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Inhibition of a family 18 chitinase by chitooligosaccharides

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Abstract

Inhibition of family 18 chitinases is emerging as a target for pest and fungal control as well as asthma and inflammatory therapy. To this regard, it is desirable to have access to non-toxic inhibitors that are easy to produce, and have high specificity and efficiency. Chitooligosaccharides (CHOS) that are partially *N*-acetylated have the potential to fulfill these requirements. In this work, a high molecular weight chitosan with a degree of acetylation of 0.65 was enzymatically degraded by chitinase C, a family 18 endochitinase from *Serratia marcescens*, to a degree of scission (the fraction of cleaved glycosidic linkages) of 0.2. The resulting CHOS were purified with respect to degree of polymerization (DP). CHOS of DP 5, 6, and 8, respectively, were allowed to interact with another type of family 18 chitinase from *S. marcescens*, chitinase B, used as a model enzyme for a group of family 18 chitinases with deep active site grooves that includes human enzymes. Products obtained after 7 h were isolated and their structures were determined using mass spectrometry. The IC_{50}-values of the resulting CHOS solutions for ChiB were in the lower micromolar range (15–18 μM).

Keywords: Family 18 chitinases; Inhibition; Chitooligosaccharides

1. Introduction

Chitin, an insoluble linear polysaccharide consisting of repeated units of β-1,4-*N*-acetylglucosamine, 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 important in antiparasite responses by lower life forms (Herrera-Estrella & Chet, 1999; Palli & Retnakaran, 1999; Shibata, Foster, Bradfield, & Myrvik, 2000). Even though chitin and chitin synthases have not been found in humans, we produce different chitinases that are believed to be a part of host antiparasite responses (Elias, Homer, Hamid, & Lee, 2005; Herrera-Estrella & Chet, 1999). One of these chitinases, acidic mammalian chitinase (AMCase), a family 18 chitinase, is induced during T_H2 inflammation through an interleukin (IL)-13 dependent mechanism (Zhu et al., 2004). Inhibition of the AMCase with the well known chitinase inhibitor allosamidin, reduced the inflammation (Zhu et al., 2004). The fact that chitinases are a factor in host antiparasite response and asthmatic T_H2 inflammation support the hypothesis that asthma may be a parasite independent antiparasite response (Elias et al., 2005). This suggests that inhibition of AMCase and other human chitinases is a potential target for asthma therapy (Donnelly & Barnes, 2004; Elias et al., 2005; Kawada, Hachiya, Arihiro, & Mizoguchi, 2007; Zhu et al., 2004). In addition, inhibition of family 18 chitinases is also a target for the development of pesticides (Banat, Kameyama, Yoshioka, & Koga, 1999; Blattner, Gerard, & SpindlerBarth, 1997; Saguez et al., 2006), fungicides (Izumida, Nishijima, Takadera, Nomoto, & Sano, 1996; Takaya, Yamazaki, Horiuchi,

Abbreviations: ChiB, chitinase B of *Serratia marcescens*; CHOS, heterochitooligosaccharides; F_A, degree of acetylation; DP, degree of polymerization; ChiC, chitinase C of *Serratia marcescens*.

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Ohta, & Takagi, 1998), and anti-malarials (Filho et al., 2002; Vinetz et al., 1999, 2000).

Based on initial observations by Letzel, Synstad, Eijsink, Peter-Katalinic, and Peter (2000) and recent work by Cederkvist et al. (2006), we are exploring the use of partially *N*-acetylated chitiooligosaccharides (CHOS) as easily accessible inhibitors of family 18 chitinases with high specificity and efficiency. CHOS inhibitors, being based on the substrate, hold a tremendous advantage in being very specific towards chitinases, and hence not likely to interfere with other enzymatic systems. CHOS can either be substrates or inhibitors of chitinases depending on the sequence of acetylated (**A**) and deacetylated (**D**) sugar units in the oligomer. As an example, the pentamer **AADAD** is a substrate that is readily hydrolyzed by chitinase B (ChiB) a family 18 chitinase produced by the soil bacterium *Serratia marcescens* while the isomer **DADAA** forms a strong binding complex with the same enzyme that is stable for periods over 3 h (Cederkvist et al., 2006). CHOS of different degree of polymerization (DP) and fraction of acetylated units (F_A), and sequence can be obtained through enzymatic degradation of chitosans, which are water soluble forms of chitin with varying F_A, followed by purifications using standard chromatographic techniques (Sørbotten, Horn, Eijsink, & Vårum, 2005). Relevant enzymes are various types of chitosanases as well as chitinases belonging to family 18 and family 19 of glycoside hydrolases. These enzymes usually have 4–6 sugar binding subsites and they differ with respect to how productivity of enzyme–substrate complexes is affected by the presence of acetylated or deacetylated sugars in each of these subsites. Thus, the outcome of a degradation experiment depends on the degree of deacetylation of the chitosan and on the type of enzyme that is used. Even enzymes belonging to the same enzyme family may show large differences with respect to their preferences for certain acetylation degrees and patterns (Horn, Sørbotten et al., 2006). In the context of the present study, it is of importance to note that productive binding of a substrate to all family 18 chitinase requires that an acetylated residue is bound in the −1 (glycon) subsite because the *N*-acetyl group of this sugar plays an essential role during catalysis (Synstad et al., 2004; Sørbotten et al., 2005; Terwisscha van Scheltinga et al., 1995; van Aalten et al., 2001).

In principle, it would be possible to use one type of chitinase or chitosanase variant to degrade chitosan and to produce CHOS that inhibit another type of chitinase. While this procedure will yield rather crude mixtures of CHOS possibly containing relatively minor amounts of the compound(s) of interest, it is still attractive because of simplicity and low costs. Furthermore, CHOS mixtures may be enriched for interesting compounds using purification procedures or by degrading non-interesting compounds through additional enzyme treatments.

In this study, we have pursued a strategy for production of inhibitors to family 18 chitinases, using ChiB as a model target enzyme, a chitinase that has been studied to much detail. Available data include crystal structures of wild-type and mutant enzymes with and without inhibitors, kinetic parameters for various substrates, and structural and binding data for interactions with a variety of inhibitors and CHOS (Cederkvist et al., 2006; Horn, Sørlie et al., 2006; Krokeide et al., 2007; Synstad et al., 2004; Sørbotten et al., 2005; Vaaje-Kolstad, Houston et al., 2004; Vaaje-Kolstad, Vasella et al., 2004; van Aalten et al., 2000, 2001). Like the human chitinases, ChiB contains the so-called α/β domain insertion in its catalytic $(\beta/\alpha)_8$ domain (Fusetti et al., 2002; van Aalten et al., 2000). Consequently, ChiB has a deep active site groove which is thought to be responsible for the processive mode-of-action of this enzyme (Horn, Sørbotten et al., 2006). In this work, a CHOS mixture was obtained through degradation of a high molecular weight chitosan with F_A of 0.65 with another type family 18 chitinase, chitinase C (ChiC) from *S. marcescens* (Suzuki et al., 1999). This endo-acting non-processive enzyme is expected to have a shallow active site groove, as in the family 18 chitinase hevamine (Terwisscha van Scheltinga, Hennig, & Dijkstra, 1996) because it lacks the so-called α/β domain. Previous studies had shown that, as expected from the differences in sequence and structure, ChiC and ChiB differ considerably with respect to their affinities for inhibitors and oligomeric substrates (Horn, Sørlie et al., 2006). ChiC was primarily selected as the degrading enzyme of chitosan because it had been shown to produce CHOS with two consecutive acetylated sugars (−**AA**) at their reducing ends (Horn, Sørbotten et al., 2006) and because CHOS pentamers ending with −**AA** previously had been found to bind strongly to ChiB (Cederkvist et al., 2006). CHOS mixtures derived form the reactions with ChiC were incubated with active ChiB with the aim of enriching for strong binding, non-degradable compounds. The sequences of the resulting CHOS were analyzed through derivatization with 2-aminoacridone (AMAC) at the reducing ends followed by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/TOF-MS/MS) (Bahrke et al., 2002).

2. Materials and methods

2.1. Protein expression and purification

ChiB and ChiC wild-type from *S. marcescens* were over expressed in *Escherichia coli* and purified as described elsewhere (Brurberg, Nes, & Eijsink, 1996; Horn, Sørlie et al., 2006). Enzyme purity was analyzed by SDS–PAGE and found to be >95% in all cases. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), with bovine serum albumin as a standard.

2.2. Enzyme activity assay

The activity of ChiB was determined using the $(GlcNAc)_3$ analog 4-methylumbelliferyl-β-D-*N*-*N*'-diacetylchi-

tobioside[4-MU-$(GlcNAc)_2$] as substrate, essentially as described previously (Brurberg et al., 1996). In a standard assay, 100 μL of a mixture containing 2.5 nM enzyme, 20 μM substrate, 50 mM citrate/phosphate buffer, pH 6.1, and 0.1 mg/mL bovine serum albumin was incubated at 37 °C for 10 min, after which the reaction was stopped by adding 1.9 mL of 0.2 M Na_2CO_3. The amount of 4-MU-$(GlcNAc)_2$ released was determined using a DyNA 200 Fluorimeter (Hoefer Pharmacia Biotech, San Francisco, CA, USA).

IC_{50}-values for CHOS solutions were determined using standard assay conditions using enzyme concentrations of 2.5 nM, a substrate concentration of 20 μM, and varying concentrations of the CHOS solution. The IC_{50}-values were estimated by plotting the relative specific activity as function of the inhibitor (CHOS) concentration. The concentration of the CHOS was based on their average molecular mass.

2.3. Chitooligosaccharide preparation

The preparation of the CHOS samples (DP 4, 5, 6, and 8) was made essentially as described earlier (Sørbotten et al., 2005). The depolymerization reaction of the chitosan ($F_A = 0.65$, $M_n = 160{,}000$) was started by adding 10 μg of chitinase C from *S. marcescens* to 10 mg of chitosan in 1 ml ammonium acetate buffer, pH 5.5. When the reaction had proceeded to a degree of scission (α) of 0.2 (verified by NMR, as described in Sørbotten et al. (2005)), the reaction was stopped by adjusting pH to 2.5 with 1.0 M HCl and the CHOS were fractionated by DP using size exclusion chromatography as described previously (Sørbotten et al., 2005). Resulting CHOS preparations (approximately 300 nmol) were subsequently incubated with 3 μM of ChiB for 7 h in ammonium acetate buffer, pH 6.1. The enzymatic digestion was stopped by adding 1 volume of 100% acetonitrile and the precipitated protein was removed by centrifugation. The resulting sugar solution was lyophilized and the dried material was stored at −20 °C.

2.4. 2-Aminoacridone derivatization of chitooligosaccharides

The reductive amination of hetero-chitooligosaccharides with 2-aminoacridone (AMAC) was performed as described by Bahrke et al. (2002).

2.5. Matrix assisted laser desorption/ionization mass spectrometry

MS and MS/MS spectra were acquired using an Ultraflex™ 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 isolated AMAC-CHOS and 2 μL of matrix solution (15 mg/mL DHB) were mixed and 1 μL of the mixed solution was spotted on a target plate. The spotted samples were dried at room temperature. The MS experiments were conducted using an accelerating potential of 20 kV in the reflector mode. In the TOF/TOF-MS/MS analysis, precursor ions were accelerated at 8 kV, and fragment ions generated from these precursors were subsequently accelerated to 19 kV.

3. Results

3.1. Production of chitooligosaccharides

A chitosan with M_n of 160,000 and F_A of 0.65 was firstly incubated with ChiC until 20% of the glycosidic bonds were cleaved (Fig. 1). The resulting CHOS were separated with respect to DP using size exclusion chromatography. Fractions containing DP of 5, 6, and 8 were further used in experiments described below.

3.2. Structure determination of the original chitooligosaccharides

For initial structure determination, the DP5, DP6, and DP8 CHOS fractions were derivatized with AMAC at the reducing ends and analyzed using MALDI-TOF/TOF-MS/MS. In general, fragmentation of linear oligosaccharides yields predominantly B- and Y-ions, from the non-reducing and the reducing end, respectively, were the glycosidic oxygen will reside on the Y-ion (Domon & Costello, 1988). The B- and Y-ions are essential for the sugar sequence analysis (Domon & Costello, 1988). The mass increment of 194 Da at the reducing end, due to the AMAC modification, allows for unambiguous identification of Y-type ions, allowing clear cut sequence determination of CHOS (Bahrke et al., 2002; Okafo et al., 1997). Because B-type ion fragmentation can occur at random within the sugar chain, they are of little diagnostic value (Domon & Costello, 1988).

Fig. 2 shows MALDI-TOF-MS spectra that reveal the dominating species in the DP 5, 6, and 8 fractions, respectively. Each preparation contains a mixture of CHOS that vary with respect to F_A. Each sugar species occurs in one or more of three dominating types of charged forms,

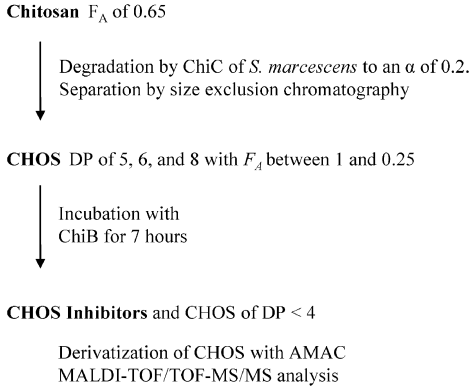

Fig. 1. A schematic overview of the production of CHOS.

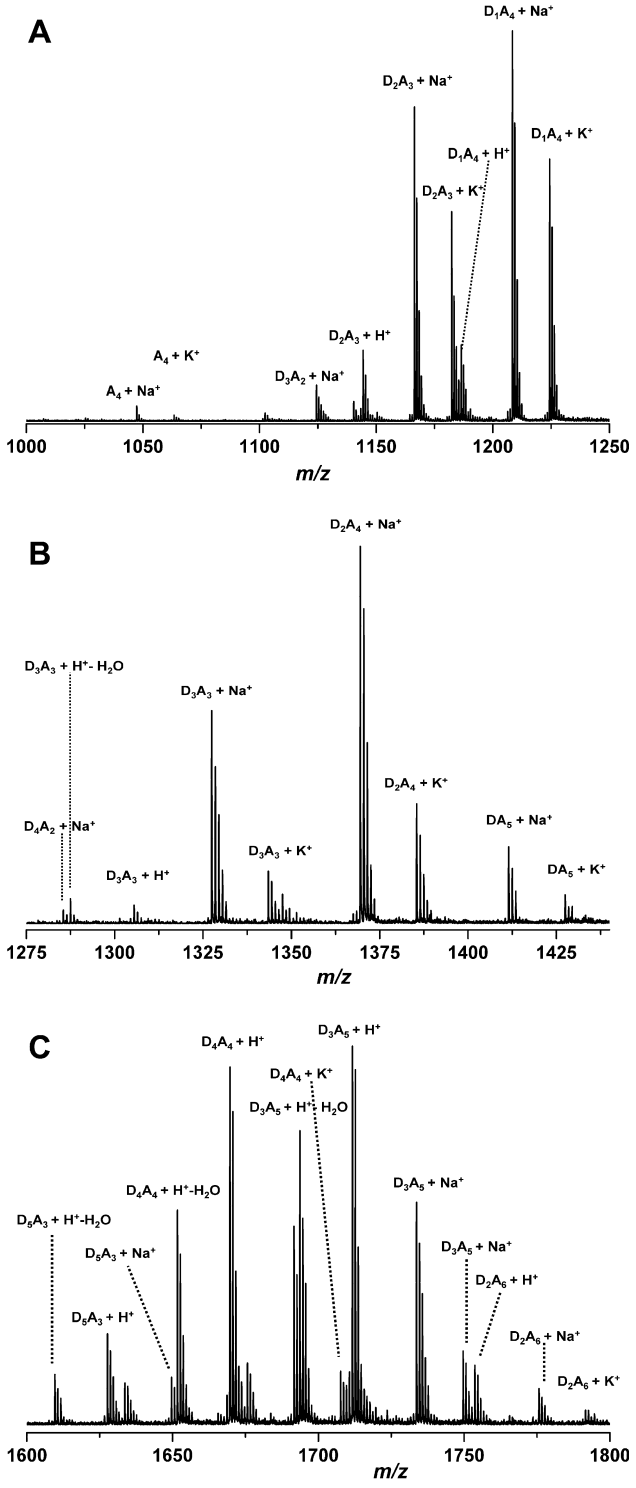

Fig. 2. Composition analysis of the AMAC derivatized hetero-chitooligosaccharide solutions by MALDI-TOF-MS. (A) DP 5, (B) DP 6, and (C) DP 8.

$[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$. It is important to note that each mass peak corresponds to several isomers of CHOS. For example, the D_2A_3 pentamer can, theoretically, contain 10 different isomers.

In order to get sequence information, MALDI-TOF/TOF-MS/MS analysis was applied on sodiated and/or protonated species depending upon their relative abundance and *m/z* overlap with non-identical species. Fig. 3 illustrates the sequencing of a D_3A_5 octamer ($[M + H]^+$ 1711.73 *m/z*). The mass spectrum shows only one Y_1-type ion (*m/z* 415.91) and one Y_2 ion (*m/z* 618.99) indicating that all D_3A_5 octamers have **AA** at the reducing end. There are two Y_3-type ion peaks appearing at *m/z* 779.88 and 821.67, respectively, corresponding to octamers with **DAA** and **AAA** at the reducing end. The Y_4-type ion at *m/z* 983.03 matches both **ADAA** (the addition of an **A** to **DAA**) and **DAAA** (the addition of a **D** to **AAA**). Furthermore, there are Y_5-type ions at *m/z* 1143.97 and 1185.9, respectively, where *m/z* = 1143.97 is the addition of a **D** to both **ADAA** and **DAAA** and *m*/z = 1185.9 is the addition of an **A** to the same structures. The mass difference between the two Y_5-type ions and the single Y_6-type ion shows that all the four isomers produce an Y_6-type ion corresponding to an A_4D_2 composition, that is **DADAA** and **DDAAA** are added an **A** and **ADAAA** and **AADAA** are added a **D**. The Y_7-type ion peak at *m/z* 1508.38 shows that all isomers have a **D** as the seventh residue. Finally, the mass difference of the parent ion and the Y_7-type ion leaves an **A** residue as the only option for the eighth residue from the reducing end. Thus, as illustrated in Fig. 3, the octamer fraction consisted of maximally 4 isomers. A complete overview over all species detected in the DP5, DP6, and DP8 fractions is provided in Figs. 4–6. As expected the largest numbers of different CHOS isomers were found in the octamer fraction. The sequence analyses show that ChiC has a very strong preference to cleave next to two consecutive **A** sugars. This is in agreement with conclusions previously drawn from experiments in which NMR analyses were used for sequence analysis of newly formed reducing ends upon degradation of a $F_A = 0.65$ chitosan with ChiC (Horn, Sørbotten et al., 2006).

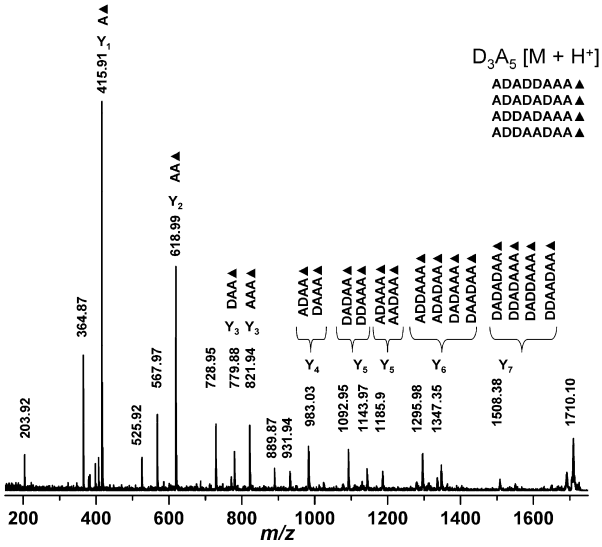

Fig. 3. MALDI-TOF/TOF-MS/MS of a D_3A_5 sugar 1711.80 *m/z*. (▲ AMAC tag at the reducing end of the CHOS sugars).

CHOS	$M_{theo.}$ [M + Na]$^+$ [M + H]$^+$	$M_{exp.}$ [M + Na]$^+$ [M + H]$^+$	Original Solution	After Incubation
D_1A_4	1208.47 1186.49	1208.41 1186.41	**ADAAA**	**Not Present**
D_2A_3	1166.46 1144.49	1166.41 1144.44	**ADDAA** **DDAAA** **DADAA**	**ADDAA** **DDAAA** **DADAA**
D_3A_2	1124.45 1102.92	1124.39 1102.46	**DDDAA**	**DDDAA**
A_4	1047.40 ----------	1047.33 ----------	**AAAA**	**Not Present**
D_1A_3	1005.39 983.41	1005.36 983.40	**Not Present**	**ADAA**
D_2A_2	963.38 941.40	963.35 941.37	**Not Present**	**DDAA**
D_1A_2	802.31 780.33	802.28 780.31	**Not Present**	**DAA**
D_2A_1	--------- 738.32	--------- 738.31	**Not Present**	**DDA**

Fig. 4. Sequence analysis of detected CHOS species in the pentamer solution (DP 5), before and after incubation with ChiB. The sequence analysis was done by MALDI-LIFT-TOF/TOF-MS/MS. ($M_{theo.}/M_{exp.}$ is the monoisotopic mass. **A**, is *N*-acetylglucosamine, and **D**, is glucosamine; --------, not used in the sequence analysis). The sequences provided are all sequences that are compatible with the MS data. See text for an example.

CHOS	$M_{theo.}$ [M + Na]$^+$ [M + H]$^+$	$M_{exp.}$ [M + Na]$^+$ [M + H]$^+$	Original Solution	After Incubation
D_1A_5	1411.55 ----------	1411.46 ----------	**ADAAAA**	**Not Present**
D_2A_4	1369.54 1347.55	1369.46 1347.48	**ADDAAA** **ADADAA** **DADAAA** **DAADAA**	**Not Present**
D_3A_3	1327.53 1305.55	1327.45 1305.47	**DADDAA** **DDDAAA** **ADDDAA**	**DADDAA** **DDDAAA** **ADDDAA**
D_4A_2	1285.52 ----------	1285.43 ----------	**DDDDAA**	**DDDDAA**
D_2A_3	1166.46 1144.49	1166.43 1144.45	**Not Present**	**ADDAA**
D_3A_2	1124.45 1102.92	1124.42 1102.45	**Not Present**	**DDDAA** **DADDA**
D_1A_3	1005.39 ----------	1005.37 ----------	**Not Present**	**ADAA**
D_2A_2	963.38 941.40	963.35 941.37	**Not Present**	**ADDA** **DDAA**
D_1A_2	802.31 780.33	802.29 780.30	**Not Present**	**ADA** **DAA**
D_2A_1	760.30 738.32	--------- 738.33	**Not Present**	**DDA**

Fig. 5. Sequence analysis of detected CHOS species in the hexamer solution (DP 6), before and after incubation with ChiB. The sequence analysis was done by MALDI-TOF/TOF-MS/MS. ($M_{theo.}/M_{exp.}$ is the monoisotopic mass. **A**, is *N*-acetylglucosamine, and **D**, is *N*-glucosamine, --------, not used in the sequence analysis). The sequences provided are all sequences that are compatible with the MS data. See text for an example.

3.3. Treatment of the DP 5 fraction with ChiB

CHOS from the DP 5 fraction were allowed to interact with ChiB for 7 h. The remaining oligosaccharides were derivatized with AMAC at the reducing ends and analyzed using MALDI-TOF/TOF-MS/MS (Fig. 4). As expected, the **ADAAA** and **AAAA** sequences were both hydrolyzed by ChiB since they are excellent substrates. All D_2A_3 isomers present in the original solution (Fig. 4) showed considerable resistance towards hydrolysis and were detected after the 7 h incubation. Among these is the **DADAA** sequence that has previously been observed to have a tendency to bind non-productively to ChiB (Cederkvist et al., 2006). **DDDAA**, the only D_3A_2 isomer present, was also resistant towards hydrolysis. The CHOS solution obtained after 7 h also contained hydrolytic products of DP 4 and DP 3. The tetramer **ADAA** can only originate from the hydrolysis of **ADAA-A**, while the **DDAA**, and **DAA** are products from hydrolysis of **DDAA-A** and **DA-DAA**, respectively. The **ADDAA** and **DDDAA** sequences can not be a source for any of the hydrolytic products in the DP 5 solution and thus seem not to be degraded at all.

3.4. Treatment of the DP 6 fraction with ChiB

CHOS from the DP 6 fraction were also allowed to interact with ChiB for 7 h. Again, the remaining oligosaccharides were derivatized with AMAC at the reducing ends and analyzed using MALDI-TOF/TOF-MS/MS. Oligosaccharides present before and after interaction are shown in Fig. 5. The species with high degrees of acetylation (D_1A_5 and D_2A_4) completely disappeared during incubation, while the D_3A_3 and D_4A_2 were still present in the solution after incubation. Judged from the product profile and as expected on the basis of known ChiB substrate preferences (Sørbotten et al., 2005), **DDDDAA** was not hydrolyzed at all. The hydrolytic products include pentamers that were found to be resistant to hydrolysis in the studies with the DP 5 fraction. The pentamer **DDDAA** can come from either **A-DDDAA** or **DDDAA-A** while **DADDA** is a hydrolytic product from **DADDA-A**. The original DP 6 sugar mixtures therefore contained at least two of the three possible D_3A_3 sequences detected in the starting DP 6 preparation.

3.5. Treatment of the DP 8 fraction with ChiB

The sequence analysis of the oligosaccharides in the DP 8 fraction before and after incubation with ChiB showed a complex mixture of isomers (Fig. 6). As observed for the fractions of DP 5 and 6, the species with most **A** sugars, such as D_2A_6, were degraded. Interestingly though, the four isomers with as few as 3 **D** sugars already showed considerable resistance to hydrolysis. For D_4A_4, there are 11 possible isomers and all of these were not completely hydrolyzed by ChiB after 7 h, nor were the two possible D_5A_3 isomers. Due to ambiguities in the sequencing procedure (see example described above) it is not certain whether all isomers shown in Fig. 6 are present in the original solution (several Y-ions can correspond to different isobars, that is CHOS with same numbers of **D** and **A** and different sequence). In the case of the DP 8 fraction, even analysis of the hydrolysis products gives little further information. For

CHOS	$M_{theo.}$ [M + Na]$^+$ [M + H]$^+$	$M_{exp.}$ [M + Na]$^+$ [M + H]$^+$	Original Solution	After Incubation
D_2A_6	--------- 1753.72	--------- 1753.69	ADADAAAA	Not Present
D_3A_5	1733.69 1711.71	1733.80 1711.73	ADADDAAA ADADADAA ADDADAAA ADDAADAA	ADADDAAA ADADADAA ADDADAAA ADDAADAA
D_4A_4	1692.02 ----------	1691.71 ----------	ADADDDAA ADDADDAA ADDDADAA ADDDDAAA DDAADDAA DDADADAA DDADDAAA DAADDDAA DADADDAA DADDADAA DADDDAAA	ADADDDAA ADDADDAA ADDDADAA ADDDDAAA DDAADDAA DDADADAA DDADDAAA DAADDDAA DADADDAA DADDADAA DADDDAAA
D_5A_3	---------- 1627.68	---------- 1627.59	DDDDADAA DADDDDAA	DDDDADAA DADDDDAA
D_4A_3	1466.62	1466.62	Not Present	DADDDAA
D_1A_5	1411.55 ----------	1411.46 ----------	Not Present	Not Present
D_2A_4	1369.54 1347.55	1369.60 1347.48	Not Present	ADADAA ADAADA ADDAAA
D_3A_3	---------- 1305.55	---------- 1305.56	Not Present	ADADDA ADDADA ADDDAA
D_4A_2	1285.52 ----------	1285.52 ----------	Not Present	DDDDAA DDDADA
D_3A_2	1124.45 1102.92	1124.39 1102.46	Not Present	DDADA DDDAA
D_2A_2	963.38 ---------	963.36 ---------	Not Present	DADA DDAA
D_1A_2	802.31 ---------	802.30 ---------	Not Present	ADA DAA

Fig. 6. Sequence analysis of detected CHOS species in the octamer solution (DP 8), before and after incubation with ChiB. The sequence analysis was done by MALDI-LIFT-TOF/TOF-MS/MS. ($M_{theo.}$/$M_{exp.}$ is the monoisotopic mass. **A**, is *N*-acetylglucosamine, and **D**, is *N*-glucosamine, --------, not used in the sequence analysis). The sequences provided are all sequences that are compatible with the MS data. See text for an example.

example, 6 (**ADADDDAA**, **DDAADDAA**, **DDADADAA**, **DAADDDAA**, **DADADDAA**, and **DADDDAAA**) out of the 11 possible isomers in the D_4A_4 component can produce the observed hydrolytic products. However, as observed for the DP 6 fraction, hydrolysis of the DP 8 fraction yields several CHOS isomers with high resistance towards further hydrolysis.

3.6. Inhibition studies of digested CHOS solution

The stability of certain CHOS towards hydrolysis by ChiB strongly suggests that the mixtures arising during the incubations described above contain inhibitors of this family 18 chitinase. After incubation of the DP 5 and DP 8 fractions with ChiB for 7 h, the remaining oligosaccharides were isolated and their IC_{50}-values were determined using fresh batches of ChiB. The remaining CHOS from the DP 5 fraction had an apparent IC_{50}-value of 16 μM while the same value was 18 μM for the CHOS from the DP 8 fraction. This indicates that the IC_{50}-value for the most active components must be lower, possibly in the sub micromolar range since much of the CHOS in the tested fractions are trimers, dimers and monomers that will not have much inhibitory effect. To check for this, CHOS derived from a DP 4 solution incubated with ChiB for 7 h, determined to contain a mixture of monomers, dimers, and trimers only, were shown to have an IC_{50}-value > 500 μM. Letzel et al. (2000) have previously shown that the IC_{50}-values of partially acetylated oligomers with a DP of four or lower for ChiB are in the millimolar range.

ChiC is an endo-acting non-processive enzyme that over the time period of 7 h hydrolyzes the CHOS used in this study. CHOS with DP of 8 and with a low degree of acetylation (D_5A_3) are degraded slower, still remains after 2 h, than those of high degree of acetylation (D_3A_5).

CHOS remaining in the reaction mixtures at the end of the incubation period are not necessarily inert towards further hydrolysis. Instead, they may be hydrolyzed very slowly because the initial preferred binding is unproductive and compete with inhibitors that are being formed during the incubation. This in accordance with the fact that indeed partial conversion of some of the resistant CHOS species is observed (see description of the results presented in Figs. 4–6, above and see below for further discussion).

4. Discussion

Inhibition of family 18 chitinases is receiving attention in fields ranging from medicine to agriculture. In many potential applications of chitinase inhibitors, it is essential that inhibitors are non-toxic and easily accessible at low cost, and that they have high specificity and efficiency towards chitinases. An additional challenge is that an excellent inhibitor towards one specific chitinase may be inefficient with another. It is important to be able to produce specific inhibitors to specific chitinases. As an example in humans, the chitinase called chitotriosidase has a (putatively protective and beneficial) fungistatic effect (van Eijk et al., 2005) while inhibition of the acidic mammalian chitinase was reported to reduce inflammation (Zhu et al., 2004). Thus, an inhibitor of acidic mammalian chitinase is of interest but should preferably not inhibit the chitotriosidase. Chitooligosaccharides with some of the sugar moieties deacetylated have the potential to satisfy the listed criteria. The present strategy shows an example of combining degradation of a highly *N*-acetylated and high molecular weight chitosan with two chitinases in sequence to produce inhibitory CHOS.

The primary cause of the inhibitory effect of CHOS comes from the fact that family 18 chitinases have an absolute preference for an *N*-acetylglucosamine sugar in the −1 subsite. Therefore, any CHOS that preferably binds to a family 18 chitinase in such a way that a **D** ends up in the crucial −1 subsite will act as an inhibitor. Clearly, the initial CHOS mixtures contain numerous species that potentially can bind in such a way, especially since several of

the other subsites do not have strong **A/D** preferences (Horn, Sørlie et al., 2006; Sørbotten et al., 2005). However, many of the initial **D**-containing species can also bind productively and do so, as is observed. At the end of the 7 h incubation period with ChiB the CHOS mixture is enriched for species that cannot be degraded at all or that are not good enough substrates to compete with the inhibiting CHOS species that emerge. To fully appreciate the resistance the remaining CHOS towards hydrolysis, it is important to note that 1000 μM **A**4 (an excellent substrate; $k_{cat} = 28\ s^{-1}$ (Krokeide et al., 2007)) would be completely hydrolyzed after 30 s under the conditions used for the ChiB treatment, i.e., with an enzyme concentration as high as 3 μM.

Interestingly, several of the species remaining after the 7 h incubation period seem good substrates for ChiB, e.g. all octamers ending with −**AAA**. While it is possible that these are not cleaved due the presence of strong inhibitors in the reaction mixtures, it is also possible that lack of cleavage is due to the fact that these oligomers have a strong preference for binding non-productively, due to their specific pattern of **A** and **D** sugars. For example, we have previously shown that the pentamer **DADAA** has a very strong preference of binding non-productively to the −3 to +2 subsites, whereas it does not engage in (productive) binding to the −2 to +3 subsites (Cederkvist et al., 2006). Such non-productive binding of potential substrates is fully plausible for two reasons. Firstly, in the case of ChiB, both experimental data (Sørbotten et al., 2005) and structural information on enzyme–substrate complexes (van Aalten et al., 2001) show that the −2 subsite has a strong preference for an acetylated sugar (Fig. 7), which would promote non-productive binding in the **DADAA** case. The structural data show that the oxygen atom in the acetamido group forms a bifurcated hydrogen bond with Trp403 and Gln407, whereas the methyl group packs tightly in an apolar environment provided by the side chains of Tyr292 and Ile339 (van Aalten et al., 2001). Secondly, from a pure binding affinity point of view, it is likely that in the −1 subsite a **D** is more favorable than an **A**. Binding of an **A** in the −1 subsite is accompanied by distortion of the sugar. Such a distortion may be energetically highly unfavorable and it is well known, e.g. from early work on lysozyme (Schindler, Assaf, Sharon, & Chipman, 1977), that binding to the −1 subsite contributes negatively to the overall binding affinity of oligomeric substrates. In the case of family 18 chitinases, distortion of the −1 sugar is in part driven by tight interactions between the *N*-acetylgroup and the enzyme (van Aalten et al., 2001) and removal of the *N*-acetylgroup would certainly remove some of the distortion stress.

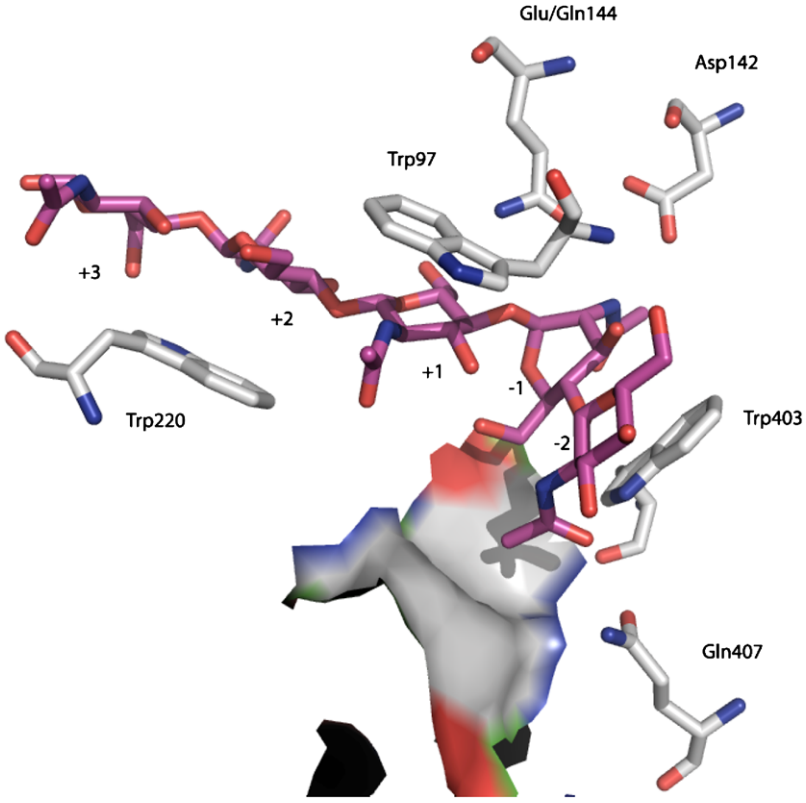

Fig. 7. Structure of the active site of ChiB with $(GlcNAc)_5$ bound (van Aalten et al., 2001) (PDB code 1e6n). $(GlcNAc)_5$ binds from +3 to −2 in the active site. Trp220 and Trp97 stack with the sugar moieties in the +2 and +1 subsites, respectively. Glu144 (mutated to Gln in this complex) is the catalytic acid that protonates the glycosidic linkage before hydrolysis, while Asp142 participates in a hydrogen bond with Glu144 and the acetamido group of the −1 sugar. The oxygen atom in the acetamido group of the −2 sugar forms a bifurcated hydrogen bond with Trp403-Nε1 and Gln407-Nε2 and the methyl group packs tightly in an apolar environment provided by the side chains of Tyr292 and Ile339, illustrated by the CPK-colored surface in this figure.

In the present study, we have explored a potential strategy for production of chitinase inhibitors. The strategy can easily be extrapolated to bulk quantities, since chitosan is available in large amounts and since the enzymatic steps involved are straightforward, standard biocatalytic conversions. The expensive and complicated chromatographic separation of the original CHOS mixture that we used in this study does not necessarily need to be implemented in an industrial process since the enrichments steps such as the ChiB treatment will do the same job (i.e., enriching for CHOS with optimal lengths and inhibitory power, as illustrated by the similar IC_{50}-values obtained from the DP 5 and the DP 8 fraction). Output of inhibitory power may be maximized by choosing optimal process conditions for the initial chitosan degradation reaction (degree of acetylation, degree of hydrolysis, type of enzyme), as discussed in detail by Sikorski et al. (2005). Chitosan hydrosylates may be rapidly sampled for their potential as source for inhibitors, either using the strategy used in this study (i.e., incubation with a target enzyme followed by MS analysis to determine if there are any CHOS sequences that remain resistant towards hydrolysis), or by direct MS-based analysis of non-covalent enzyme–ligand complexes, possibly coupled to *Top-Down* sequencing of the bound ligand (Cederkvist et al., 2006). Finally and most importantly, identification of the most potent inhibiting species is a major future target and experiments to do so are currently in progress in our laboratory. Identification of these species will not only help in fine-tuning production processes but also provide new and valuable general insight in the mode-of-action of family 18 chitinases and possible routes towards inhibitor design.

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