Human Chitotriosidase Catalyzed Hydrolysis of Chitosan

Journal:	*Biochemistry*
Manuscript ID:	bi-2011-015585.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a

Complete List of Authors:	Eide, Kristine; The Norwegian University of Life Sciences Norberg, Anne Line; The Norwegian University of Life Sciences Heggset, Ellinor; NTNU, Biotechnology Lindbom, Anne Rita; The Norwegian University of Life Sciences Varum, Kjell; Norwegian University of Science and Technology, Biotechnology Eijsink, Vincent; Norwegian University of Life Sciences, Chemistry, Biotechnology and Food Science Sørlie, Morten; The Norwegian University of Life Sciences

Human Chitotriosidase Catalyzed Hydrolysis of Chitosan

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

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

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

AUTHOR INFORMATION

Corresponding author

*To whom correspondence should be addressed: Telephone: +47 64965902. Fax: +47 64965901. E-mail address: morten.sorlie@umb.no

Funding information

This work was supported by Grants 164653/V40, 178428/V40, and 177542/V30 from the Norwegian Research Council.

RECEIVED DATE (to be automatically inserted after your manuscript is accepted if required according to the journal that you are submitting your paper to)

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 substrate-binding cleft of HCHT extends over one face of the enzyme and is lined with solvent exposed aromatic residues (Fig 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 partially acetylated chito-oligosaccharides (CHOS). CHOS whose preferred binding mode places a deacetylated unit in subsite –1 will bind non-productively, 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 in between subsequent hydrolytic reactions (*31*). Another variable within the family 18 chitinases concerns the binding affinities and selectivity of their individual subsites. To analyze these characteristics, studies on the degradation of chitosan, the water soluble partially deacetylated polymeric chitin analogue, have shown to be useful (*32-35*).

Being 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 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 harvested through centrifugation for 30 minutes at 3500 rpm and 20 ºC. HCHT is secreted into the culture medium and is present in the supernatant after centrifugation. The supernatant was filtered through a 0.22 µm filter and concentrated using a Vivaflow 200 PES, 10 000 MWCO, until a total volume of 30-50 mL. Concentrated supernatant was dialyzed against 50 mM sodium acetate pH 4.2 at 4 ºC for 72 hours in order to get rid of 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 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 minutes in Amicon centrifuge tubes 10 000 MWCO. Enzyme purity was analyzed by SDS-PAGE and found to be over 95% in all cases (Figure S1). Protein concentrations were determined by using the Quant-It protein assay kit and a Qubit fluorometer from Invitrogen (CA, USA).

Degradation of High Molecular Mass Chitosan with F_A = 0.62, F_A = 0.49, F_A = 0.35 and F_A = 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 F_A = 0.62 was depolymerized by adding 0.075 µg HCHT pr mg chitosan. Samples were taken at various time points between 2.5 min. and 9 days after starting the reaction and enzyme activity was stopped by adjusting the pH to 2.5 with 5 M HCl followed by boiling for 2 minutes. Chitosans with F_A = 0.49, 0.35 or 0.18 were depolymerized (as described above) to a maximum degree of scission (α). The degree of scission was determined by 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^{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).

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

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 substrates with lower F_A values (Figure 4). It has 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 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 should be noted, since such chitosans have several (potential) applications in human food (*45*).

Determination of Endo/exo Mode. By studying the relative viscosity of the chitosan solution during chitinase-catalyzed hydrolysis, it is possible to determine whether the enzymes act in an endo- or an 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* for which highly detailed studies have shown that endo-type of action is predominant when acting on chitosan. Figure 5 displays relative viscosity over time for a chitosan solution ($F_A = 0.62$) hydrolyzed by acid, ChiB, and HCHT. In all three cases 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 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, respectively, solvent exposed aromatic amino acids (Figure 1A and 1D), which the tested isoform of HCHT does not have, that 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 represent the number of cuts (N_{cuts}) per formation of an enzyme-substrate complex (*34*). The observed number of cuts is expressed as 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 enzyme-substrate complex during hydrolysis of a chitosan with F_A of 0.62. The same numbers are 9.1 and 3.4 cuts per formation of 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 on in the reaction with chitosan (*21, 35, 50*). HCHT showed this clear dominance of even-numbered only very early in the reaction (insert in Figure 3). The ratio between the size of an even-numbered peak and an odd-number peak may serve for a relative quantification of processivity; in this study, the DP6 and DP7 peaks were used. At α below 0.01 the DP6/DP7 ratio was about 3, but it rapidly decreased via 1.5 at α = 0.03 to about 1.3 at α = 0.08 (Figure 3). For ChiA and ChiB the DP6/DP7 ratios at α ≈ 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 non-processive endo-acting family 18 chitinases such as ChiC from *S. marcescens* where the polymer peak disappears at $\alpha \approx 0.05$ (*33*). For ChiA and ChiB, the polymer peak disappears at $\alpha \approx 0.20$. The combination of 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 enzyme-substrate 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 increase transglycosylation activity (*51*). This Trp is naturally present in HCHT, whose active site is highly similar to that of the 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 increased occurrence 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 due to 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 to understand enzyme properties such as endo- vs. exo-action, processivity, and substrate binding preferences. This information is important for further work on 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 to 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 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

The *Pichia pastoris* cells producing the 39 kDa form of HCHT gene were a kind gift from Prof. Daan M.F. van Aalten, University of Dundee.

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

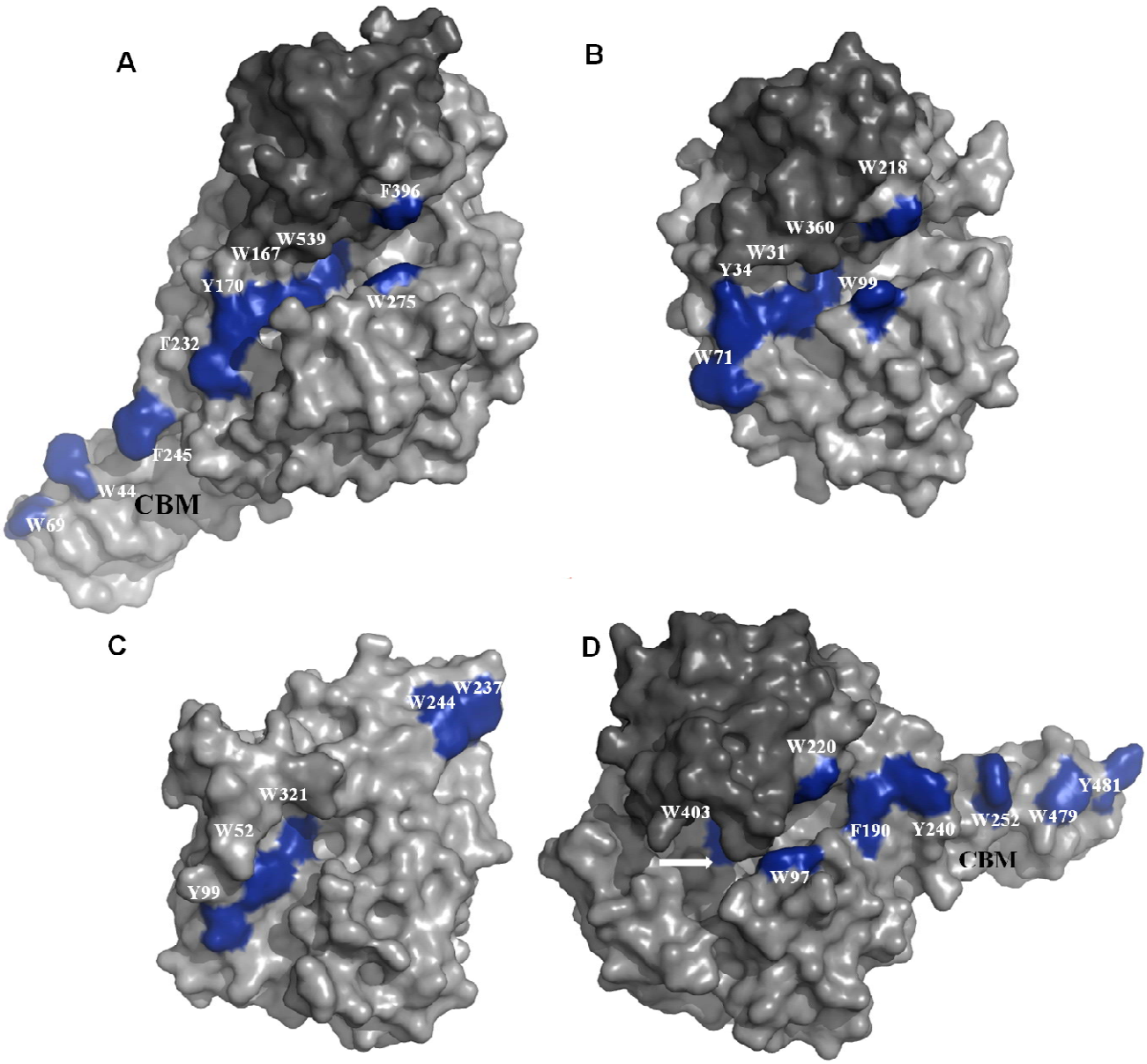


Figure 2

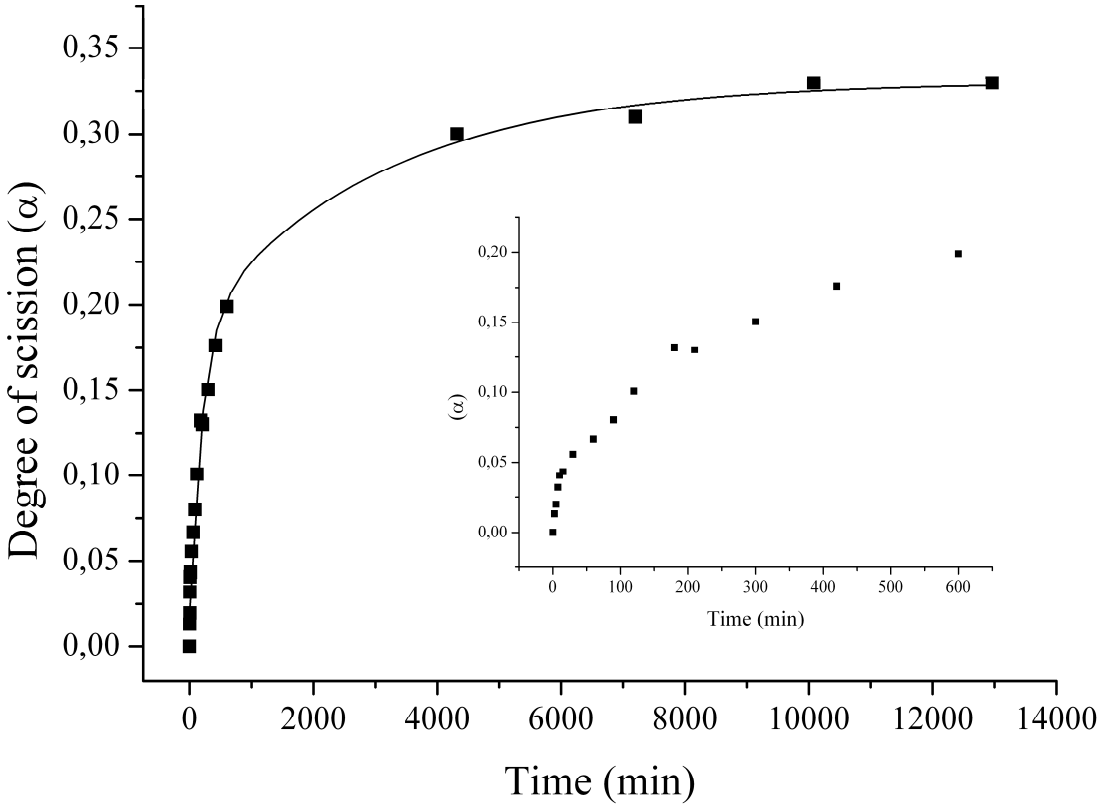

Degree of scission (α)
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
0
2000
4000
6000
8000
10000
12000
14000
Time (min)
(α)
0,20
0,15
0,10
0,05
0,00
0
100
200
300
400
500
600
Time (min)

Figure 3

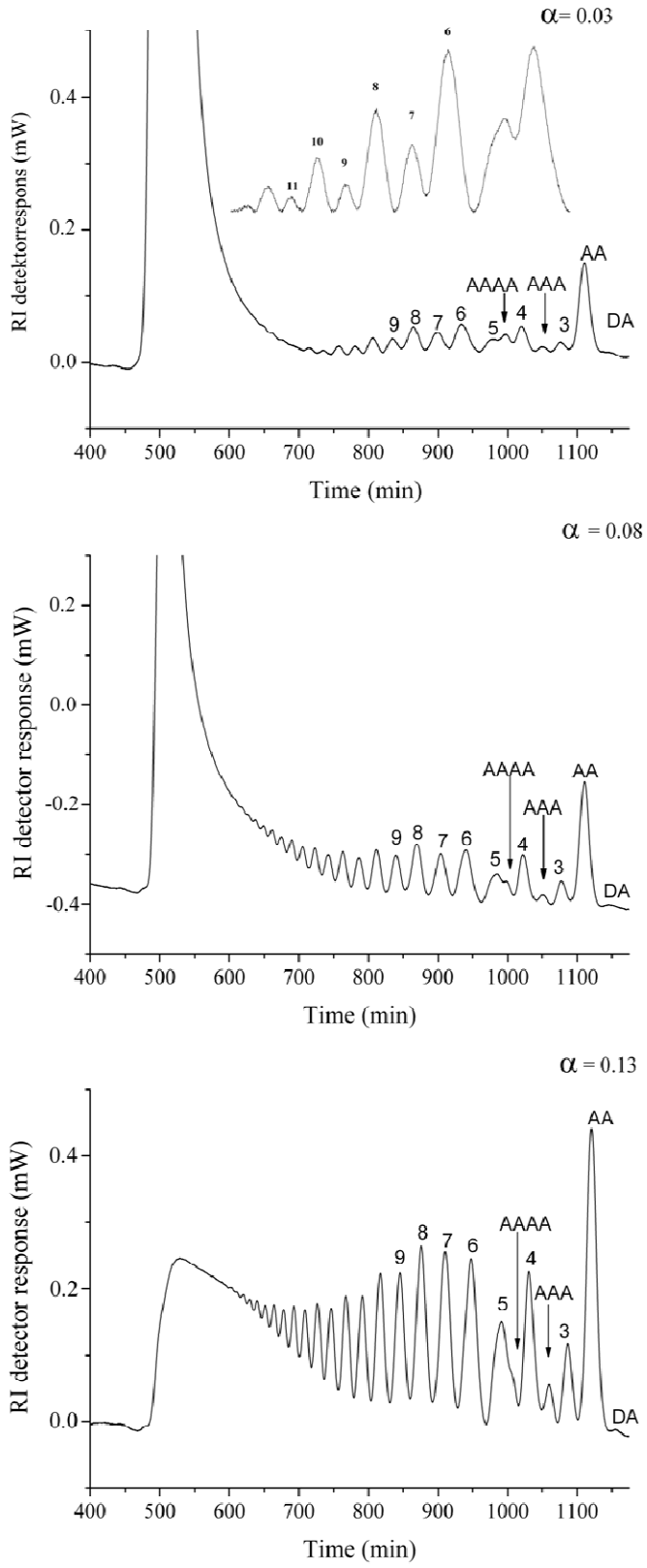

α= 0.03
RI detektorrespons (mW)
Time (min)
AA
AAAA
AAA
DA
α = 0.08
RI detector response (mW)
α = 0.13

Figure 4

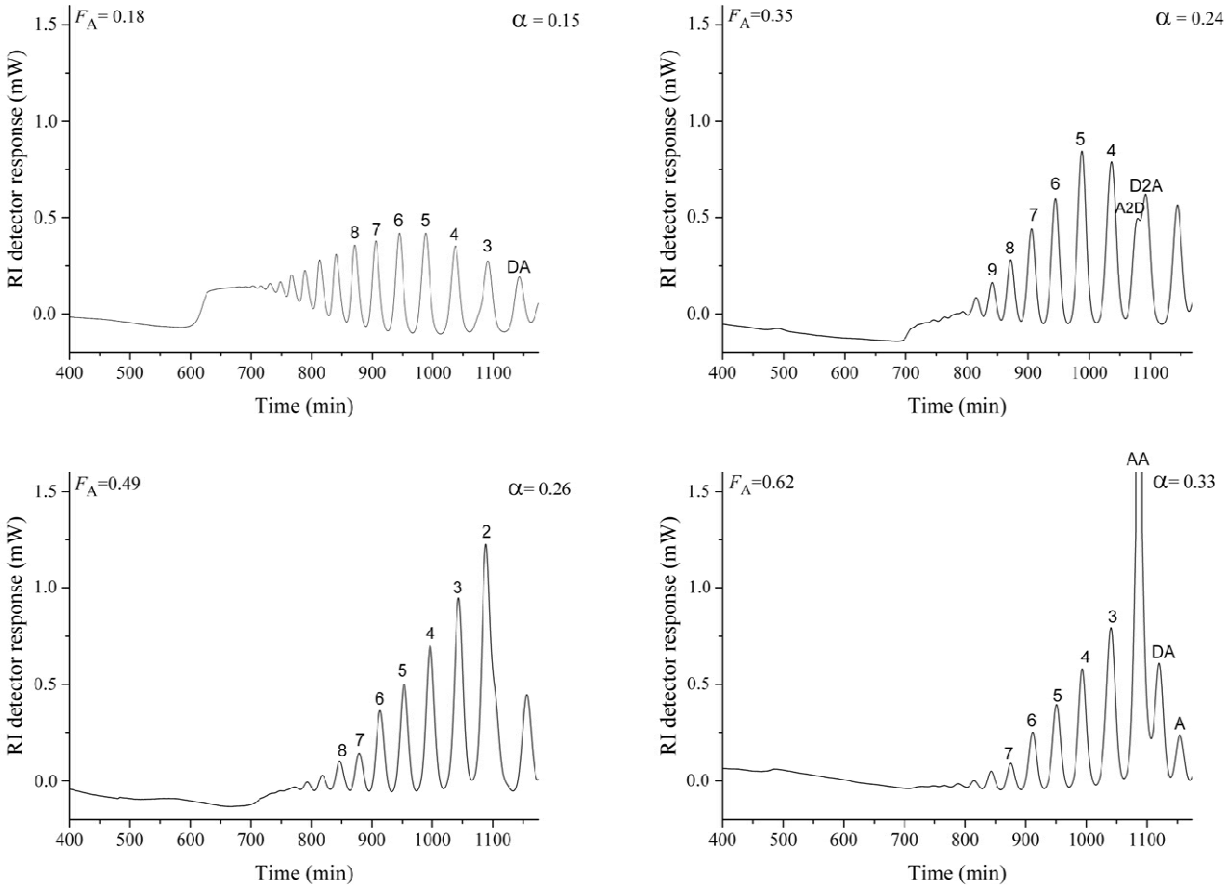

F_A= 0.18
α = 0.15
RI detector response (mW)
Time (min)
8 7 6 5 4 3 DA
F_A=0.35
α = 0.24
9 8 7 6 5 4 D2A A2D
F_A=0.49
α= 0.26
8 7 6 5 4 3 2
F_A=0.62
α= 0.33
AA
7 6 5 4 3 DA A

Figure 5

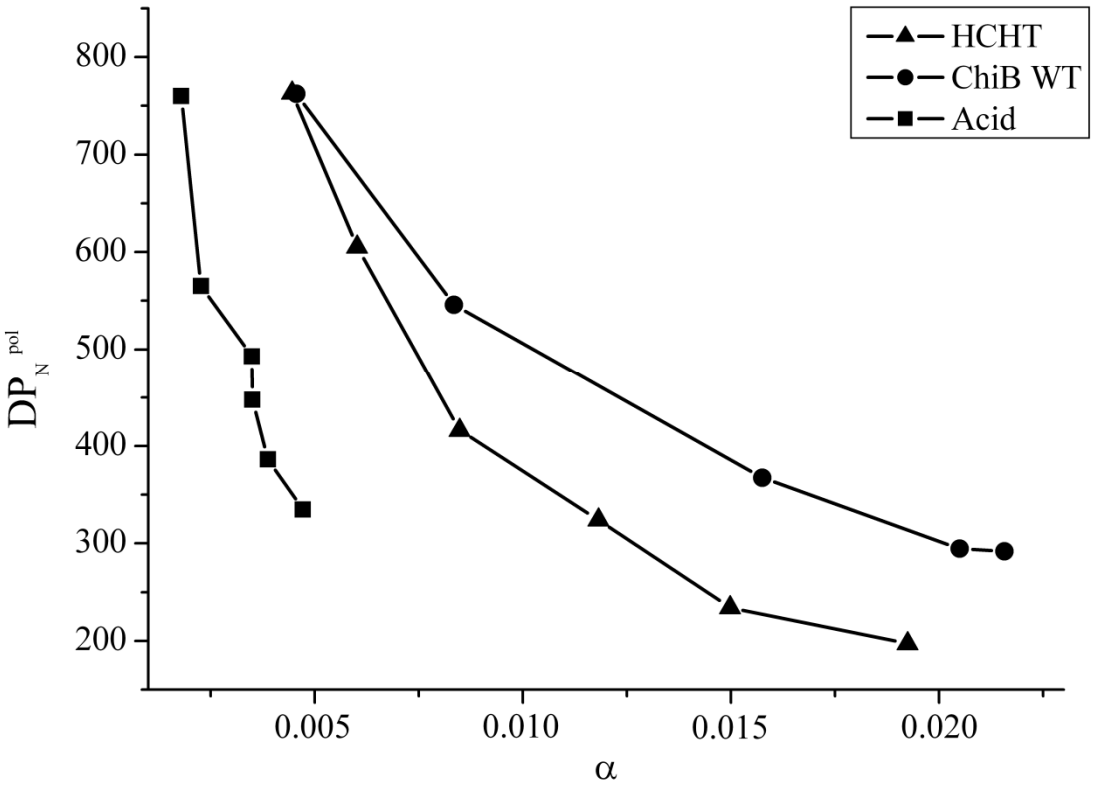
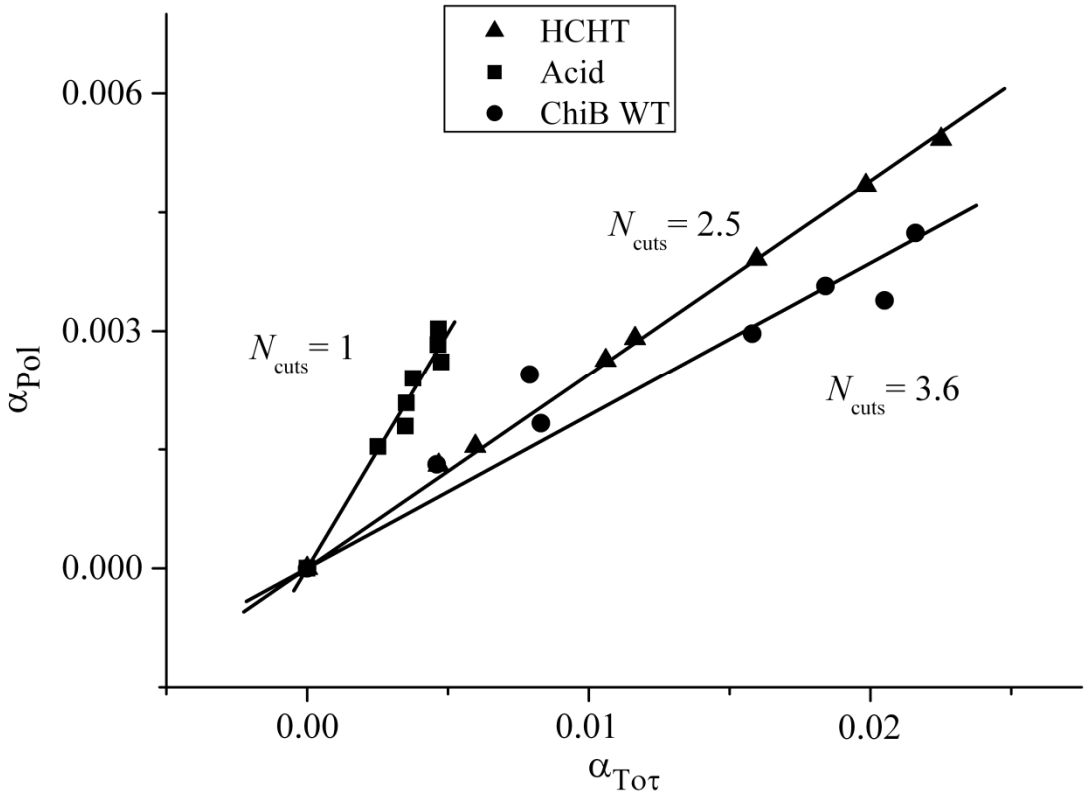

HCHT
ChiB WT
Acid
800
700
600
500
400
300
200
DP_N^{pol}
0.005
0.010
0.015
0.020
α

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}

REFERENCES

1. Herrera-Estrella, A., and Chet, I. (1999) Chitinases in biological control, *EXS 87*, 171-184.

2. Palli, S. R., and Retnakaran, A. (1999) Molecular and biochemical aspects of chitin synthesis inhibition, *EXS 87*, 85-98.

3. Shibata, Y., Foster, L. A., Metzger, W. J., and Myrvik, Q. N. (1997) Alveolar macrophage priming by intravenous administration of chitin particles, polymers of N-acetyl-D-glucosamine, in mice, *Infect.Immun. 65*, 1734-1741.

4. van Eijk, M., van Roomen, C. P. A. A., Renkema, G. H., Bussink, A. P., Andrews, L., Blommaart, E. F. C., Sugar, A., Verhoeven, A. J., Boot, R. G., and Aerts, J. M. F. G. (2005) Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity, *Int.Immun. 17*, 1505-1512.

5. Henrissat, B., and Davies, G. (1997) Structural and sequence-based classification of glycoside hydrolases, *Curr Opin Struct Biol 7*, 637-644.

6. Boot, R. G., Blommaart, E. F. C., Swart, E., Ghauharali-van der Vlugt, K., Bijl, N., Moe, C., Place, A., and Aerts, J. (2001) Identification of a novel acidic mammalian chitinase distinct from chitotriosidase, *J. Biol. Chem. 276*, 6770-6778.

7. Renkema, G. H., Boot, R. G., Muijsers, A. O., Donker-Koopman, W. E., and Aerts, J. M. F. G. (1995) Purification and Characterization of Human Chitotriosidase, a Novel Member of the Chitinase Family of Proteins, *J. Biol. Chem. 270*, 2198-2202.

8. Elias, J. A., Homer, R. J., Hamid, Q., and Lee, C. G. (2005) Chitinases and chitinase-like proteins in TH2 inflammation and asthma, *J.Allergy Clin.Immunol. 116*, 497-500.

9. van Eijk, M., Scheij, S. S., van Roomen, C., Speijer, D., Boot, R. G., and Aerts, J. (2007) TLR- and NOD2-dependent regulation of human phagocyte-specific chitotriosidase, *FEBS Lett. 581*, 5389-5395.

10. Musumeci, M., Bellin, M., Maltese, A., Aragona, P., Bucolo, C., and Musumeci, S. (2008)

Chitinase levels in the tears of subjects with ocular allergies, *Cornea 27*, 168-173.

11. Ramanathan, M., Lee, W. K., and Lane, A. P. (2006) Increased expression of acidic mammalian chitinase in chronic rhinosinusitis with nasal polyps, *Am. J. Rhinol. 20*, 330-335.

12. Zhu, Z., Zheng, T., Homer, R. J., Kim, Y. K., Chen, N. Y., Cohn, L., Hamid, Q., and Elias, J. A. (2004) Acidic Mammalian Chitinase in Asthmatic Th2 Inflammation and IL-13 Pathway Activation, *Science 304*, 1678-1682.

13. Hollak, C. E. M., Vanweely, S., Vanoers, M. H. J., and Aerts, J. (1994) Marked Elevation of Plasma Chitotriosidase Activity - a Novel Hallmark of Gaucher Disease, *J. Clin. Invest. 93*, 1288-1292.

14. Bargagli, E., Margollicci, M., Perrone, A., Luddi, A., Perari, M. G., Bianchi, N., Refini, R. M., Grosso, S., Volterrani, L., and Rottoli, P. (2007) Chitotriosidase analysis in bronchoalveolar lavage of patients with sarcoidosis, *Sarcoidosis Vasc. Diff. 24*, 59-64.

15. Brunner, J., Scholl-Burgi, S., and Zimmerhackl, L. B. (2007) Chitotriosidase as a marker of disease activity in sarcoidosis, *Rheumatol. Int. 27*, 1171-1172.

16. Artieda, M., Cenarro, A., Ganan, A., Lukic, A., Moreno, E., Puzo, J., Pocovi, M., and Civeira, F. (2007) Serum chitotriosidase activity, a marker of activated macrophages, predicts new cardiovascular events independently of C-reactive protein, *Cardiology 108*, 297-306.

17. Karadag, B., Kucur, M., Isman, F. K., Hacibekiroglu, M., and Vural, V. A. (2008) Serum chitotriosidase activity in patients with coronary artery disease, *Circ. J. 72*, 71-75.

18. Kucur, M., Isman, F. K., Balci, C., Onal, B., Hacibekiroglu, M., Ozkan, F., and Ozkan, A. (2008) Serum YKL-40 levels and chitotriosidase activity as potential biomarkers in primary prostate cancer and benign prostatic hyperplasia, *Urol. Oncol. 26*, 47-52.

19. Malaguarnera, L., Rosa, M. D., Zambito, A. M., dell'Ombra, N., Marco, R. D., and Malaguarnera, M. (2006) Potential role of chitotriosidase gene in nonalcoholic fatty liver disease evolution, *Am. J. Gastroenterol. 101*, 2060-2069.

20. Wajner, A., Michelin, K., Burin, M. G., Pires, R. F., Pereira, M. L. S., Giugliani, R., and Coelho, J. C. (2004) Biochemical characterization of chitotriosidase enzyme: comparison between normal individuals and patients with Graucher and with Niemann-Pick diseases, *Clin. Biochem. 37*, 893-897.

21. Gorzelanny, C., Poppelmann, B., Pappelbaum, K., Moerschbacher, B. M., and Schneider, S. W. (2010) Human macrophage activation triggered by chitotriosidase-mediated chitin and chitosan degradation, *Biomaterials 31*, 8556-8563.

22. Renkema, G. H., Boot, R. G., Strijland, A., DonkerKoopman, W. E., vandenBerg, M., Muijsers, A. O., and Aerts, J. (1997) Synthesis, sorting, and processing into distinct isoforms of human macrophage chitotriosidase, *Eur. J. Biochem. 244*, 279-285.

23. Fusetti, F., von Moeller, H., Houston, D., Rozeboom, H. J., Dijkstra, B. W., Boot, R. G., Aerts, J. M. F. G., and van Aalten, D. M. F. (2002) Structure of Human Chitotriosidase. Implications for Specific Inhibitor Design and Function of Mammalian Chitinase-Like Lectins, *J. Biol. Chem. 277*, 25537-25544.

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

25. van Aalten, D. M. F., Synstad, B., Brurberg, M. B., Hough, E., Riise, B. W., Eijsink, V. G. H., and Wierenga, R. K. (2000) Structure of a two-domain chitotriosidase from Serratia marcescens at 1.9-angstrom resolution, *Proc.Natl.Acad.Sci.U.S.A 97*, 5842-5847.

26. Synstad, B., Gåseidnes, S., van Aalten, D. M. F., Vriend, G., Nielsen, J. E., and Eijsink, V. G. H. (2004) Mutational and computational analysis of the role of conserved residues in the active site of a family 18 chitinase, *Eur.J.Biochem. 271*, 253-262.

27. Tews, I., Terwisscha van Scheltinga, A. C., Perrakis, A., Wilson, K. S., and Dijkstra, B. W. (1997) Substrate-assisted catalysis unifies two families of chitinolytic enzymes, *J. Am. Chem.*

Soc. 119, 7954-7959.

28. Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Isogai, A., Henrissat, B., and Dijkstra, B. W. (1995) Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and X-ray structure of a complex with allosamidin: evidence for substrate assisted catalysis, *Biochemistry 34*, 15619-15623.

29. Cederkvist, F. H., Parmer, M. P., Vårum, K. M., Eijsink, V. G. H., and Sørlie, M. (2008) Inhibition of a Family 18 Chitinase by Chitooligosaccharides, *Carbohyd.Polym. 74*, 41-49.

30. Aam, B. B., Heggset, E. B., Norberg, A. L., Sørlie, M., Vårum, K. M., and Eijsink, V. G. H. (2010) Production of Chitooligosaccharides and Their Potential Applications in Medicine, *Marine Drugs 8*, 1482-1517.

31. Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J. K., and Jones, T. A. (1990) Three-dimensional structure of cellobiohydrolase II from Trichoderma reesei, *Science 249*, 380-386.

32. Horn, S. J., Sikorski, P., Cederkvist, J. B., Vaaje-Kolstad, G., Sørlie, M., Synstad, B., Vriend, G., Vårum, K. M., and Eijsink, V. G. H. (2006) Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides, *Proc.Natl.Acad.Sci U.S.A 103*, 18089-18094.

33. Horn, S. J., Sørbotten, A., Synstad, B., Sikorski, P., Sørlie, M., Vårum, K. M., and Eijsink, V. G. H. (2006) Endo/exo mechanism and processivity of family 18 chitinases produced by Serratia marcescens, *FEBS J. 273*, 491-503.

34. Sikorski, P., Sørbotten, A., Horn, S. J., Eijsink, V. G. H., and Vårum, K. M. (2006) *Serratia marcescens* chitinases with tunnel-shaped substrate-binding grooves show endo activity and different degrees of processivity during enzymatic hydrolysis of chitosan, *Biochemistry 45*, 9566-9574.

35. Sørbotten, A., Horn, S. J., Eijsink, V. G. H., and Vårum, K. M. (2005) Degradation of chitosans with chitinase B from Serratia marcescens. Production of chito-oligosaccharides and insight into enzyme processivity, *FEBS J. 272*, 538-549.

36. Boot, R. G., Renkema, G. H., Strijland, A., van Zonneveld, A. J., and Aerts, J. M. F. G. (1995) Cloning of a cDNA Encoding Chitotriosidase, a Human Chitinase Produced by Macrophages, *J. Biol. Chem. 270*, 26252-26256.

37. Hackman, R. H. (1954) Studies on chitin. 1. Enzymic degradation of chitin and chitin esters, *Aust. J. Biol. Sci. 7*, 168-178.

38. Sannan, T., Kurita, K., and Iwakura, Y. (1976) Studies on chitin: 2. Effect of deacetylation on solubility, *Makromol. Chem. 177*, 3589-3600.

39. Brurberg, M. B., Eijsink, V. G. H., Haandrikman, A. J., Venema, G., and Nes, I. F. (1995) Chitinase B from Serratia marcescens BJL200 is exported to the periplasm without processing, *Microbiology 141*, 123-131.

40. Bahrke, S., Einarsson, J. M., Gislason, J., Haebel, S., Letzel, M. C., Peter-Katalinic, J., and Peter, M. G. (2002) Sequence analysis of chitooligosaccharides by matrix-assisted laser desorption ionization postsource decay mass spectrometry, *Biomacromolecules 3*, 696-704.

41. Vårum, K. M., Anthonsen, M. W., Grasdalen, H., and Smidsrød, O. (1991) High-field NMR spectroscopy of partially *N*-deacetylated chitins (chitosans). 1. Determination of the the degree of *N*-acetylation and the distribution of *N*-acetyl groups in partially *N*-deacetylated chitins (chitosans) by high-field NMR-spectroscopy., *Carbohyd. Res. 211*, 17-23.

42. Vårum, K. M., Holme, H. K., Izume, M., Stokke, B. T., and Smidsrød, O. (1996) Determination of enzymatic hydrolysis specificity of partially N-acetylated chitosans, *Biochim. Biophys. Acta-Gen. Subjects 1291*, 5-15.

43. Horn, S. J., and Eijsink, V. G. H. (2004) A reliable reducing end assay for chito-oligosaccharides, *Carbohyd.Polym. 56*, 35-39.

44. Heggset, E. B., Hoell, I. A., Kristoffersen, M., Eijsink, V. G., and Vårum, K. M. (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.

45. Synowiecki, J., and Al-Khateeb, N. A. (2003) Production, properties, and some new applications of chitin and its derivatives, *Crit.Rev.Food Sci. 43*, 145-171.

46. Davies, G., and Henrissat, B. (1995) Structures and mechanisms of glycosyl hydrolases, *Structure 3*, 853-859.

47. Hult, E. L., Katouno, F., Uchiyama, T., Watanabe, T., and Sugiyama, J. (2005) Molecular directionality in crystalline beta-chitin: hydrolysis by chitinases A and B from Serratia marcescens 2170, *Biochem. J. 388*, 851-856.

48. Zakariassen, H., Eijsink, V. G. H., and Sørlie, M. (2010) Signatures of activation parameters reveal substrate-dependent rate determining steps in polysaccharide turnover by a family 18 chitinase, *Carbohyd.Polym. 81*, 14-20.

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

50. Eijsink, V. G. H., Vaaje-Kolstad, G., Vårum, K. M., and Horn, S. J. (2008) Towards new enzymes for biofuels: lessons from chitinase research, *Trends Biotechnol. 26*, 228-235.

51. Zakariassen, H., Hansen, M. C., Jøranli, M., Eijsink, V. G. H., and Sørlie, M. (2011) Mutational effects on transglycosylating activity of family 18 chitinases and construction of a hypertransglycosylating mutant, *Biochemistry 50*, 5693-5703.

52. Team, M. G. C. P. (2002) Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences, *Proc. Natl. Acad. Sci. U S A 99*, 16899-16903.

53. Krokeide, I. M., Synstad, B., Gåseidnes, S., Horn, S. J., Eijsink, V. G. H., and Sørlie, M. (2007) Natural substrate assay for chitinases using high-performance liquid chromatography: A comparison with existing assays, *Anal. Biochem. 363*, 128-134.

54. Brurberg, M. B., Nes, I. F., and Eijsink, V. G. H. (1996) Comparative studies of chitinases A and

B from Serratia marcescens, *Microbiology 142*, 1581-1589.

55. Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B., Chet, I., Wilson, K. S., and Vorgias, C. E. (1994) Crystal structure of a bacterial chitinase at 2.3 A resolution, *Structure. 2*, 1169-1180.

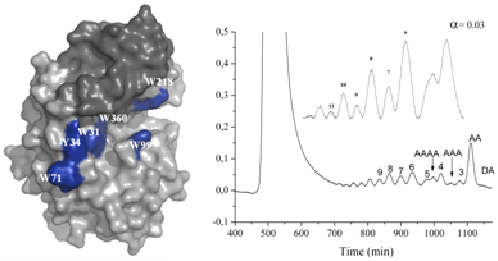
56. Uchiyama, T., Katouno, F., Nikaidou, N., Nonaka, T., Sugiyama, J., and Watanabe, T. (2001) Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase a from Serratia marcescens 2170, *J. Biol. Chem. 276*, 41343-41349.

57. Berth, G., and Dautzenberg, H. (2002) The degree of acetylation of chitosans and its effect on the chain conformation in aqueous solution, *Carbohydr. Polym. 47*, 39-51.

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



"For Table of Contents Use Only"