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Human Chitotriosidase-Catalyzed Hydrolysis of Chitosan

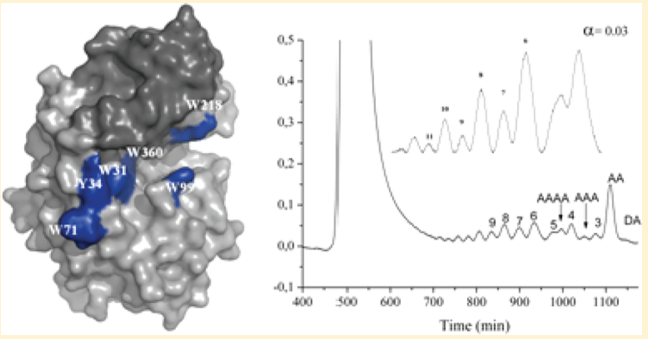
Kristine Bistrup Eide,[†] Anne Line Norberg,[†] Ellinor Bævre Heggset,[‡] Anne Rita Lindbom,[†] Kjell Morten Vårum,[‡] Vincent G. H. Eijsink,[†] and Morten Sørlie*,[†]

[†]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway

[‡]Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway

Supporting Information

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 the properties of HCHT by analyzing how the enzyme acts on high-molecular weight chitosans, soluble copolymers 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 endoprocessive mechanism, as would be expected on the basis of the structural features of its substrate-binding cleft. The preferences of HCHT subsites for acetylated versus nonacetylated 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, and +1 subsites, respectively. The latter information is important for the design of inhibitors that are specific for the human chitinases and also provides insight into what kind of products may be formed in vivo upon administration of chitosan-containing medicines or food products.



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 antiparasite 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 on the basis of 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 antiparasite responses.[8,9] While AMCase is found in the stomach,[6] in tears,[10] in the sinus mucosa,[11] and in the lungs,[12] HCHT is primarily expressed in activated human macrophages.[13]

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

Family 18 chitinases employ a substrate-assisted catalytic mechanism in which the *N*-acetyl group of the sugar bound in the −1 subsite.[24,26−28] Because of this, family 18 chitinases have an absolute preference for acetylated units in the −1 subsite. This may be exploited in the design of inhibitors based on

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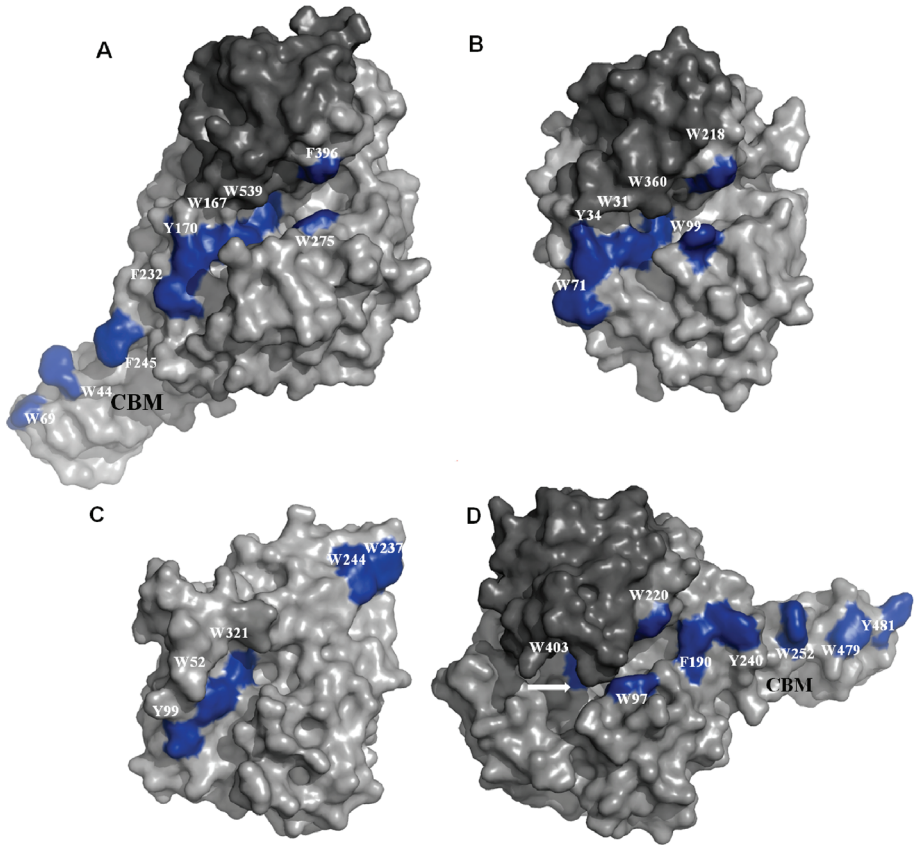

Figure 1. Crystal structures of (A) ChiA from *Serratia marcescens* (PDB entry 1ctn[55]), (B) the 39 kDa from of HCHT (PDB entry 1guv[23]), (C) the catalytic domain of ChiC from *Lactobacillus lactis* (PDB entry 3ian; this domain is 67% identical in sequence with ChiC from *S. marcescens*), and (D) ChiB from *S. marcescens* (PDB entry 1e15[25]). 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 (darker gray) 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 colored 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, and W31), the same Trp-Trp motif in the +1 and +2 subsites as ChiB (W99 and 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). 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 (denoted with an arrow). Both ChiA and ChiB have chitin-binding domains (indicated by CBM) with opposite orientations relative to that of the catalytic domain that contains solvent-exposed aromatic amino acids.

partially acetylated chito-oligosaccharides (CHOS). CHOS whose preferred binding mode places a deacetylated unit in subsite −1 will bind nonproductively and hence serve as an inhibitor.[29] CHOS bear great promise as building blocks for chitinase inhibitors, because they are natural products and potentially highly selective.[30]

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

Because it is a part of the innate immune system and associated with so many diseases, detailed knowledge of the mechanistic properties of HCHT is of great interest. Several studies 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 into 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, because of 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 into 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 the action of HCHT 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 homogeneous de-N-acetylation of chitin.[38] The characteristics of the chitosans used in this study

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are listed in Table 1. Chitinase B (ChiB) from *Serratia marcescens* was purified as described previously.[39]

Table 1. Characterization of Chitosans[a]

chitosan (F_a)	[η] (mL/g)	MW
0.18	800	257000
0.35	730	233000
0.49	746	238000
0.62	865	280000

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

HCHT Expression and Purification. *Pichia pastoris* cells expressing the 39 kDa form of HCHT were grown in 100 mL of buffered glycerol complex (BMGY) medium at 28 °C for 24 h, and 10 mL of this culture was used to inoculate 500 mL of fresh BMGY. After incubation for 48 h at 30 °C and 200 rpm, cells were harvested via centrifugation at 3500 rpm for 30 min at 20 °C. Subsequently, pellets were resuspended in 500 mL of fresh BMGY and incubated for an additional 120 h at 30 °C and 200 rpm. Every 24 h, 5 mL of high-quality methanol was added to the culture. After four additions of methanol, cells were harvested via centrifugation for 30 min at 3500 rpm and 20 °C. HCHT was secreted into the culture medium and present in the supernatant after centrifugation. The supernatant was filtered through a 0.22 μm filter and concentrated using a Vivaflow 200 PES [10000 molecular weight cutoff (MWCO)] until a total volume of 30−50 mL was reached. The concentrated supernatant was dialyzed against 50 mM sodium acetate (pH 4.2) at 4 °C for 72 h to eliminate components from the medium. HCHT was then purified using ion exchange chromatography with a HiTrap CM FF 5 mL column (GE Healthcare), using 50 mM sodium acetate (pH 4.2) as running buffer and a flow rate of 5 mL/min. The protein was eluted from the column by applying a linear gradient to 100% 50 mM sodium acetate (pH 6.5) over 20 column volumes and detected using a UV detector. The contents of the collected fractions were analyzed using SDS−PAGE. Fractions containing HCHT were pooled and concentrated to approximately 2 mg/mL by centrifugation at 4000 rpm for approximately 20 min in Amicon centrifuge tubes (10000 MWCO). Enzyme purity was analyzed by SDS−PAGE and found to be >95% in all cases (Figure S1 of the Supporting Information). Protein concentrations were determined by using the Quant-It protein assay kit and a Qubit fluorometer from Invitrogen.

Degradation of High-Molecular Mass Chitosan with F_A Values of 0.62, 0.49, 0.35, and 0.18. Chitosan was dissolved in 80 mM sodium acetate buffer (pH 5.5) to a final concentration of 10 mg/mL.[35] Chitosan with an F_A of 0.62 was depolymerized by addition of 0.075 μg of HCHT/mg of chitosan. Samples were taken at various time points between 2.5 min and 9 days after the reaction had started, and enzyme activity was stopped by adjusting the pH to 2.5 with 5 M HCl followed by boiling for 2 min. Chitosans with F_A values of 0.49, 0.35, and 0.18 were depolymerized (as described above) to a maximal degree of scission (α). The degree of scission was determined by nuclear magnetic resonance (NMR) (see below) and was considered maximal after it had been established that addition of fresh enzyme to the reaction mixtures did not yield a further increase in the degree of scission.

Analytical Instrumentation. Oligomers produced from the enzymatic depolymerization of chitosan were separated on three columns packed with Superdex 30 from GE Healthcare, coupled in series (overall dimensions of 2.60 cm × 180 cm).[35] Fractions of 4 mL were collected for further analyses of the depolymerization products. Using this method, oligomers were separated by DP only, except for oligomers with lower DPs (<5), where there also is some separation according to sugar composition (see Results and Discussion).

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 recorded at 85 °C and 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-d_4 (0.00 ppm). 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 the 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 version 4.1. For sample preparation, 1 μL of the reaction products was mixed with 1 μL of 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 reflectron mode.

RESULTS AND DISCUSSION

Degradation of High-Molecular Mass Chitosans with HCHT; Subsite Preferences. High-molecular weight chitosan (M_r = 140000) with an F_A of 0.62 was degraded with HCHT to different degrees of scission (α), which is the fraction of glycosidic linkages that have been cleaved by the enzyme. The degree of scission at any time point of the reaction was determined by monitoring the increase in the number of reducing end resonances relative to the number of resonances from internal protons in a ^{1}H NMR spectrum of the reaction mixture.[35] Figure 2 shows the time course for the reaction in which the observed maximum α value was found to be 0.33. The initial phase of hydrolysis (Figure 2, inset) suggested biphasic kinetics. The experimental data were fitted to double-exponential equations (eq 1)

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

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

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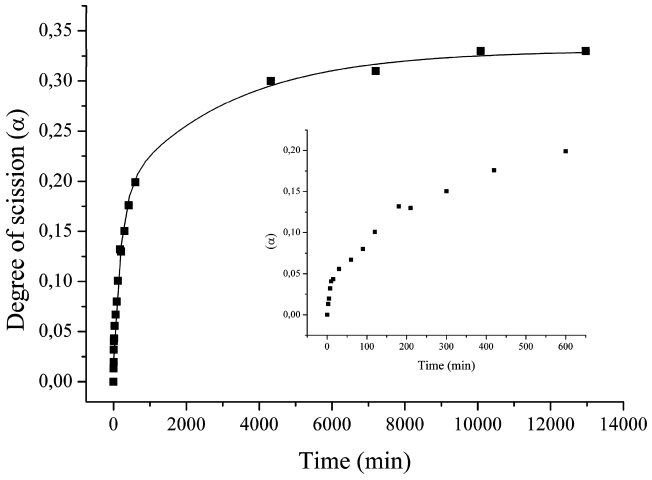

Figure 2. Time course for degradation of F_A = 0.62 chitosan with HCHT. The graph shows the degree of scission (α is the fraction of cleaved glycosidic bonds) as a function of time. The first 600 min of the degradation is shown as an inset.

represents the end point of the hydrolysis reaction (maximum α). The fit yielded apparent rate constants (k_{cat}^{app}) of 102 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 an F_A of 0.62 to α values of 0.03, 0.08, and 0.13; Figure 4 shows a chromatogram for an α of 0.33. High-molecular weight chitosan (DP > 40) is eluted in the void peak at approximately 550 min, while chitosan oligomers are eluted in separate peaks from 700 to 1200 min. Generally, oligomers are separated by DP only, but at low DP values, some separation according to sugar composition (acetylated, A, versus deacetylated, D) is observed as indicated in Figure 3. The DP_3–DP_6 fractions were subjected to sequence analysis, and the results are listed in Table 2. The reducing ends of the observed products reflect binding preferences in the negative subsites, whereas the nonreducing ends of the products reflect binding preferences in the positive subsites. Combining the data in Figure 3 with the sequence data in Table 2 shows that early 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 and Table 2). Significant amounts of products with a D at the nonreducing 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 progresses, the reaction will decelerate because the number of optimal cleavage sites, containing the AA-A stretch as well as perhaps adjacent sequence features that cannot be resolved from these data, will decrease.

Three other high-molecular weight chitosans with F_A values of 0.49, 0.35, and 0.18 were also incubated with HCHT and extensively depolymerized to maximum α. As expected on the basis of the clear preferences for acetylated units discussed above, the size distribution of the product mixtures shifted toward higher oligomer lengths and the maximum α became lower for substrates with lower F_A values (Figure 4). It has

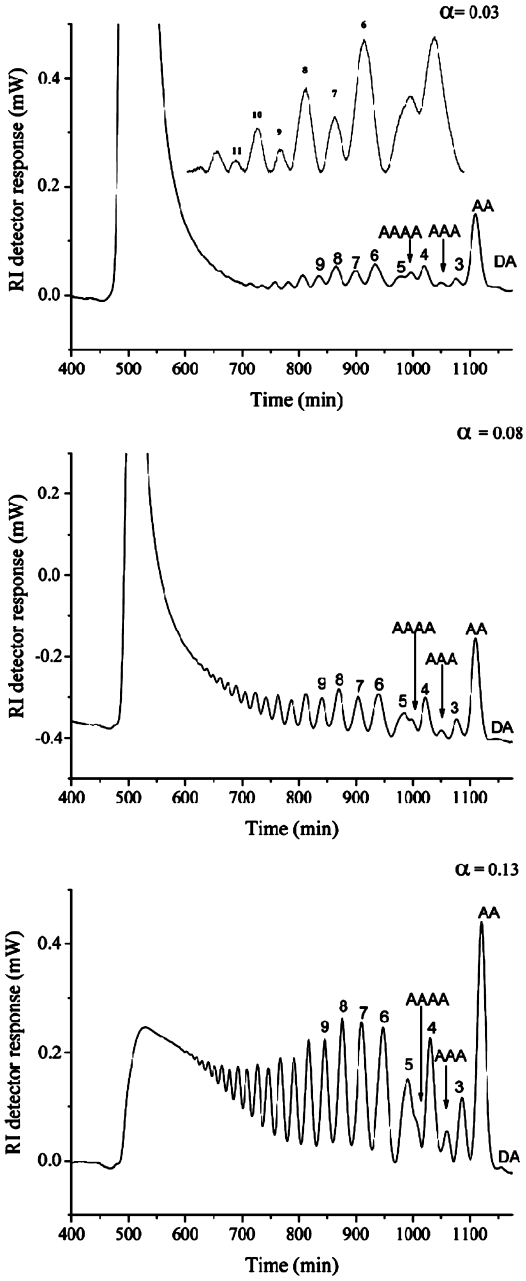

Figure 3. Size exclusion chromatograms of oligomers obtained after degradation of F_A = 0.62 chitosan at different degrees of scission (α) by HCHT. Peaks are labeled with DP values or (in the case of monocomponent peaks with known content) with the sequence of the oligomer; the large peak at the left 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 inset for the SEC chromatogram for α = 0.03 results in oligomers with an α of <0.01. A picture for maximally degraded chitosan (α = 0.33) is provided in Figure 4.

previously been shown that chitinases that use aromatic side chains to interact with their substrate are more "tolerant" for deacetylation than chitinases that primarily bind the substrate through specific hydrogen bonds involving polar side chains.[44] This is due to the fact that aromatic residues stack with the hydrophobic faces of the sugars, an interaction type that is less specific than hydrogen bonds that may involve the *N*-acetyl groups. Clearly, both the structural data shown in Figure 1 and

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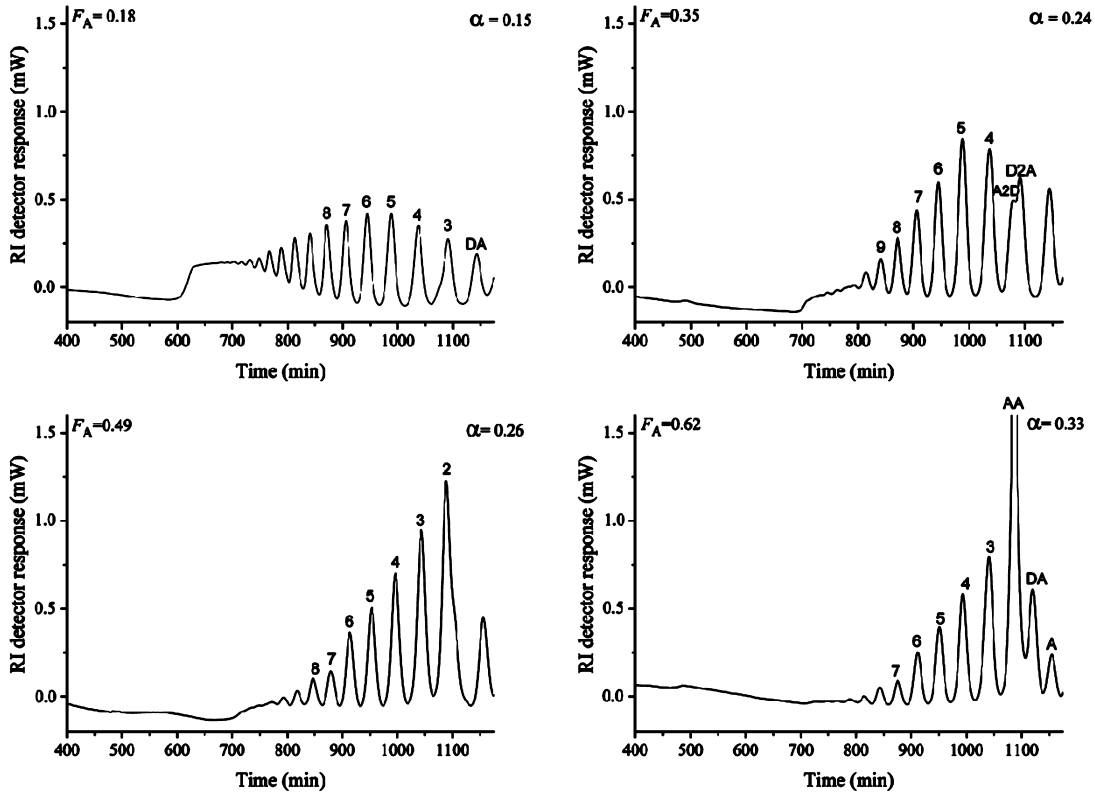

Figure 4. Degradation of chitosans with varying F_A values at a maximum degree of scission. To ensure that maximum α values were reached, samples were collected after it had been established that addition of enzyme to the reaction mixtures did not produce a further increase in α.

the observations displayed in Figure 4 show that HCHT belongs to the former category. The ability of HCHT to degrade chitosans with low F_A values should be noted, because such chitosans have several (potential) applications in human food.[45]

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

Figure 1 shows that HCHT, chitinase A (ChiA) from *S. marcescens*, and ChiB have relatively deep substrate-binding clefts, a property that is often considered to be indicative of exo activity and/or processivity.[46] Nevertheless, all three enzymes were found to predominantly act in an endo mode when hydrolyzing chitosan (Figure 5 and Sikorski et al.[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 nonreducing 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 with a soluble substrate is much less energetically demanding than association with 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 greatly 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,D) 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 subsites +1 and +2, respectively, are important for the enzyme's processive action on chitosan.[32] ChiA also has aromatic residues at these positions (Trp-275 and Phe-396), but their mutation had an only limited effect 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 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 have chitin binding domains containing three and four solvent-

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Table 2. Sequences of the Isolated Oligomers of Different Lengths Obtained after Hydrolysis of High-Molecular Weight 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
DP_3	A3	AAA	AAA	AAA	
	A2D	DAA	DAA	DAA	DAA
					ADA
	AD2				ADD
DP_4	A4	AAAA	AAAA	AAAA	
	A3D	DAAA	DAAA	DAAA	
				ADAA	ADAA
	D2A2				DDAA
DP_5	A4D	AADAA	ADAAA	ADAAA	
		ADAAA		AADAA	
	A3D2		DADAA	DADAA	ADDAA
			DDAAA	DDAAA	
	A2D3				DDDAA
DP_6	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 toward the nonreducing end. The sequences listed are those that are compatible with the mass spectra, and not all listed sequences may actually occur. For example, the pentamer fraction at an α of 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.

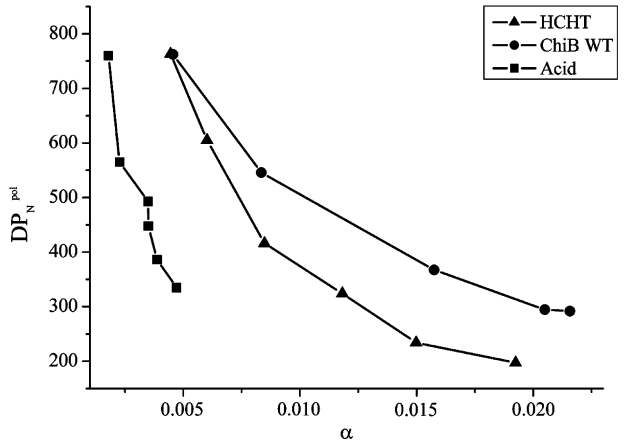

Figure 5. Changes in DP_n^{pol} as a function of extent of reaction α.

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

The degree of processivity of HCHT was assessed by plotting the relative viscosity of the polymer solution from which the α of the polymer fraction, α_{pol}, may be calculated, versus the total number of reducing ends (α_{tot}) (Figure 6). The inverse of the slopes of the lines shown in Figure 6 represents

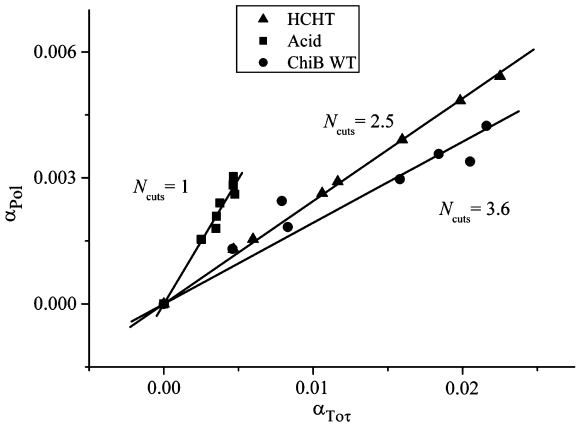

Figure 6. Degree of scission of the polymer fraction (α_{pol}) as a function of the total degree of scission (α_{tot}).

the number of cuts (N_{cuts}) per formation of an enzyme–substrate complex.[34] The observed number of cuts is expressed as a relative number, where N_{cuts} observed for acid hydrolysis is set to 1. The results indicate that HCHT is processive with an average of 2.5 cuts per formation of the enzyme–substrate complex during hydrolysis of a chitosan with an F_A of 0.62. The same numbers are 9.1 and 3.4 cuts per formation of the enzyme–substrate complex for ChiA and ChiB, respectively.[34] As a control, the value for ChiB was also determined and found to be 3.6 (Figure 6), in good accordance with the work of Sikorski et al.

Processivity in family 18 chitinases leads to a diagnostic product profile dominated by even-numbered products early in the reaction with chitosan.[21,35,50] HCHT showed this clear dominance of even-numbered products only very early in the reaction (inset in Figure 3). The ratio between the size of an even-numbered peak and an odd-number peak may serve as a relative quantification of processivity; in this study, the DP_6 and DP_7 peaks were used. When $\alpha < 0.01$, the DP_6/DP_7 ratio is ~3, but it rapidly decreased from 1.5 at an α of 0.03 to ~1.3 at an α of 0.08 (Figure 3). For ChiA and ChiB, the DP_6/DP_7 ratios at an α of ≈0.08 are approximately 4 and 3, respectively.[33] The initial dominance of even-numbered products for HCHT has also been detected by Gorzelanny et al.[21] using a different approach based on the use of electrophoresis and MS. Another characteristic feature of endo-acting processive enzymes is the slow disappearance of the polymer peak. This is indeed the case for HCHT, where disappearance of this peak is much slower [at $\alpha > 0.13$ (Figure 3)] than for nonprocessive endo-acting family 18 chitinases such as ChiC from *S. marcescens* where the polymer peak disappears when $\alpha \approx 0.05$.[33] For ChiA and ChiB, the polymer peak disappears when $\alpha \approx 0.20$. The combination of the slow disappearance of the polymer peak (Figure 3) and a clear endo activity (Figure 5) coupled with an initial dominance of even-numbered products and an estimated 2.5 cuts per formation of the enzyme–substrate complex suggest that HCHT is processive, albeit possibly to a lesser degree than ChiA and ChiB.

It is conceivable that the analysis of processivity in HCHT to some extent is disturbed by transglycosylation. HCHT is known to have relatively high transglycosylation activity, and recent mutational work on ChiA has shown that the introduction of a Trp at position +2 drastically increases transglycosylation activity.[51] This Trp is naturally present in HCHT, whose active site is highly similar to that of the

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engineered hypertransglycosylating ChiA mutant. Perhaps the rapid disappearance of the dominance of even-numbered products during the course of the reaction is somehow linked to the increased frequency of transglycosylation. Furthermore, the isoform of HCHT tested in this work does not contain the C-terminal chitin binding domain that contains eight aromatic amino acids[52] (unknown if these are solvent-exposed because of a lack of crystal structure for this isoform), and it is conceivable that the presence of this chitin binding domain may increase the degree of processivity.

In summary, we provide insight into how HCHT acts on chitosan, which is useful for understanding enzyme properties such as endo versus exo action, processivity, and substrate binding preferences. This information is important for further work aimed at understanding the roles of human chitinases, the faith of chitosan-containing food products or medicines, and the development of inhibitors that are specific for certain chitinases. HCHT acts on fungal cell walls,[4] and it is conceivable that its processive mechanism contributes to its fungistatic effect. Association with the insoluble polymer is the rate-determining step in chitin hydrolysis,[48] and a processive mode allows for more hydrolytic events to take place upon each association compared to a nonprocessive mechanism. The ability to bind in an endo mode may also promote substrate binding because the enzyme may not be dependent on finding chain ends.

HCHT is called chitotriosidase most likely because in the original studies it was found to release 4-methylumbelliferyl from the artificial substrate 4-methylumbelliferyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose. In retrospect, it is clear that the 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 our data clearly show that the main hydrolysis product is chitobiose, i.e., the AA dimer (Figure 4, $F_A = 0.62$ experiment). This is fully consistent with HCHT acting as a "normal" processive enzyme. Formally, in analogy with the nomenclature used in the cellulose field, it would probably be better to refer to the enzyme as chitobiohydrolase.

■ ASSOCIATED CONTENT

Supporting Information

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

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +47 64965902. Fax: +47 64965901. E-mail: morten.sorlie@umb.no.

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■ ABBREVIATIONS

ChiA, chitinase A from *S. marcescens*; ChiB, chitinase B from *S. marcescens*; ChiC, chitinase C from *S. 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.

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