

Conclusion

ScCsn46A degradation of chitosans of varying degrees of acetylation has revealed information on the enzyme's subsite preferences and the chito-oligosaccharides products. ScCsn46A has a relatively low subsite specificity toward acetylated/deacetylated units but with a preference for deacetylated units.

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Supporting Information Available. Full ^{13}C NMR spectra (Figure 5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Paper VI

Degradation of Chitosans with a family 75 chitosanase from *Streptomyces avermitilis*

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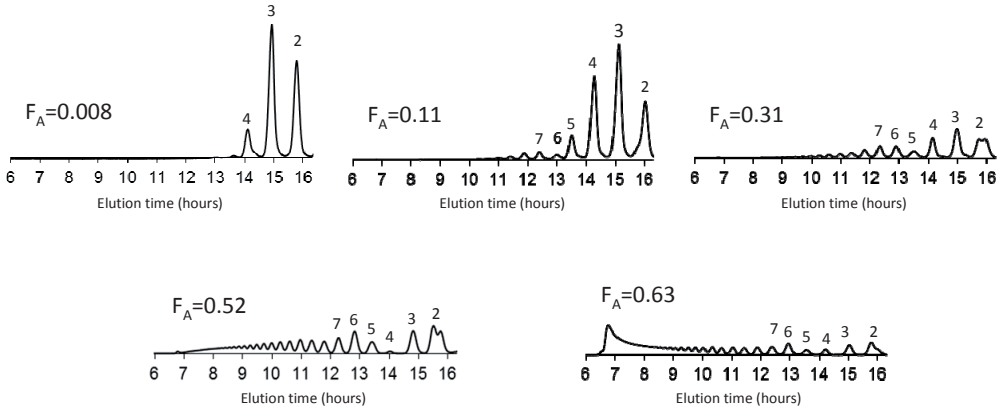
Synopsis TOC



Abstract

Chitooligosaccharides (CHOS) are oligomers composed of glucosamine and *N*-acetylglucosamine with several interesting bioactivities that can be produced from enzymatic cleavage of chitosans. By controlling the degree of acetylation of the substrate chitosan, the enzyme, and the extent of enzyme degradation, CHOS preparations with limited variation in length and sequence can be produced. We here report on the degradation of chitosans with a novel family 75 chitosanase, SaCsn75A from *Streptomyces avermitilis* . By characterizing the CHOS preparations, we have obtained insight into the mode of action and subsite specificities of the enzyme. The degradation of a fully deacetylated and a 31% acetylated chitosan revealed that the enzyme degrade these substrates according to a non-processive, endo mode of action. With the 31% acetylated chitosan as substrate, the kinetics of the degradation showed an initial rapid phase, followed by a second slower phase. In the initial faster phase, an acetylated unit (**A**) is productively bound in subsite -1, whereas deacetylated units (**D**) are bound in the -2 subsite and the +1 subsite. In the slower second phase, **D**-units bind productively in the -1 subsite, probably with both acetylated and deacetylated units in the -2 subsite, but still with an absolute preference for deacetylated units in the +1 subsite. Chitooligosaccharides produced in the initial phase are composed of deacetylated units with an acetylated reducing end. In the slower second phase, higher amounts of low DP fully deacetylated oligomers (dimer and trimer) are produced, while the higher DP oligomers are dominated by compounds with acetylated reducing ends containing increasing amounts of internal acetylated units. The degradation of chitosans with varying degrees of acetylation to maximum extents of degradation showed that increasingly longer oligomers are produced with increasing degree of acetylation, and that the longer oligomers contain sequences of consecutive acetylated units interspaced by single deacetylated units. The

catalytic properties of SaCsn75A differ from the properties of a previously characterized family 46 chitosanase from *S. coelicolor* (ScCsn46A).

Keywords: Chitosanase, chitosan degradation, chitooligosaccharides, subsite analysis

Abbreviations: DP; degree of polymerization, **A**-units; GlcNAc-units, **D**-units; GlcN-units, CHOS; chitooligosaccharides, F_A; fraction of acetylated units, α; degree of scission

Introduction

Chitin is an aqueous insoluble structural polysaccharide composed of (1→4)-linked units of 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc, **A**-unit), occurring mainly in crustacean shells, the cuticle of insects, and in the cell walls of fungi. Chitosans are produced by deacetylation of chitin, and are binary heteropolysaccharides that in addition to **A**-units may contain widely varying amounts of potentially positively charged 2-amino-2-deoxy-β-D-glucopyranose (GlcN, **D**-unit) and that are soluble in acidic solutions [1]. Variation in the chemical composition and chain length of chitosans affects properties such as solubility [2], susceptibility to degradation by chitinases, chitosanases and lysozyme [3-7], as well as functional properties in gene delivery [8-11] and drug delivery systems [12-13]. Chitosans prepared by homogeneous de-*N*-acetylation of chitin, such as those used in the present study, have a random (Bernoullian) distribution of *N*-acetyl groups [14-16].

Chitinases and chitosanases are glycoside hydrolases that catalyze the hydrolysis of chitin and chitosan to low molecular weight products, chitooligosaccharides (CHOS) [17]. Chitosanases (EC 3.2.1.132) occur in six different families of the glycoside hydrolases (family 5, 7, 8, 46, 75 and 80; see Cantarel *et al*., 2009 for classification), and they are produced by various organisms like e.g. fungi and bacteria [18]. Three of the families, GH46, GH75 and GH80, contain exclusively chitosanases, while the families GH5, GH7 and GH8 primarily contain non-chitosanases.

Glycoside hydrolases operate either according to a retaining "double-displacement" mechanism or an inverting "single displacement" mechanism [19-21]. The polymeric substrates can be degraded from one of the chain ends (exo attack) or from a random point along the polymer chain (endo attack), and each of these mechanisms may occur in combination with a processive mode of action. While processivity so far has not been found among real chitosanases, two family

18 chitinases from *Serratia marcescens* have recently been found to degrade highly acetylated chitosans according to an endo-processive mode of action [4, 22-23].

We have previously studied the conversion of chitin and chitosan into CHOS using chitinases [4-6, 22-25], and recently we also reported on the degradation of chitosans with widely varying F_A using a GH family 46 chitosanase, ScCsn46A from *Streptomyces coelicolor* A3(2) [7]. These studies have shown that the kinetics of the degradation reactions is such that product profiles change considerably during the degradation. This means that both the chain length and the chemical composition (including sequence) of CHOS change as a function of the degree of scission. Progress curves showed multiphasic kinetics, as the enzymes have very different affinities for different sequences on the substrate, and showed, not unexpectedly, that the outcome of a degradation reaction also depends on the degree of acetylation (F_A) of the substrate. All in all these observations show that widely varying CHOS preparations, varying with respect to chain lengths and composition/sequence, can be obtained with the same enzyme. Using chitinases/chitosanases of varying subsite specificities even larger variations in CHOS can be obtained. Having the tools to produce a wide variety of CHOS is important for the testing and exploitation of their many proven or putative biological applications [17].

By October 2011, the GH75 chitosanase family contained only 47 entries in the CAZy database (http://www.cazy.org/) [18], and this family has not been studied to the same extent as the GH46 chitosanase family. Family 75 chitosanases are found in fungi such as *Aspergillus* and *Fusarium*, and also in prokaryotes mainly belonging to the genus *Streptomyces*. One of the most studied family 75 chitosanases is the endo-chitosanase from *Aspergillus fumigatus* [26-27]. According to a study by Cheng *et al*. (2006), the *A. fumigatus* chitosanase uses an inverting mechanism. Using site-directed mutagenesis these authors further showed that Asp^{160} and Glu^{169} are essential for the catalytical action of the chitosanase [26]. Figure 1 shows a sequence alignment

of the *A. fumigatus* chitosanase (Csn) and a putative GH75 chitosanase from *Streptomyces avermitilis* that is the subject of the present study. The sequence alignment indicates that the two catalytic amino acids (Asp^{141} and Glu^{150}, respectively) are conserved in SaCsn75A..

In this study we have expressed this putative GH75 chitosanase from *S. avermitilis*, and according to current CAZY nomenclature the enzyme has been named SaCsn75A. We further report on a study of the degradation of chitosans by SaCsn75A to gain insight into the mode of action and the productive subsite binding preferences of the enzyme. The results reveal the substrate-binding preferences of the enzyme, reveal how the enzyme could be used to produce various types of CHOS mixtures, and add a novel well-characterized enzyme to the enzymatic toolbox for CHOS production.

Experimental

Chitosans

Chitin was isolated from shrimp shells by the method of Hackman [28], and milled in a hammer mill to pass through a 1.0 mm sieve. Chitosans, with various degrees of *N*-acetylation of 63 %, 52 %, 31 %, 11 %, and 0.8 % (i.e. fractions of acetylated units (F_A) of 0.63, 0.52, 0.31, 0.11 and 0.008, respectively) were prepared by homogeneous de-*N*-acetylation of chitin [29], except for the F_A = 0.008 chitosan that was prepared by further heterogeneous deacetylation of a highly deacetylated commercial chitosan. The properties of chitosans are summarized in Table 1.

SaCsn75A

DNA techniques:

Streptomyces avermitilis NRRL8165, obtained from the ARS Culture Collection, was grown in TSB-medium (Tryptone Soya Broth) at 37°C with stirring at 225 rpm. Two day old cultures were used for preparation of genomic DNA using the E.Z.N.A Bacterial DNA Kit (Omega Bio-Tek) according to the manufacturer's instructions. The *SaCsn75A* gene, without the part encoding a putative 26-residue N-terminal leader peptide, was amplified from genomic DNA [30] with primer *SaCsn75A*-F: 5'GGTATTGAGGGTCGCATGTCCCCGTCCGGCACCAAGGCCC 3' and primer *SaCsn75A*-R: 5'AGAGGAGAGTTAGAGCCTCAGTTGTTCTGGAGGAACTGCTT 3'. PCR reactions were conducted with *Phusion* DNA polymerase (Finnzymes, Espoo, Finland) in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany).

The amplification protocol consisted of an initial denaturation cycle of 30 seconds at 98°C, followed by 30 cycles of 10 seconds at 98°C, 30 seconds at 55°C, and 30 seconds at 72°C,

followed by a final step of 10 minutes at 72°C. The 657 bp amplified DNA fragment was excised from an agarose gel with the E.Z.N.A gel extraction kit (Omega Bio-Tek) following the manufacturer's instructions. The PCR-fragment was cloned without the need for restriction digestion or ligation into a Xa/LIC vector (pET30) as described in the Xa/LIC Cloning Kit (Novagen). The Xa/LIC vector contains an N-terminal His_6-tag followed by a factor Xa cleavage site to precisely remove all vector-encoded amino acids. The final construct was transformed into *E. coli* BL21 Star (DE3) (Invitrogen). DNA sequencing was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer / Applied Biosystems, Foster City, CA, USA) and an ABI PRISM® 3100 Genetic Analyser (Perkin Elmer / Applied Biosystems).

Production and purification of recombinant protein

200 ml *E. coli* BL21Star (DE3) transformants containing the pET30-*SaCsn75A* construct were grown at 37°C in LB-medium with 100 μg ml^{-1} kanamycin, with stirring at 225 rpm, to a cell density of 0.6 at 600 nm. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM, and the cells were further incubated for 3 hours at 37°C, followed by harvesting by centrifugation (9829 x g, 10 minutes at 4°C).

The cell pellet was resuspended in 5 mL 100 mM Tris-HCl, pH 8.0. To weaken the cell walls, 0.1 mg/mL lysozyme and 1 μL/mL DNAseI were added, and the mixture was incubated at room temperature for 30 minutes with occasional stirring. The cells were lysed by sonication at 30% amplitude on ice (15 pulses of 10 s each, with 10 s delay between pulses), using a Vibra cell Ultrasonic Processor, converter model CV33, equipped with a 3 mm probe (Sonics, Newtown, CT, USA). After sonication, the cell-lysate was centrifuged (7741 x g, 10 minutes at 4°C), and the supernatant containing SaCsn75A was pressed through a 0.20 μm sterile filter, and stored at 4°C.

Extract from 200 mL culture of SaCsn75A was purified on a Ni-NTA column (1 cm in diameter, packed with 1.5 mL column material; Qiagen, Venlo, The Netherlands) using a flow rate of 2 mL/min. The column was equilibrated in 100 mM Tris-HCl buffer pH 8.0, containing 20 mM imidazole. After loading the protein-sample, the column was washed with the starting buffer. The His-tagged protein was then eluted with 100 mM Tris-HCl buffer pH 8.0, containing 100 mM imidazole.

After purifying the protein, the buffer was exchanged to a factor Xa-buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl and 5 mM $CaCl_2$, using an amicon centricon filter device with 10 000 MW cut-off. Factor Xa was added to a concentration of 2U/mg SaCsn75A, and the mixture was incubated at room temperature overnight. The digested mixture was then applied onto a Ni-NTA column equilibrated in 20 mM Tris-HCl buffer pH 8.0. The flow-through fraction, now containing factor Xa and the SaCsn75A protein with no $(His)_6$-tag, was then applied onto a Heparin Sepharose column equilibrated in 20 mM Tris-HCl buffer pH 8.0. The flow-through fraction contained SaCsn75A with no $(His)_6$-tag was stored at 4^oC and used directly for further experiments. Purity was confirmed by SDS-PAGE. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, USA), with bovine serum albumin as a standard.

This procedure typically yielded about 0.5 mg of pure, non-His-tagged SaCsn75A per 100 ml of culture. An alternative approach for expression and purification, based on the use of the pBAD/HisB vector (Invitrogen, USA), was also explored. This procedure, described in the supplementary material, yielded only about 0.2 mg of pure His-tagged SaCsn75A per 100 ml of culture, which, however, had a slightly higher specific activity and better storage stability than the non His-tagged enzyme described above. This His-tagged SaCsn75A was used in part of the

(non-quantitative) experiments depicted in Figures 8 & 9. In all other experiments, the non-His-tagged SaCsn75A was used.

Enzymatic degradation of the Chitosans

Chitosan with $F_A = 0.31$ (as the hydrochloride salt) was dissolved in water to a concentration of 20 mg/ml and incubated with shaking in room temperature, overnight. Subsequently, an equivalent volume of 0.08 M sodium acetate buffer, pH 5.5 (containing 0.2 M NaCl) and 0.2 mg BSA was added. The chitosan solutions were then incubated in a shaking water bath at 37 °C for 30 minutes. The degradation reactions were started by adding 0.5 µg purified SaCsn75A per mg chitosan, and samples were withdrawn at regular time intervals from 12 to 504 hours. The reactions were stopped by boiling for 5 minutes, followed by a centrifugation (to remove potential precipitates). As negative control, chitosan ($F_A = 0.31$) was dissolved in the same manner, and incubated for 72 hours without adding SaCsn75A

For analysis of the maximum degree of degradation of chitosans with varying degree of *N*-acetylation (F_A of 0.008, 0.11, 0.31, 0.52 and 0.63), reactions were performed using the same conditions except that the amount of added enzyme was increased to 4 µg SaCsn75A per mg chitosan, and the incubation time was two weeks (336 hours). For the almost fully deacetylated chitosan (F_A of 0.008), samples at varying time intervals were taken.

Control experiments were performed to determine whether SaCsn75A was stable during the incubation. The experiment was started as described for the reactions testing maximum degradation, but after one week of incubation a sample was taken from the reaction and the size-distribution and quantity was analysed by SEC (see below). Subsequently, more enzyme (4.0 µg SaCsn75A per mg chitosan) was added to the reaction tube, and the reaction was further

incubated for one more week. The SEC chromatogram showed that adding additional enzyme had no effect on the product profile (results not shown).

Size Exclusion Chromatography (SEC)

Chitosan and CHOS were separated on three XK 26 columns, connected in series, packed with Superdex^{TM} 30 (Pharmacia Biotech, Uppsala, Sweden), with an overall dimension of 2.60 x 180 cm. The running buffer, 0.15 M ammonium acetate, pH 4.5, was applied at a flow rate of 0.8 mL/min using a LC-10ADvp pump (Shimadzu GmbH, Duisburg, Germany). Products were monitored with a refractive index (RI) detector (Shodex RI-101, Shodex Denko GmbH, Dusseldorf, Germany), coupled to a CR 510 Basic Data logger (Campbell Scientific Inc, Logan, UT, U.S.A.). For analytical analysis of DP-distributions in product mixtures, typically 10 mg of enzyme-degraded chitosan was applied. If the separation was to be followed by ^{1}H-NMR spectroscopy of individual oligomer fractions, typically samples of 100 mg enzyme-degraded chitosan were applied and fractions of 3.2 mL were collected using a fraction collector.

NMR Spectroscopy

Samples for ^{1}H-NMR and ^{13}C-NMR were dissolved in D_2O and 0.1 M DCl/NaOD was used to adjust the pD to 4.2 and 4.6, respectively. The deuterium resonance was used as a field-frequency lock, and the chemical shifts were referenced to internal sodium 3-(trimethylsilyl-)propionate-d_4 (0.00 ppm). ^{1}H-NMR spectra were obtained at 300 or 400 MHz at 90 °C, and ^{13}C-NMR spectra were obtained overnight at 100 MHz at a temperature of 90 °C, as previously described [5, 15-16].

To determine the sequence of shorter oligomers purified by SEC and to calculate the number average degree of polymerization, DP_n, of the enzyme-degraded chitosans, ^{1}H-NMR spectroscopy was used, as described previously [4]. The degree of scission, α, which gives the fraction of cleaved glycosidic linkages, was calculated as the inverse value of DP_n.

Results

Degradation of chitosan with $F_A = 0.31$

Subsite specificity in subsites -1 and -2

Progress in the degradation of chitosan ($F_A = 0.31$) with SaCsn75A was followed by analysing ^{1}H-NMR spectra that allowed determination of the increase in new reducing end resonances relative to internal H-1 protons, for determination of the extent of degradation (α). The identity of the new reducing ends (**A**- or **D**-unit) in the ^{1}H-NMR spectra reflects productive binding preferences in subsite -1 of the enzyme. In addition, it is possible to get information about the sugar binding specificity in the -2 subsite, since the chemical shift of the β-anomer proton in **A**-units depends on whether the neighbouring unit is an **A** or **D**-unit [4]. When a **D**-unit binds productively in the -1 subsite, it is not possible to discriminate between the identities of the neighbouring sugar next to the reducing end [6].

Figure 2 shows the anomer region of the ^{1}H-NMR spectra of the chitosan degraded to α-values between 0.04 and 0.27. At α-values below 0.07, the new reducing ends are almost exclusively acetylated with deacetylated neighbours, as only resonances at 5.19 ppm (for –**A**(α)) and 4.742 ppm (for –DA(β)) are detected. As the chitosan becomes more degraded (α-values above 0.07), new deacetylated reducing ends appear (signals at 5.43 ppm for –**D**(α) and 4.92 ppm for –**D**(β)). This shows that SaCsn75A has a preference for acetylated units in the -1 subsite, but is also capable of productive binding of deacetylated units in this subsite. The neighbouring sugar next to an acetylated reducing end, i.e. the sugar unit bound productively in the -2 subsite is always deacetylated; even upon prolonged degradation only the signal at 4.742 ppm is seen, whereas there is no sign of the resonance corresponding to an acetylated reducing end with an acetylated neighbouring unit (-AA(β)) which is expected at 4.705 ppm.

Time course of the degradation reaction

The kinetics of the degradation of the F_A = 0.31 chitosan shows a rapid linear phase at α-values below 0.11, followed by a slower second kinetic phase up to α-values of about 0.2 (Figure 3). In the fast initial phase mainly **A**-units are bound productively in subsite -1. The reduction of the reaction rate at higher α-values coincides with **D**-units binding productively in the -1 subsite (Figure 2). Upon extended degradation using high enzyme concentrations, about 1 in 3 of the glycosidic linkages in this chitosan substrate can be cleaved, as the maximum α-value obtained is 0.27.

Subsite specificity in subsite +1

From analysis of product mixtures with ^{13}C-NMR spectroscopy, the identity of the new non-reducing ends can be determined [5], which yields information about the specificity in the +1 subsite. Parts of the ^{13}C-NMR spectra (carbon 3 and 5 region from 73 to 79.5 ppm) for product mixtures obtained upon degrading F_A = 0.31 chitosan to α = 0.07 and α = 0.27 are shown in Figure 4. The spectra show almost exclusively deacetylated units at the new non-reducing ends, also for the extensively degraded chitosan (to α = 0.27), showing that SaCsn75A has an absolute preference of productive binding for deacetylated units in subsite +1.

A similar experiment that was performed with a chitosan with a higher degree of acetylation (F_A = 0.63), still only revealed **D**-units at the new non-reducing ends, confirming the absolute specificity in the +1 subsite (results not shown).

Size-distribution of products as a function of α

The chitosan (F_A = 0.31) was degraded to increasing extents of degradation (α-values from 0.04 to 0.27), and product mixtures were separated according to size (Figure 5). Identification of the DP of the peaks was performed by comparing these chromatograms with chromatograms of standard samples of fully acetylated and fully deacetylated oligomers, as described previously [4]. From the chromatograms shown in Figure 5 it can be seen that the void peak (DP > 50) gradually disappears as the reaction proceeds, and that a continuum of shorter oligomers appears with no preference for even-numbered oligomers at the lower α-values. This indicates that SaCsn75A operates according to an endo-mode of action with no processivity.

Upon extensive degradation, increasing amounts of shorter oligomers appear. The chromatograms show some interesting and uncommon irregularities in that certain DP-values are present in remarkably low or high amounts. Examples include the relatively low amount of pentamer at α = 0.27 and the high amount of tetramer at α = 0.21. The dimer fraction is separated into two peaks, identified by ^{1}H-NMR spectroscopy as DA and DD, whereas the dimers AD and AA are not observed. This is compatible with the preferences for a **D** in -2 and +1 derived from the NMR data of Figures 2 and 4. At α = 0.16 there is a slight dominance of the dimer DA, indicative of the preference for **A** in -1. However, as the reaction proceeds, the two dimers occur in almost equal amounts, and at α = 0.27 the dominating dimer is DD. At α = 0.27, i.e. at maximum degree of depolymerisation, the product mixture still contains significant amounts of oligomers with DP>10, although the dominating oligomers are dimers, trimers and tetramers.

Chemical composition of oligomeric products

Individual oligomer fractions obtained after degradation of the $F_A = 0.31$ chitosan degraded to α-values of 0.07 and 0.27 were collected (see Figure 5) and characterized by ^{1}H-NMR spectroscopy to determine their chemical composition and partial sequence. The results discussed below are summarized in Table 2.

Oligomers obtained after initial degradation (α = 0.07). Figure 5 shows that insignificant amounts of dimers were present. Figure 6 shows the anomer regions of the ^{1}H-NMR spectra of the trimer, tetramer and pentamer fractions. The trimer fraction had a DP of 3.2 and a F_A of 0.19, and the ^{1}H-NMR spectrum (Figure 6) showed exclusively **D**-units as the neighboring unit to the reducing end and no internal **A**-units. Thus, this fraction consists of DDA (59%) and DDD (41%). The tetramer fraction had a DP of 4.0 and a F_A of 0.23. The anomer region of the ^{1}H-NMR spectrum (Figure 6) is dominated by acetylated reducing ends (82%), and the main tetramer thus is DDDA. However, DDDD and small amounts of DADD and/or DDAD must also be present, since the NMR spectrum shows deacetylated reducing ends and internal **A**-units (Figure 6). The pentamer fraction (Figure 6) had a DP of 4.8 and a F_A of 0.21, and acetylated units at the reducing end are even more dominating in this fraction (87%) than in the tetramer and trimer fractions (Figure 6). The dominating pentamer thus is DDDDA, whereas the presence of resonances for deacetylated reducing ends and internal **A**-units (Figure 6) indicate the presence of small amounts of fully deacetylated pentamers and pentamers with one internal **A**-unit. The internal **A**-units resonate at 4.63 ppm, which is indicative for internal **A**-units but exclusively with a deacetylated neighbouring unit (see next paragraph) [16].

For the higher DP oligomers (results not shown), the NMR spectra became too complicated to derive sequence information. DP values calculated from spectra for the hexamers,

heptamers and octamers corresponded well with the expected DP and the determined F_A-values were 0.21, 0.22 and 0.21, respectively.

Oligomers obtained after extensive degradation (α = 0.27). ^{1}H-NMR analysis of the last sugar containing fraction eluting from the column gave a DP of 2.0 and a F_A of 0.11 (Figure 7; DP2b), confirming that this fraction mainly contains the fully deacetylated dimer (DD). The fraction eluting just before DD had a DP-value of 2.1 and a F_A of 0.46, and the NMR spectrum (Figure 7; DP2a) only showed acetylated reducing ends, identifying this dimer as DA. ^{1}H-NMR analysis of the next fraction gave a DP-value of 3.0 and a F_A of 0.15, with acetylated and deacetylated reducing ends occurring in about equal amounts, identifying the trimers as DDA and DDD. Analysis of the tetramer fraction gave a DP- and F_A-value of 4.0 and 0.23, respectively. The anomer region of the ^{1}H-NMR spectrum shows that 81% of the reducing ends are acetylated and 19% are deacetylated, as well as complete absence of internal acetylated units. This indicates that the dominating tetramer is DDDA (appr. 80%), while the remaining 20% mainly is DDDD. The pentamer fraction gave a DP-value of 5.4 and an F_A-value of 0.30. The percentage of acetylated units at the reducing end was 82%. The NMR spectrum shows internal **A**-units, with resonances between 4.55 and 4.65 ppm, which can be divided into 2 groups, those with an acetylated neighbouring unit (A<u>A</u>; at the lower ppm-values) and those with a deacetylated neighbouring unit (A<u>D</u>; at the higher ppm-values) [16]. The AA and AD sequences for internal **A**-units occur in approximately equal amounts, meaning that one or all of the three possible pentamers containing the internal AA sequence (DAADD, DAADA and DDAAD) must occur in much higher quantities compared to the more numerous possible pentamers containing only the internal AD sequence (DADDA, DDADA, DADAD, DADDD, DDADD and DDDAD). Thus, the pentamer fraction contains DDDDA in addition to DDDDD, DAADD, DAADA and DDAAD.

For the higher oligomers (results not shown), the NMR spectra became too complicated to derive sequence information. The DP values derived from the spectra generally corresponded with the values expected from their elution from the columns. The F_A-values for the hexamer, heptamer and octamer, were high relative to the substrate and amounted to 0.44, 0.41 and 0.41, respectively. These longer oligomers thus had considerable and increasing amounts of internal **A**-units with increasing DP.

In order to check whether the deacetylated monomer was produced, the salt containing fractions eluting at around 17 hours were collected and the presence of sugars was verified with ^{1}H-NMR spectroscopy. This revealed the presence of the D monomer, albeit in very low quantities.

Degradation of fully deacetylated chitosan (F_A = 0.008)

A fully deacetylated chitosan (F_A = 0.008) was degraded with SaCsn75A to different degrees of scission. Analysis of the products (Figure 8) shows that the void peak (DP>50) gradually disappears while a continuum of shorter CHOS appears, with no dominant production of dimers. This indicates that SaCsn75A degrades the fully deacetylated substrate according to a non-processive endo-mode of action, confirming the results from the degradation of the chitosan with F_A = 0.31 (Figure 5). At maximum α (α = 0.42), the product mixture almost exclusively consisted of dimers, trimers and tetramers, the dimer and the trimer being the dominating products.

SaCsn75A degradation of chitosans with varying F_A

Chitosans with widely varying degrees of acetylation (F_A = 0.008, 0.11, 0.31, 0.52, and 0.63) were degraded to maximum α-values of 0.42, 0.34, 0.27, 0.21, and 0.14, respectively. Figure 9 shows SEC chromatograms for the product mixtures. The chromatograms illustrate what the

maximum α–values show: as the degree of acetylation decreases, degradation by SaCsn75A becomes more extensive. All five product mixtures contain oligomers down to dimers, but the DP-distributions vary widely between the chitosans. The increasing presence of **A**-units in oligomers with increasing degree of acetylation of the substrate is clearly visible in the split dimer peak, showing an increasing fraction of DA (left dimer peak) relative to DD (right dimer peak; Figure 9). The profile for the chitosan with F_A = 0.52 shows large amounts of very long oligomers; in the case of the F_A = 0.63 chitosan, a considerable fraction of these oligomers has a DP > appr. 50 and elutes in the void (polymer) peak.

Chemical composition of oligomers obtained after extensive degradation of a F_A = 0.52 chitosan

The chemical composition of the CHOS produced after degradation of the chitosan with F_A= 0.52 was analyzed by ^{1}H-NMR spectroscopy (spectra shown in the supporting information) and the results are included in Table 2. In terms of composition and sequence, products were similar to those obtained with the F_A = 0.31 chitosan, but, as expected, the relative abundance of **A** containing products increased. The yield of tetramer (DP-value of 4.2, F_A-value of 0.30) was remarkably low (Figure 9), and much lower compared to the tetramer yield obtained after extensive degradation of the chitosan with F_A of 0.31. The yields of pentamer and hexamer were, however, higher as compared to the yields obtained from extensive degradation of the chitosan with F_A of 0.31 (Figure 9). These pentamers and hexamers had higher contents of internal acetylated units and of the AA diad, reflecting increased relative occurrence of non-cleavable sequences containing acetylated units.

Discussion

This is the first in-depth study of the chitosan-degrading ability of a family 75 chitosanase, providing new insight into the specificity of these enzymes and into their potential to produce various mixtures of CHOS. Our data add to a much more limited dataset on the cleaving specificity of the family 75 chitosanase from *A. fumigatus*, which was concluded to cleave **A**-**D** and **D**-**D** linkages based on degradation of a 60% acetylated chitosan [26].

Experiments with two different high-molecular weight chitosans, one fully deacetylated and the other 31 % acetylated, revealed that the enzyme operates according to a non-processive endo-mode of action, similar to what has been observed for a family 46 chitosanase, a family 19 chitinase and one member (but not all members) of the family 18 chitinases [6-7, 22]. NMR analyses showed that SaCsn75A has an absolute preference for **D**-units in subsite +1. Subsite -1 can accommodate both **A** and **D** in a productive manner, but has a preference for **A**. For products with an acetylated reducing end, an absolute preference for a deacetylated unit in the -2 subsite was observed. This indicates a preference for **D** in -2, but, since the neighbouring residue in products with a **D** at the reducing end could not be determined, productive binding of an **A** to subsite -2 cannot be excluded (hence products with –AD at the reducing end are not excluded from Table 2). These data on subsite binding preferences were confirmed by the compositional and sequence data collected for isolated oligomeric degradation products formed at low and high extents of degradation (Figures 6, 7; Table 2), showing that product mixtures initially are dominated by oligomers with acetylated reducing ends and that products with a **D** at the reducing end become more prominent later during the reaction. It should be noted though that considerable amounts of products with a deacetylated reducing end appear early in the reaction (e.g. DDD at α = 0.07; Table 2) and that the initial dominance of products with acetylated reducing ends is most

clearly manifested in the longer products (DP > 3). It is important to note that the preference for **A** in subsite -1 is far from absolute. This is further illustrated by the fact that SaCsn75A could degrade a fully deacetylated chitosan (F_A = 0.008) extensively (degree of scission of 0.42), with trimers and dimers being the dominating products (Figure 8).

Products from F_A = 0.31 chitosan with higher DP showed higher F_A values and increasing amounts of internal **A** residues (Figure 7 and Table 2) and so did the increasingly longer oligomers that are produced upon extended degradation of chitosans with increasing degree of acetylation (Figure 9). These longer oligomers will contain sequences of consecutive acetylated units interspaced by single deacetylated units, which are not cleavable by SaCsn75A due to the absolute specificity for a **D** in subsite +1 together with the same absolute specificity in subsite -2 when there is an **A**-unit in -1. This can be illustrated with the non-cleavable pentamer sequence AADAA.

We recently reported a similar study of a GH46 chitosanase, ScCsn46A from *S. coelicolor* A3(2), which had an initial preference for **D**-units in subsite -1 [7], meaning that at low extents of degradation, the two chitosanases will produce quite different CHOS-mixtures. The differences between the two chitosanases are illustrated in Table 3 (for further comparison, data for a GH19 chitinase [6] are included in this Table). As an example, the dominating tetramer obtained during the initial phase of degradation is DDDA for SaCsn75A and DDDD for ScCsn46A. The α-values obtained after extensive degradation of chitosans with F_A of 0.3 and 0.6 have been included in the table to further illustrate differences between these chitinolytic enzymes. Another difference concerns the levels of dimers and trimers at the lower degrees of scission ($\alpha \leq 0.16$; Figure 5), which are much lower for SaCsn75 than for ScCsn46A acting on the same chitosan substrate [7]. This can be explained by the initial difference in the preferences for acetylated/deacetylated units in subsite -1. Since SaCsn75A initially prefers **A**-units in -1 subsite, the probability of finding

another **A**-unit two or three units away, which is necessary to form the dimer/trimer, is relatively low with a substrate dominated by **D**-units ($F_A = 0.31$). ScCsn46A prefers **D**-units in the -1 subsite and the probability of finding another **D**-unit two or three units away is much higher in this substrate.

Yet another difference between the two enzymes concerns the yield of fully deacetylated oligomers with a given DP obtained upon degrading a fully deacetylated chitosan. SaCsn75A reaches a maximum α of 0.42, whereas ScCsn46A reached an α-value as high as 0.59[7] . Consequently, the fully deacetylated products obtained with SaCsn75A are generally longer (compare Figure 8 with Figure 2 in reference 7). At maximum α, the most abundant product obtained with SaCsn75A is the trimer, whereas ScCsn46A produces mainly dimers and a considerable amount of monomers.

Another difference between the two enzymes lies in their specific activities. From the data in Figure 3 it can be calculated that SaCsn75A has a specific activity against the F_A 0.31 chitosan of 6.5 min^{-1} for the initial degradation phase ($\alpha \leq 0.11$), and 1 min^{-1} for the following degradation phase, which is very different to the initial specific activity of 325 min^{-1} of the family 46 chitosanase, ScCsn46A degrading the same chitosan substrate [7]. In other studies, the specific activities of two chitinases, ChiG (GH19) and ChiB (GH18) on a F_A 0.64 chitosan were found to be 2280 and 970 min^{-1}, respectively [6]. Clearly, SaCsn75A is an ineffective enzyme compared to these other enzymes.

Fukamizo *et al*. have classified chitosanases into three subclasses (I-III) according to the substrate specificity towards chitosan [31]. In this classification system, all three subclasses hydrolyze the linkage between two **D**-units, and chitosanases belonging to subclass I are in addition able to cleave the glycosidic linkage between **A**-**D** while chitosanases belonging to

subclass III can hydrolyze the **D-A** linkage. Obviously, since specificities are not absolute, this classification has limited value. This is illustrated by our previous study on ScCsn46A, which showed that this enzyme can cleave any linkage (**A-D**, **D-D**, **A-A** and **D-A**), albeit with different efficiency. For SaCsn75A, the situation is clearer; this enzyme belongs to subclass I.

In conclusion, this study shows how a family 75 chitosanase converts chitosans with different degrees of acetylation into mixtures of CHOS and reveals information about the subsite specificities of SaCsn75A. Compared to ScCsn46A [7], SaCsn75A shows higher subsite specificity and, consequently, yields clearly less complex mixtures of CHOS. The differences between the chitosanases and between the chitosanases and previously studies chitinases [4, 6-7, 22-24] are relevant in an applied context, since quite different CHOS-mixtures may be produced using the various enzymes.

Acknowledgement

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Table 1. Characterization of chitosans. The Table shows the fraction of acetylated units (F_A), intrinsic viscosities ([η]) and molecular weight (M_W) of the chitosans. The molecular weights were calculated using the intrinsic viscosity vs. molecular weight relationship [32].

Chitosan (F_A)	[η] (mL/g)	M_w (g/mol)
0.008	590	185000
0.11	1200	399000
0.31	740	236000
0.52	1310	439000
0.63	865	280000

Table 2. Composition of isolated CHOS. Chemical composition and sequence of isolated oligomers obtained after degradation of a chitosan with F_A 0.31, degraded to a degree of scission (α) of 0.07 and 0.27, and a chitosan with F_A 0.52 degraded to an α-value of 0.21. Dominating compounds are shown in bold. The relative abundance in compounds in each of the oligomer fractions is either given in percent (dimer) or may be partly deduced from the overall F_A values for the fractions that are included in the Table. IA; Insignificant Amounts

Chitosan substrate	**α**	**Dimer**		**Trimer**	**Tetramer**	**Pentamer**
F_A = 0.31	0.07	IA		F_A = 0.19 **DDA** **DDD**	F_A = 0.23 **DDDA** DDDD DDAD* DADD*	F_A = 0.21 **DDDDA** DDDDD DADDD* DDADD* DDDAD* DADAD*
F_A = 0.31	0.27 (max)	**DD** 52%	**DA** 48%	F_A = 0.15 **DDD** **DDA**	F_A = 0.23 **DDDA** DDDD	F_A = 0.30 **DDDDA** DDDDD DAADD* DDAAD* DAADA*
F_A = 0.52	0.21 (max)	**DD** 44%	**DA** 56%	F_A = 0.21 **DDA** DDD	IA	F_A = 0.51 **DAADA** DAADD* DDAAD*

* Indicates species with internal **A**-units that do not necessarily all occur, but all the given sequences are compatible with the experimental data

Table 3. Subsite specificities. The table summarizes the subsite specificities for SaCsn75A (GH family 75 chitosanase), ScCsn46A (GH family 46 chitosanase)[7] and ChiG (GH family 19 chitinase)[6]. In the cases where both **A**- and **D**-units productively bind to a subsite, the preferred sugar is printed in upper case. The α-values obtained after extensive degradation of two different chitosans (F_A of 0.3 and 0.6) are also shown.

Enzyme	GH family	Subsite specificity			Sequence of the dominating tetramer, obtained at low extents of degradation	α-value after extensive degradation of a F_A 0.3	α-value after extensive degradation of a F_A 0.6
		-2	-1	+1			
SaCsn75A	75	D/A	A/D	D	DDDA[a]	0.27	0.14
ScCsn46A	46	D/A	D/A	D/A	DDDD[b]	0.44	0.32
ChiG	19	A	A/D	A	ND	0.12	0.33

[a] F_A = 0.31 chitosan; α = 0.07

[b] F_A = 0.32 chitosan; α = 0.10

Figure Legends

Figure 1. Sequence alignment of a family 75 chitosanase from *A. fumigatus* (Csn; Uniprot code Q87519) and SaCsn75A from *S. avermitilis*. The conserved catalytically important Asp and Glu residue[26] are printed in red. The sequence alignment was made with ClustalW [33].

Figure 2. Anomer region of the ^{1}H-NMR spectrum of a F_A = 0.31 chitosan after degradation with SaCsn75A to α-values between 0.04 and 0.27. The reducing end of the α-anomer of an **A**-unit resonates at 5.19 ppm, while the β-anomer from the same unit resonates at 4.742 ppm when the neighboring residue is **D** (present in the spectrum) or at 4.705 ppm if the neighboring residue is an **A** (not present in the spectrum). The α-anomer of a **D**-unit resonates at 5.43 ppm and the β-anomer at 4.92 ppm. The resonance of **D**β is only partially resolved from the resonance for the internal **D**-units (4.8 - 4.95 ppm) [16, 34]. Internal **A**-units resonate at 4.55 – 4.65 ppm.

Figure 3. Time course of the degradation of a F_A = 0.31 chitosan with SaCsn75A. The figure shows the degree of scission, α, as determined from ^{1}H-NMR spectra (such as those shown in Figure 2), as a function of the incubation time. The insert shows the rapid initial phase of the reaction.

Figure 4. ^{13}C NMR spectra (C5 and C3 region) of chitosan (F_A = 0.31) degraded to varying extents. The α-values were 0.07 (panel A) and 0.27 (panel B). Spectra were acquired at 100 MHz, 90 °C and pD 4.6. Resonances were assigned according to previously published ^{13}C-NMR spectra of degraded chitosan [5-6]. Deacetylated nonreducing ends of carbon 5 (C5) resonate at 79.1 ppm, while acetylated nonreducing ends of C5 resonate at 78.5 ppm; the spectra lack these latter resonances. Deacetylated nonreducing ends of carbon 3 (C3) resonate at 74.5 ppm, while acetylated nonreducing ends of C3 resonate at 76.2 ppm; the spectra lack these latter resonances.

Figure 5. Size-distribution of CHOS after degrading chitosan (F_A = 0.31) to varying extents. The SEC chromatograms show the size-distribution of oligomers obtained after degradation of chitosan (F_A = 0.31) to α-values ranging from 0.04 to 0.27. A chromatogram of undegraded chitosan is shown at the top of the figure. Peaks are labeled with the DP-values; the dimer peak is split into two (DA elutes before DD).

Figure 6. ^{1}H-NMR spectra (anomer region) of selected CHOS fractions (DP3-5) obtained after degradation of F_A = 0.31 chitosan to a low degree of scission (α = 0.07). Spectra were acquired at 300 MHz, at 90 °C and pD 4.2. See legend to Figure 2 for more information about the resonances in the anomer region.

Figure 7. ^{1}H-NMR spectra (anomer region) of selected CHOS fractions (DP2-5) obtained after extensive degradation of F_A = 0.31 chitosan (α = 0.27). Spectra were acquired at 300 MHz, at 90 °C and pD 4.2. See legend to Figure 2 for more information about the resonances in the anomer region. The two dimer fractions are labelled DP2a, eluting first, and DP2b, eluting last.

Figure 8. Size-distribution of CHOS after degradation of a F_A = 0.008 chitosan. SEC chromatograms showing the size-distribution of oligomers obtained after degradation of a fully deacetylated chitosan (F_A = 0.008) to α-values varying from 0.05 to 0.42. The peaks in the chromatograms were identified by comparing with chromatograms of standard samples of fully de-*N*-acetylated oligomers. Peaks are labeled with the DP-values.

Figure 9. Size-distribution of CHOS after extensive degradation of various chitosans. The SEC chromatograms show the size-distribution of oligomers obtained after extensive (maximal) degradation of chitosans with F_A of 0.008, 0.11, 0.31, 0.52 and 0.63 to α-values of 0.42, 0.34, 0.27, 0.21 and 0.14, respectively. Peaks are labeled with DP-values.

Figures

Figure 1

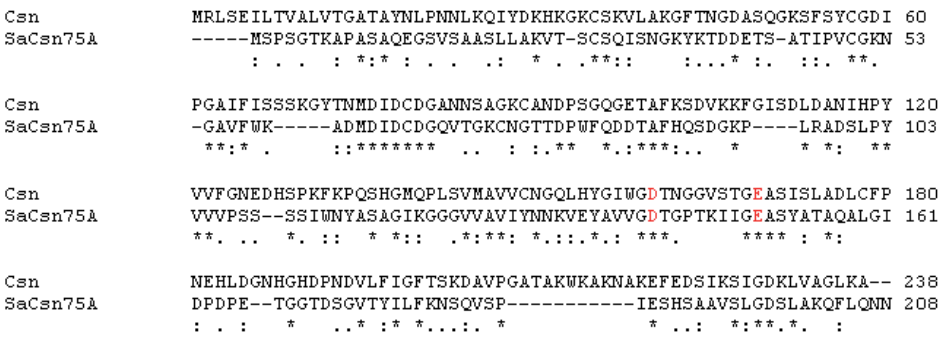
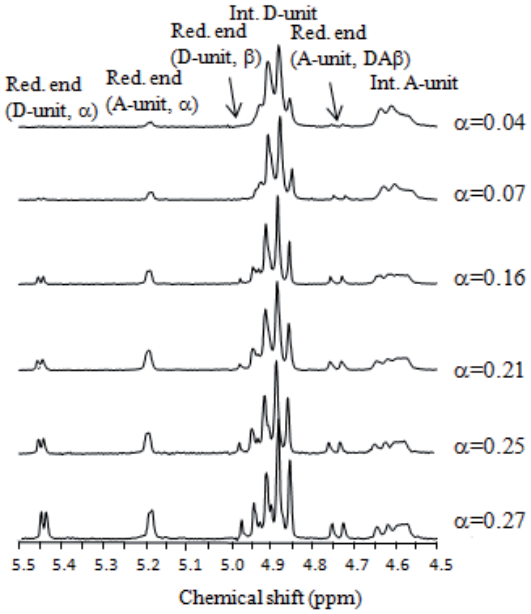


Figure 2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Red. end
(A-unit, DAβ)
Red. end
(D-unit, α)
Red. end
(A-unit, α)
Int. A-unit
α=0.04
α=0.07
α=0.16
α=0.21
α=0.25
α=0.27
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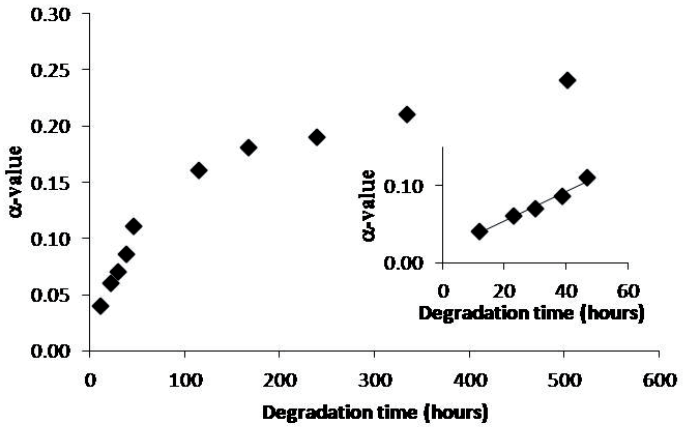
Chemical shift (ppm)

Figure 3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0.25
0.20
0.15
0.10
0.05
0.00
α-value
0
100
200
300
400
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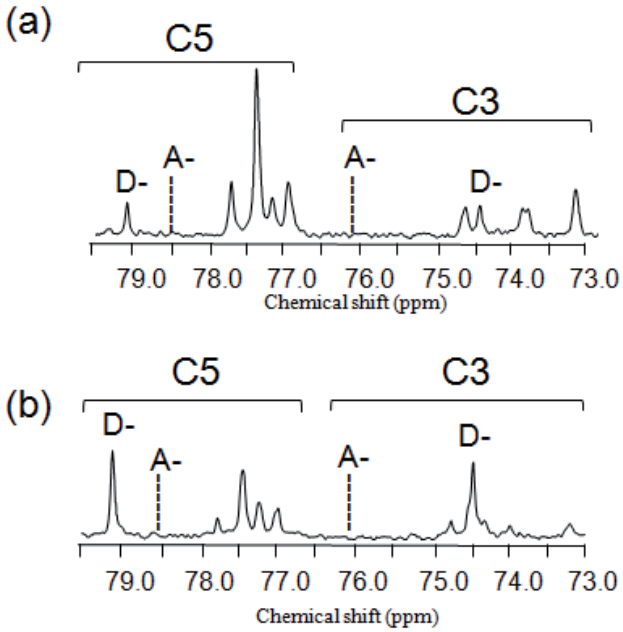
Degradation time (hours)
0.10
0.00
α-value
0
20
40
60
Degradation time (hours)

Figure 4


(a)
C5
C3
D-
A-
A-
D-
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Chemical shift (ppm)
(b)
C5
C3
D-
A-
A-
D-
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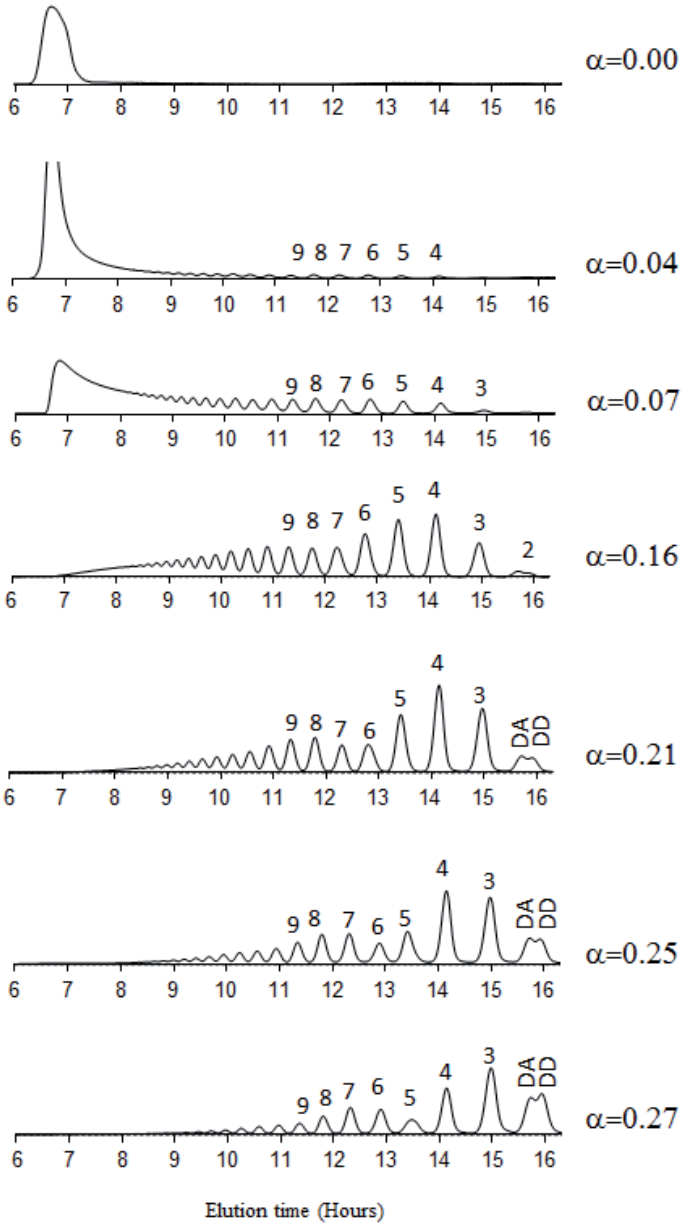
Chemical shift (ppm)

Figure 5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9 8 7 6 5 4
9 8 7 6 5 4 3
9 8 7 6 5 4 3 2
9 8 7 6 5 4 3 DA DD
9 8 7 6 5 4 3 DA DD
9 8 7 6 5 4 3 DA DD
6 7 8 9 10 11 12 13 14 15 16
Elution time (Hours)

Figure 6

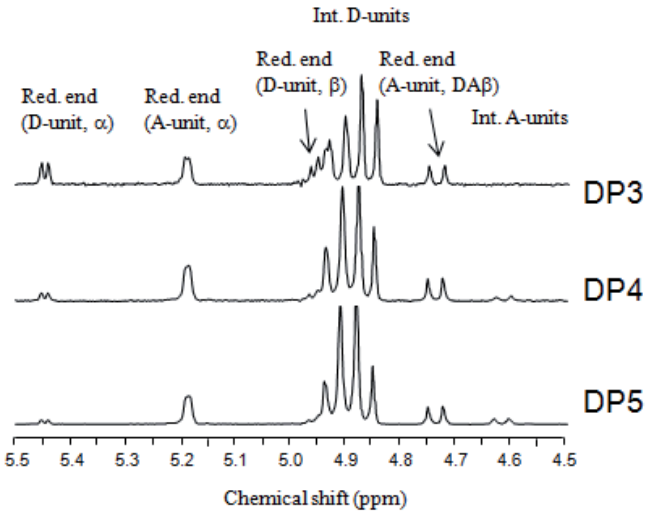

Int. D-units
Red. end
(D-unit, β)
Red. end
(A-unit, DAβ)
Red. end
(D-unit, α)
Red. end
(A-unit, α)
Int. A-units
DP3
DP4
DP5
5.5
5.4
5.3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
Chemical shift (ppm)

Figure 7

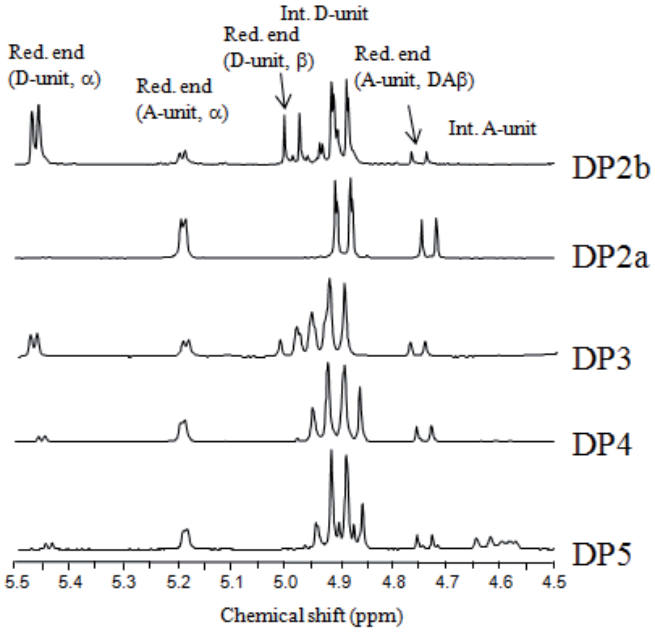

Int. D-unit
Red. end
(D-unit, β)
Red. end
(D-unit, α)
Red. end
(A-unit, α)
Red. end
(A-unit, DAβ)
Int. A-unit
DP2b
DP2a
DP3
DP4
DP5
5.5
5.4
5.3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
Chemical shift (ppm)

Figure 8

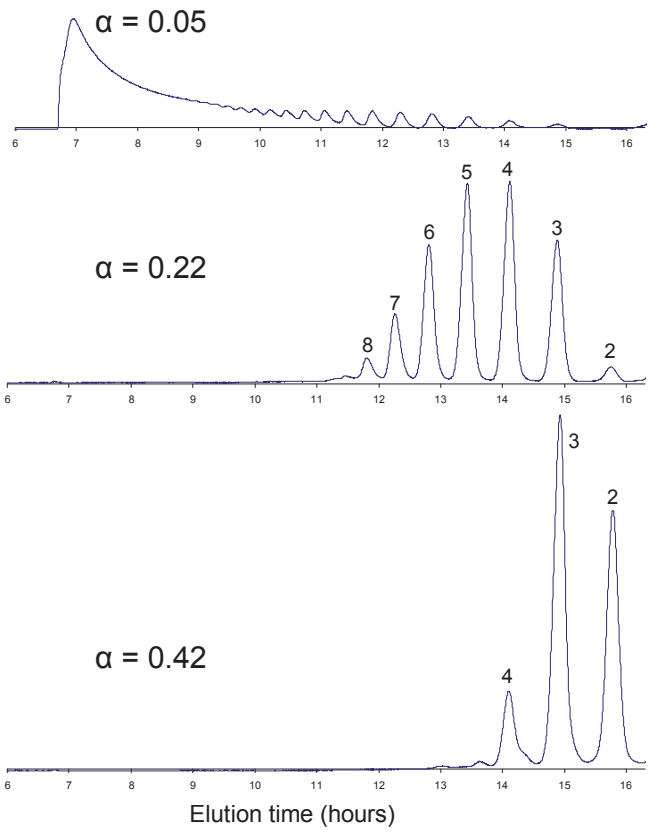
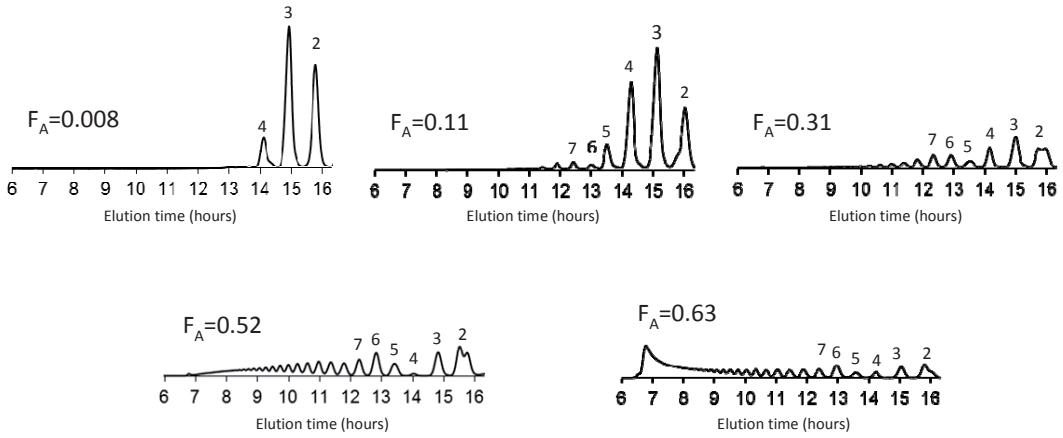


Figure 9



SUPPLEMENTARY METHOD

Alternative method for cloning, production and purification of recombinant protein

The gene encoding SaCsn75A, excluding the part coding for a putative 26-residue signal peptide, was amplified from *Streptomyces avermitilis* genomic DNA by polymerase chain reactions with the following primers (synthesized at Eurofins MWG Operon, Germany): *SaCsn75A*-F 5' CGAAGATCTTCCCCGTCCGGCACCAAGGC 3' and primer *SaCsn75A*-R 5' CAGAAGCTTTCAGTTGTTCTGGAGGAACTGC 3'. A BglII and a HindIII site were incorporated at the start and the end of the gene, respectively, to enable construction of an in-frame N-terminal His tag-fused construct in the pBAD/HisB(s) vector. This vector is a variant of the commercial vector pBAD/HisB (Invitrogen, USA) with a shortened region between the N-terminal polyhistidine tail and the down-stream multiple cloning site. In the final construct, the expression of the gene is under the transcriptional control of the arabinose-regulated araBAD promoter. The gene product consists of the sequence MAHHHHHHHRS followed by the mature SaCsn75A. The resulting plasmids were transformed into *E. coli* TOP 10 cells (Invitrogen). DNA sequencing was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer / Applied Biosystems, Foster City, CA, USA) and an ABI PRISM® 3100 Genetic Analyser (Perkin Elmer / Applied Biosystems).

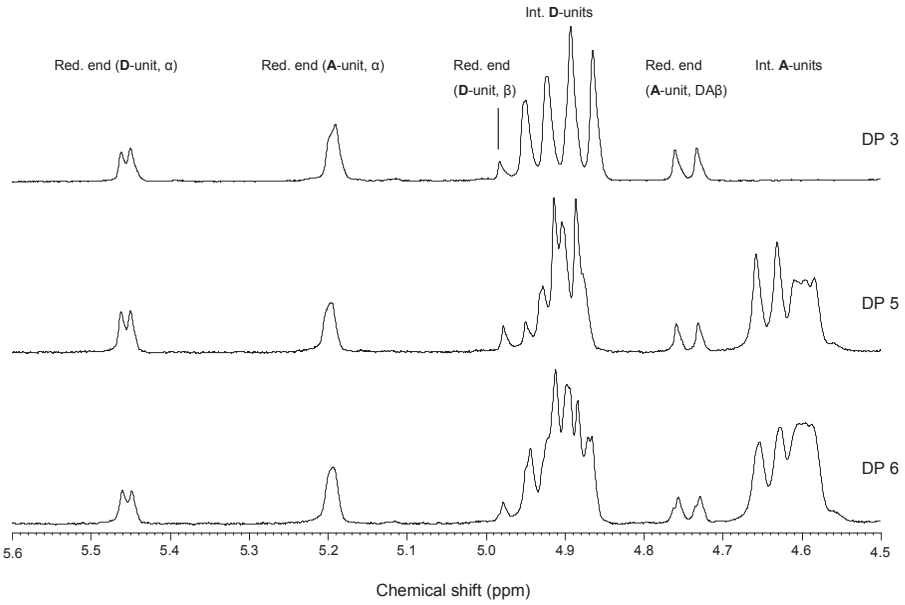
For protein expression, the transformant was grown at 37°C in 2×TY medium containing 100 mg of ampicillin per liter until the OD_{600} reached 0.5, after which gene expression was induced by adding 0.02% (w/v; final concentration) arabinose. After 40 hours incubation at 18°C, cells were harvested by centrifugation and the protein was purified to homogeneity by Ni^{2+} affinity column chromatography using a Ni-NTA Superflow Column (Qiagen, Venlo, Netherlands), and purity was confirmed by SDS-PAGE. 2 mM $CaCl_2$ and 10% Glycerol were added to all the buffers for protein purification. Protein concentrations were determined using the

Bio-Rad Protein Assay (Bio-Rad, USA), with bovine serum albumin as a standard. The protein yield was about 2 mg of pure SaCsn75A per litre of culture of *E. coli*. The protein was stored at -20^{o}C in 20 mM Tris pH 8.0, 20 mM NaCl, 2 mM $CaCl_2$, and 50% Glycerol.

Supporting information

Figure SI 1. ^{1}H-NMR spectra (anomer region) of selected CHOS fractions (DP3, 5 and 6) obtained after extensive degradation (α = 0.21) of a F_A = 0.52 chitosan. Spectra were acquired at 300 MHz, 90 °C and pD 4.2. The reducing end of the α-anomer of an **A**-unit resonates at 5.19 ppm, while the β-anomer of a **A**-unit resonates at 4.742 ppm when the neighboring residue is **D** (present in the spectrum) or at 4.705 ppm if the neighboring residue is an **A** (not present in the spectrum). The α-anomer of a **D**-unit resonates at 5.46 ppm and the β-anomer at 4.97 ppm. The reducing end resonance of **D (**β-anomer) is only partially resolved from the resonance of the internal **D**-units (4.85 - 4.97 ppm) [16, 34]. Internal **A**-units resonate at 4.55 – 4.68 ppm.

Figure SI 1


Int. D-units
Red. end (D-unit, α)
Red. end (A-unit, α)
Red. end (D-unit, β)
Red. end (A-unit, DAβ)
Int. A-units
DP 3
DP 5
DP 6
5.6
5.5
5.4
5.3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
Chemical shift (ppm)