A study of human chitinases with respect to the importance of surface exposed aromatic residues and carbohydrate-binding modules in substrate degradation and role in inflammations

En studie av humane kitinaser med hensyn på overflate-eksponerte aromatiske residuer og karbohydrat-bindende moduler ved nedbrytning av substrat og rolle i inflammasjon

Philosophiae Doctor (PhD) Thesis

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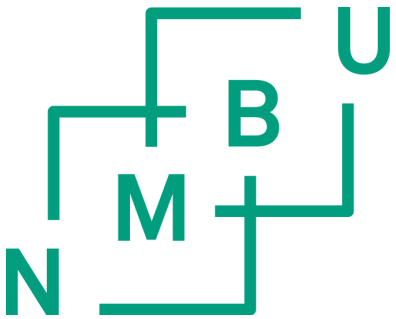

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Exercise and science share some common features.

To enjoy them, you must be able to appreciate the feelings of struggle and pain, and long for that great feeling of adrenaline rushing through your blood when you are almost at the finish line.

I do!

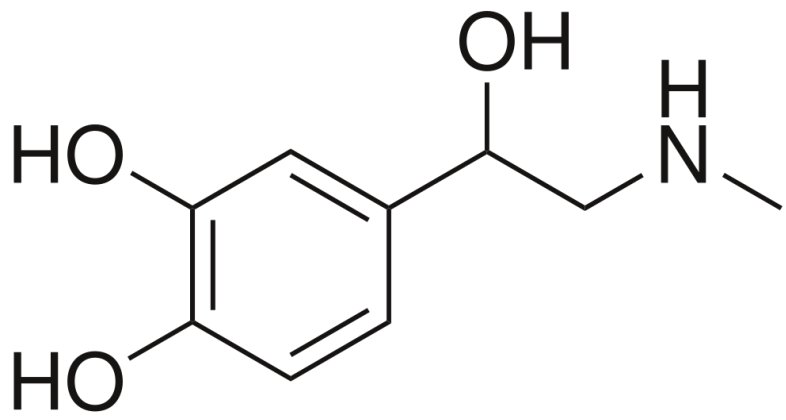

Figure X – Adrenaline, (R)-(-)-L-Epinephrine, $C_9H_{13}NO_3$

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Linn W. Stockinger

Ås, 19th June 2015

LIST OF PUBLICATIONS

This thesis is based on studies presented in the following appended papers:

Paper I: The Effect of the Carbohydrate Binding Module on Substrate Degradation by the Human Chitotriosidase

Linn Wilhelmsen Stockinger, Kristine B Eide, Anette I Dybvik, Håvard Sletta, Kjell Morten Vårum, Vincent G Eijsink, Anne Tøndervik, Morten Sørlie, *Re-submitted to Biochimica et Biophysica Acta (BBA)* 2015

Paper II: The Importance of Aromatic Residues in the Active Site in Human Chitotriosidase for Substrate Degradation

Kristine B Eide*, Linn Wilhelmsen Stockinger*, Anna Sofia Lewin, Vincent G Eijsink, Anne Tøndervik, Morten Sørlie, *Manuscript*

*The authors contributed equally to this work

Paper III: Screening of Human Chitinases and Chitinase-like Proteins in Inflammatory Disease

Linn Wilhelmsen Stockinger, Lene Therese Olsen Hult, Morten Sørlie, Tor Erling Lea, Charlotte Kleiveland, *Manuscript, ready to be submitted*

ABSTRACT

Chitin is an insoluble, linear polymer consisting of β-1, 4-linked *N*-acetyl-glucosamine units tightly packed in a crystalline structure. It is the second most abundant polysaccharide in nature, after cellulose, with an estimated annual production of about 10^{11} tons. Chitin is an essential structural component in the exoskeleton of crustaceans, arthropods, and insects, and is also found in the cell walls of certain fungi, algae, and parasitic nematodes. Enzymatic degradation of recalcitrant polysaccharides in biomass is of great biological importance. In nature, the degradation of chitin is catalyzed by chitinases, which are assigned to the glycoside hydrolase (GH) family 18 in the CAZY database (www.cazy.org). Humans have two active chitinases that are considered elements of the immune system because they degrade chitin-containing pathogens as a part of the host defense mechanism. The aim of the work presented in this thesis was to study the enzymatic mechanisms of one of the human chitinases, namely the human chitotriosidase (HCHT), to gain mechanistic insight into substrate degradation. A second goal was to study the expression of mammalian chitinases and chitinase-like proteins (CLP) in response to specific inflammatory stimuli to increase knowledge about the enzymes' roles in the immune system.

HCHT exists in two isoforms, one two-domain protein of 50 kDa (HCHT50) and one single-domain protein of 39 kDa (HCHT39). Common to both isoforms is a catalytic domain, characteristic of family 18 GHs and often referred to as a $(\beta/\alpha)_8$ TIM barrel, with a path of surface-exposed aromatic residues in the active site cleft. In addition, the 50 kDa isoform has a hinge region of 29 amino acids and a C-terminal carbohydrate-binding module (CBM). This particular CBM, consisting of 49 amino acids, is assigned to the CBM family 14 inthe CAZy database. Papers I and II describe the importance of the CBM and select surface-exposed aromatic residues in the active site of HCHT for substrate degradation. As expected, the CBM of HCHT makes enzyme degradation of insoluble chitin faster and much more efficient than HCHT without the CBM. Moreover, even though HCHT50 has a low degree of processivity, it is highly efficient at degrading insoluble chitin. Normally, processive ability correlates positively with substrate degradation efficiency for family 18 GHs. Mutations of tryptophans to alanines in the active site of either side of the catalytic acid (subsites -3, Trp^{31}, and +2, Trp^{218}) makes the enzyme less efficient with a concomitant decrease in initial hydrolysis rate. The largest effects are

observed when Trp^{31} in subsite -3 is mutated. Removal of the CBM causes larger effects than removing an aromatic residue.

Paper III investigated the mRNA and protein expression profile of mammalian chitinases and CLPs in a mouse model of acute intestinal inflammation. Chi3l1 and Chi3l3 mRNA and protein were up-regulated in the mouse colon after DSS-induced colitis. Immunohistochemistry analysis showed that increased Chi3l3 expression was mainly localized in infiltrating neutrophils and macrophages, while Chi3l1 was expressed by infiltrating neutrophils and, to some extent, epithelial cells of the colon.

SAMMENDRAG

Kitin er en uløselig, lineær polymer bestående av β-1, 4-linket *N*-acetyl-glykosamin enheter tett pakket i en krystalinsk struktur. Etter cellulose er kitin det polysakkaridet i naturen det er størst forekomst av. Kitin er en viktig strukturell komponent i skalldyr, insekter og sopp, og er også tilstede i celleveggen til enkelte sopper, alger og parasitter. Til tross for de enorme mengdene kitin som produseres årlig akkumulerer ikke kitin i naturen. Dette skyldes en mengde proteiner som effektivt er med på enzymatisk nedbrytning av kitin, kjent som kitinaser. Humane kitinaser er også involvert i immunsystemet og i nedbrytning av kitin-holdige patogener. Formålet med dette prosjektet har vært å studere de enzymatiske mekanismene til en av de humane kitinasene, human kitotriosidase (HCHT). Dette for å tilegne kunnskap om mekanismene bak nedbrytning av polysakkarider. I tillegg ble rollen til mammalske kitinaser studert i en spesifikk inflammasjon for å øke kunnskapen om disse enzymene i immunsystemet.

Artikkel I og II beskriver i detalj hvordan HCHT bryter ned løselige og uløselige polysakkarider og hvor avhengig enzymet er av et karbohydrat-bindende modul (CBM) for å kunne effektivt bryte ned polysakkarider. Denne modulen bidrar til at enzymet kan bryte ned kitin både raskere og mer effektivt enn om domenet er fjernet fra enzymet. Dette studiet har i tillegg oppdaget at begge isoformene til HCHT har tilnærmet lik, lav grad av prosessivitet. Flere mutasjoner i konserverte, aromatiske residuer i det aktive setet til HCHT er gjort for å studere effekten disse residuene har på effektiviteten og hastigheten enzymet har på nedbrytning av både løslige og uløslige polysakkarider. Dette viste at en mutasjon i subsete -3 bidrar til at enzymet blir mindre effektivt og gir lavere hastighet sammenliknet med villtypen. Når både CBM og det aromatiske residuet i -3 subsetet er fjernet vil enzymet mer eller mindre miste sin evne til å bryte ned både løslige og uløslige polysakkarider. Dette viser viktigheten av CBM, og at CBM er viktigere enn aromatiske residuer hva gjelder enzymatisk effektivitet. I Artikkel III ble ekspresjonsprofilen for mammalske kitinaser og kitinase-liknende proteiner i en musemodell for akutt intestinal inflammasjon undersøkt. mRNA og protein fra Chi3l1 og Chi3l3 ble funnet oppregulert i kolon fra mus etter DSS-indusert kolitt. Med immunohistokjemi ble det vist at økt Chi3l3 uttrykk hovedsakelig var lokalisert i infiltrerende nøytrofiler og makrofager, mens Chi3l1 kun var uttrykt i infiltrerende nøytrofiler og til en viss grad i epitelceller i kolon. I tillegg ble

det vist at ved bruk av Bioprotein ble graden av inflammasjon forminsket og nivåene av infiltrerende mononucleære celler redusert. Samtidig fant vi at mRNA og protein ekspresjonen av både Chi3l1 og Chi3l3 ble redusert. Dette viser at uttrykket av Chi3l1 og Chi3l3 endrer seg i takt med utviklingen av inflammasjon i tarm og at de derfor kan virke som gode markører for inflammasjon i tarm.

ABBREVIATIONS

α_{pol}	Alpha Polymeric
α_{tot}	Alpha total cuts
AMCase	Acidic Mammalian Chitinase
CAZy	Carbohydrate Active EnZYmes
CBP21	Carbohydrate-Binding Protein 21
CBM	Carbohydrate Binding Module
CEC	Colonic Epithelial Cells
CHOS	Chito-oligosaccharides
ChiA	Chitinase A
ChiB	Chitinase B
ChiC	Chitinase C
Chi3l1	Chitinase-3-like-1
Chi3l3	Chitinase-3-like-3
CLP	Chitinase-like-protein
DP	Degree of Polymerization
F_A	Degree of Acetylation
Fizz1	Found in Inflamatory Zone protein 1
GH	Glycoside Hydrolases
GlcNAc	*N*-acetyl glucosamine
HCHT	Human Chitotriosidase
HCHT50	Human Chitotriosidase with CBM
HCHT39	Human Chitotriosidase without CBM
IFN-γ	Interferon gamma

IL-4	Interleukin-4
IL-13	Interleukin-13
k_{cat}^{app}	Apparent K_{cat}
LP	Lamina Propria
NMR	Nuclear Magnetic Resonance
P^{app}	Apparent processivity
SI-CLP	Stabelin-1-Interacting Chitinase-Like-Protein
TGFβ	Transforming Growth Factor Beta
TNFα	Tumor Necrosis Factor Alpha
UC	Ulcerous Colitis
Å	Ångstrøm

INTRODUCTION

Carbohydrates exist in numerous forms and account for about two-third of the carbon in the biosphere, with cellulose being the most abundant biopolymer. They function as structural components and energy sources, but are also crucial molecules for life, participating in signaling and cellular communication (Yip and Withers, 2004, Sinnott, 1990). Carbohydrates are among the most ideal media for information transfer. Their abilities to encode information and act as signaling devices are direct consequences of their structural diversity. Due to the vast number of biological functions of oligosaccharides, glycoproteins and glycolipids, there are potential applications in biochemistry, medicine and biotechnology for molecules that interfere with their processing (Yip and Withers, 2004). Large amounts of carbohydrates are produced per annum, and despite the high stability of the glycosidic linkage, they do not accumulate in the biosphere (Henrissat, 1991). This is why we find it interesting to study how enzymes catalyze the hydrolysis of glycosidic linkages. To understand this, it is important to study the interaction between the carbohydrate substrate and the enzyme. This thesis describes investigations of mammalian-related glycosyl hydrolases using both biochemical methods and *in vitro/in vivo* models.

Chitin and chitosan

Chitin (Fig. 1) is the second most abundant polysaccharide in nature, after cellulose (Buck and Obaidah, 1971). Cellulose and chitin are structurally similar to each other, and about 10^{11} tons are produced each year. Chitin is an essential structural component in the exoskeleton of crustaceans, arthropods, and insects, and is also found in the cell walls of certain fungi, algae, as well as in parasitic nematodes (Palli and Retnakaran, 1999). Enzymatic degradation of recalcitrant polysaccharides in biomass, such as cellulose and chitin is of great biological and economical importance.

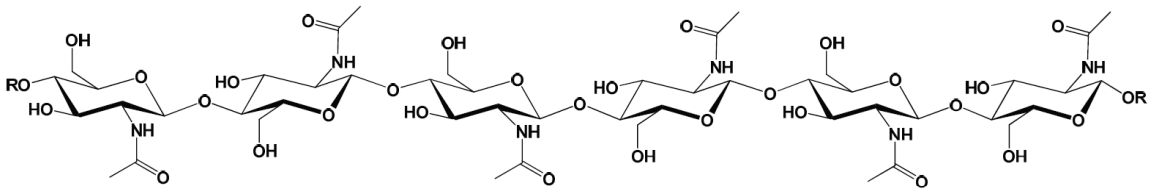

Figure 1 - The structure of chitin comprising β -1,4-linked N-acetyl-glucosamine units rotated 180° to each other

In nature, chitin occurs mainly in two different crystalline forms, α and β (Rudall and Kenching.W, 1973) (Rinaudo, 2006, Aam et al., 2010a). The dominant form, α-chitin, is composed of linear chains of GlcNAc arranged in an antiparallel manner. The less dominant form, β-chitin, consists of parallel GlcNAc chains. In addition, there is a γ-form in which two parallel strands alternate with single antiparallel strands. Chitin polymers are tightly packed with the two main forms of crystallization. In both crystalline forms, the polymeric strands are tightly held together by hydrogen bonds, mainly the strong C-O***HN bond (Merzendorfer and Zimoch, 2003). Chitin's crystalline structure makes it insoluble in water, which causes difficulties studying its degradation (Eijsink et al., 2008).(Eijsink et al., 2008). Deacetylation of chitin generates the analogue chitosan (Fig. 2). When the degree of deacetylation reaches about 70%, chitosan becomes soluble in aqueous acidic media. Solubilization occurs when the acetyl group in the C-2 position of the repeating GlcNAc unit is removed. This leaves a free NH_2-group that can act as a proton acceptor in acidic media to form a positive charge on the polymer. Characterization of chitosan describes the degree of acetylation (F_A), the pattern of acetylation (P_A), degree of polymerization (DP_n), and molecular weight distribution (Kurita, 2006, Rinaudo, 2006). Chitin and chitosan exhibit interesting biological and physiochemical characteristics, including antibacterial and antifungal activities, and therapeutic properties (Rinaudo, 2006, Kurita, 2006). They are non-toxic and biodegradable, which makes both chitin and chitosan desirable components for use in medicine (Kawada et al., 2007, Rhoades et al., 2006).

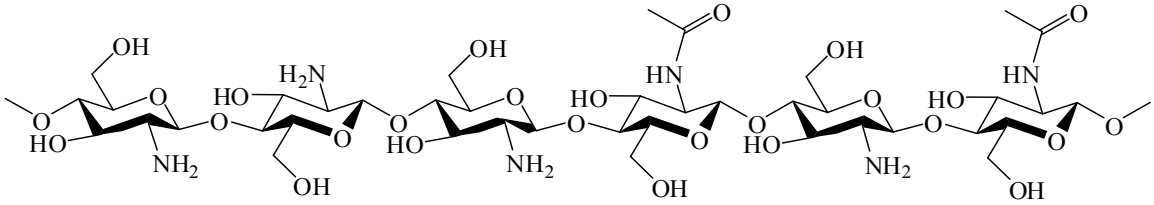

Figure 2 - The structure of chitosan comprising randomly distributed β -1,4 linked N-acetyl-glucosamine and glucosamine units rotated 180° to each other

Further hydrolysis of chitosan generates chitooligosaccharides (CHOS) of random sequence and size consisting of GlcNAc and D-glucosamine (GlcN; D) (Aam et al., 2010b). There is no chitin in humans, but human chitinases, the enzymes that catalyze the hydrolysis of glycosidic linkages in chitin, are thought to play roles in antiparasite responses of the innate immune system (van Eijk et al., 2007, Elias et al., 2005).

Enzymes that catalyze hydrolysis of glycosidic linkages – Glycoside hydrolases

Glycoside hydrolases (GHs) are enzymes that catalyze the hydrolysis of the glycosidic linkage in glycosides, leading to the formation of a sugar hemiacetal or hemiketal and the corresponding free aglycon. GHs are also referred to as glycosidases, and sometimes also as glycosyl hydrolases. To easily identify the correct subsite where binding and cleavage of the sugar takes place, the subsites are labeled from $-n$ to $+n$, with $-n$ at the non-reducing end, and $+n$ at the reducing end (Davies et al., 1997). Cleavage of the glycosidic bond takes place between subsite -1 and +1 (Davies et al., 1997). Glycoside hydrolases can catalyze the hydrolysis of O-, N-, and S-linked glycosides. Enzymatic hydrolysis of the glycosidic bond takes place via general acid catalysis that requires two critical residues: a proton donor and a nucleophile/base. This hydrolysis occurs via two major mechanisms giving rise to either an overall retention, or an inversion of the anomeric configuration (Davies and Henrissat, 1995). Both mechanisms involve oxacarbenium-ion-like transition states and a pair of carboxylic acids at the active site. When inverting (Fig. 3a) GHs, these two residues are located approximately 10 Å (+/-2 Å) apart on average (McCarter and Withers, 1994) and the reaction occurs via a single-displacement mechanism wherein one carboxylic acid acts as a general base and the other as a general acid. In between the base and the anomeric carbon, a nucleophilic water molecule is positioned and activated by the base (Henrissat and Davies, 1997). The inverting glycosidases hydrolyse the glycosidic linkage via a direct replacement of the sugar moiety in the positive subsites (Davies et al., 1997), leading to a change in anomeric configuration. When retaining enzymes (Fig. 3b), the two carboxylic

acid residues are approximately 5.5 Å apart (McCarter and Withers, 1994) and the reaction proceeds via a double-displacement mechanism (Rye and Withers, 2000). The catalyst acts first as an acid, donating a proton to the glycosyl oxygen. Concomitantly the nucleophile forms a covalent intermediate, and in the next step the deprotonated acid/base acts as a general base, receiving a proton from a water molecule. The activated water molecule then carries out a nucleophilic attack on the nucleophilic intermediate created in the first step. This results in retention of the stereochemistry at the anomeric center (Rye and Withers, 2000) (Davies and Henrissat, 1995). For a number of enzymes, binding to the substrate also depends on interaction with subsites distant from where the glycosidic bond is actually cleaved.

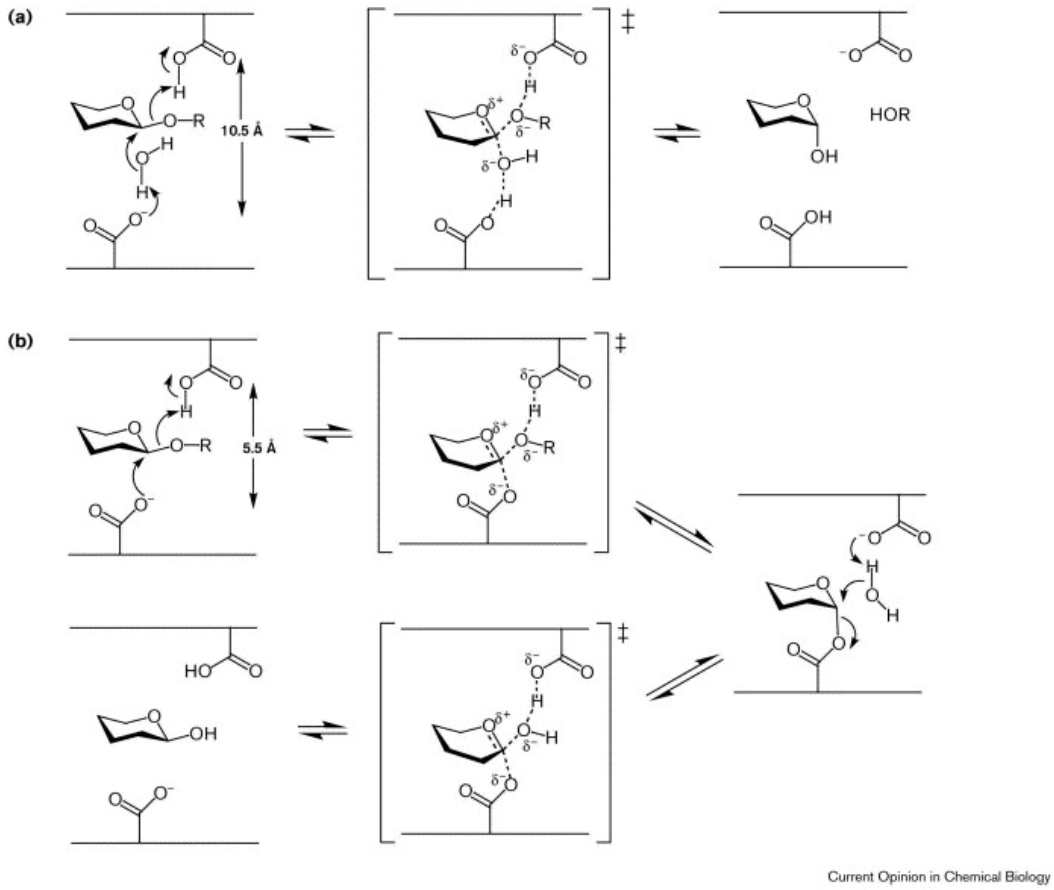

Figure 3 - General glycosidase mechanism for inverting (a) and retaining (b) glycosyl hydrolases, resulting in newly formed reducing ends possessing α - and β -anomeric configurations, respectively.

Glycosyl-hydrolases are divided into different classes. The IUBMB Enzyme nomenclature of GHs is based on their substrate specificity, and occasionally on their molecular mechanism. (http://www.cazy.org/Glycoside-Hydrolases.html). According to this system, each enzyme is given an Enzyme Commission (EC) number that is based on substrate

specificity and the type of reaction catalyzed. The EC-number of GHs is 3.2.1.x, where the first three digits indicate enzymes hydrolyzing *O*-glycosyl linkages and the x indicates the substrates and molecular mechanisms (IUBMB 1992). Such a classification does not (and was not intended to) reflect the structural features of these enzymes. Motivated by the desire for a more informative classification system, Bernard Henrissat and co-workers developed a new classification system based on amino acid sequence similarities (Henrissat, 1991). Today, GHs, polysaccharide lyases, carbohydrate esterases, carbohydrate binding modules (CBMs), and a group of redox enzymes named auxiliary activities (AAs) are also classified according to the same classification system, named Carbohydrate Active EnZYmes (CAZY) database (Lombard et al., 2014). Updated information about these families is available on the CAZY website (http://www.cazy.org). (The 3D structural level may be more conserved than the amino acid sequences, and therefore high sequence similarity within a family indicates similar folding). GHs from different families fold differently, and the overall topology of the active site can be divided into three general classes: (i) pockets, (ii) clefts, and (iii) tunnels (Henrissat and Davies, 1997). GHs with pockets recognize saccharides with several available ends, both reducing and non-reducing. The depth and shape of the pocket reflects the number of subsites that contribute to the binding of initial substrate and to the length of the leaving group (Davies et al., 1997). GHs with clefts have open structures, allowing binding at random sites of substrate polymers, and tunnel topology allows polysaccharide chains to be threaded through the active sites, giving the enzyme increased ability to catalyze numerous hydrolytic events without releasing the substrate. Clefts are mostly present in endo-acting enzymes, while pocket topology is found in exo-acting enzymes. This also forms the basis for processivity, a key factor for efficient enzymatic degradation of insoluble substrates. In addition, the enzymes can be classified on the basis of mode of action. The terms *"exo"* and *"endo"* relate to the enzyme's action on a polysaccharide, whether it attacks one of the termini of a polysaccharide or somewhere within the polymer chain, respectively. The catalytic residues of these enzymes are normally found in one of the three abovementioned locations. *Exo*-acting enzymes are specific to polysaccharide chain ends that tend to have their active site located within a pocket (Sulzenbacher et al., 1997, Sulzenbacher et al., 1996).

Importance of the carbohydrate-binding modules (CBM)

The carbohydrate-binding modules (CBM) of GHs play pivotal roles in enhancing hydrolysis activity. CBMs have two general functions for their associated catalytic modules:

i) a proximity effect, and ii) a targeting function (Boraston et al., 2004). In addition, researchers suggest that CBMs can have a disruptive function as well (Din et al., 1994). The presence of CBMs has been shown to increase the concentration of protein on the surface of the substrate, and removal of the CBM from a cellulase decreases its activity dramatically (Boraston et al., 2004). CBMs focus enzymes on to polysaccharide substrates through their sugar binding activity. Proteolytic excision or genetic truncation of CBMs from the catalytic modules results in significant decreases in enzymatic activity on insoluble, but not soluble, polysaccharides (Bolam et al., 1998). CBMs are connected to catalytic modules in the enzymes through linker sequences that are sometimes highly flexible (Tomme et al., 1988, Herve et al., 2010). CBMs and lectins share structural similarities, and bind to their target ligands through similar mechanisms. CBMs are generally found in enzymes that degrade complex carbohydrates primarily to provide nutrients, and it is this significant distinction in functionality that merits their separation into different protein groups. CBMs bring the enzyme into close proximity with the target substrate, thereby increasing the rate of catalysis (Bolam et al., 1998). CBMs are grouped into amino acid sequence-based families in the continuously updated CAZy database (Cantarel et al., 2009). The nomenclature for CBMs is adopted from that of glycoside hydrolases (Henrissat et al., 1998) in the literature. CBMs are named according to their family, but a name may also include the organism and even the enzyme from which it is derived (Boraston et al., 2004). The catalytic modules of glycoside hydrolases are classified into 96 different families based on amino acid sequence similarity. These families are grouped into 14 clans/superfamilies using the following criteria: conservation of the protein fold, catalytic machinery, and mechanism of glycosidic bond cleavage. Although fold similarities between CBMs have been demonstrated, there is no formal super-grouping of the 39 CBM families. Boraston *et al* (Boraston et al., 2004) have manually classified the structures into seven fold families, where CBMs identified as chitin-binding proteins belong to the hevein-fold family. Such groupings are not predictive CBM function. Another useful classification of CBMs is based on structural and functional similarities, where the modules are grouped into three types: surface-binding (Type A), glycan-binding (Type B), and small-sugar-binding (Type C) CBMs (Boraston et al., 2004). The CBM for mammalian chitinases belongs to the CBM14 family, according to the CAZy database (www.cazy.org, (Lombard et al., 2014, Boraston et al., 2004)). Not much is known about this particular CBM, but we do know that it interacts both with chitin, i.e. fungal cell walls, as well as chito-oligosaccharides (van Munster et al., 2013, van den Burg et al.,

2006). A typical feature for this CBM is the presence of 6 conserved cysteine residues that can form three disulphide bonds (Tjoelker et al., 2000). It also appears that Cys, Pro, and Gly residues significantly influence the structure of this CBM that is well conserved in HCHT50 and in tachycitin. Tachycitin is an invertebrate chitin-binding protein that plays a role in the innate immune defense against bacterial and fungal infections in invertebrates (Suetake et al., 2000).

Chitinolytic enzymes

In nature, chitin degradation is catalyzed by chitinases. Depending on their amino acid sequence, chitinases are divided into two of the GH families: family 18 and family 19. (Henrissat and Davies, 1997). Family 19 chitinases are found mostly in higher plants, and are thought to play a part in defense mechanisms against fungal pathogens (Akagi et al., 2006). Family 18 chitinases (GH18) are much more widespread across species and are found within several organisms including bacteria, fungi, nematodes, insects, and mammals. The function of GH18 chitinases differs between organisms, as bacteria hydrolyze chitin as an energy source, whereas in humans chitinases are part of the immune system. They are also involved in degradation of chitin-containing pathogens as a part of the host defense mechanism (Elias et al., 2005). The catalytic domain of GH18 chitinases consist of a TIM-barrel fold composing of eight β/α motifs, with the highly conserved DXXDXDXE sequence motif located in β-strand 4 and the catalytic acid located at the end of the barrel. These catalytic residues help to catalyze various chitins efficiently. Family 18 chitinases use a substrate-assisted reaction mechanism (van Aalten et al., 2001). In the enzyme-substrate complex, the acetoamido group of the -1 sugar lies away from the aldohexose ring and assumes an energetically favorable conformation. Upon binding, the enzyme imposes on the -1 sugar, which leads to conformational change. The stable "chair" conformation $^{4}C_{1}$ becomes the "boat" $^{1,4}B$. This conformational change participates in the bending and rotating of the bound oligosaccharide.

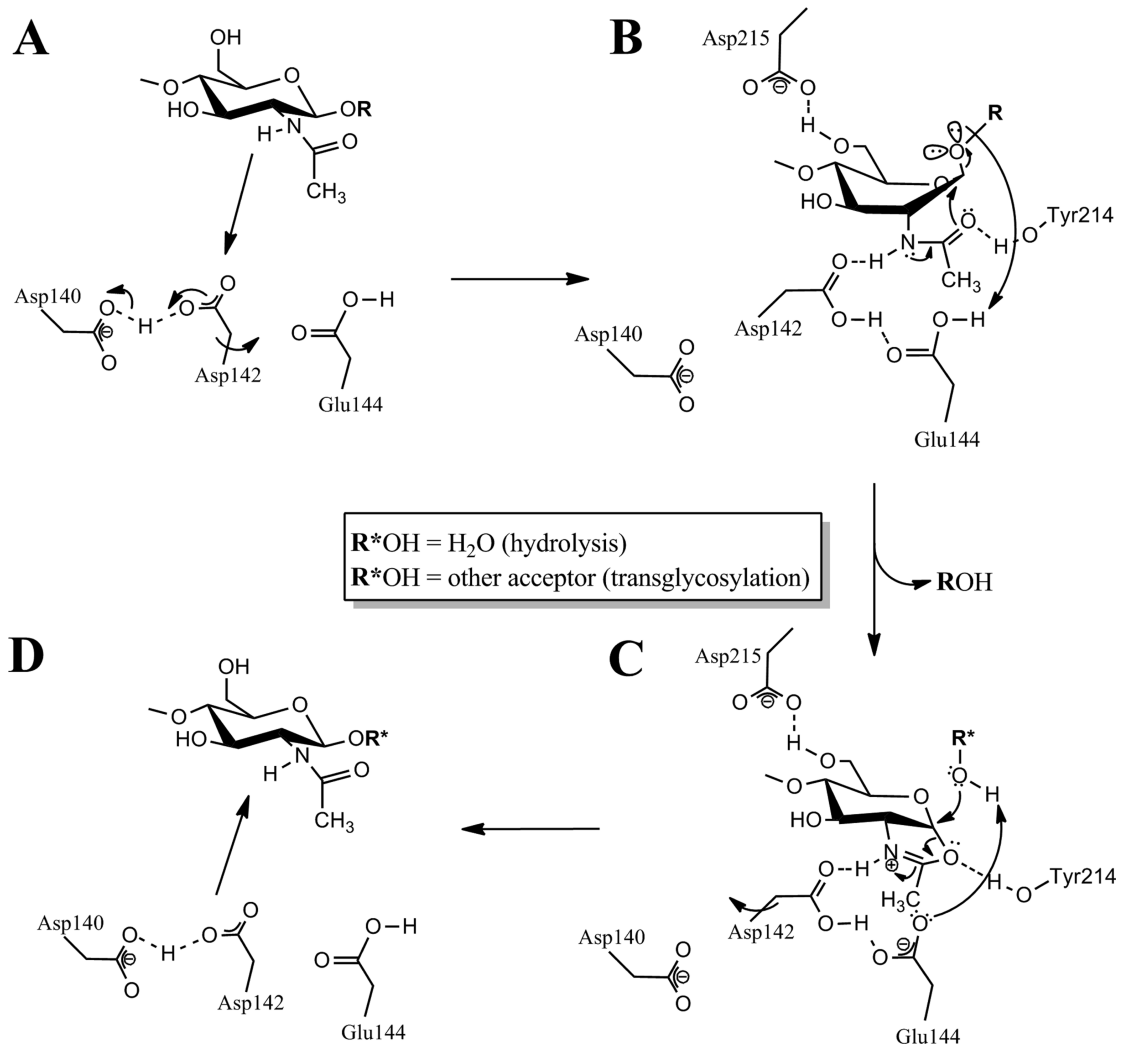

Figure 4 - Proposed catalytic mechanism. Asp-140, Asp-142, and Glu-144, conserved in most family 18 chitinases, are shown during separate stages of catalysis. A three-dimensional view of the changing interactions in the crystal structures described here is shown in Fig. 2. (*A*) Resting enzyme. Asp-142 is too far away to interact with Glu-144. (*B*) Binding of substrate (only −1 binding NAG residue is shown) causes distortion of the pyranose ring into a boat or skewed boat conformation (see also Fig. 2) and rotation of Asp-142 toward Glu-144, enabling hydrogen bond interactions between the hydrogen of the acetamido group, Asp-142, and Glu-144. (*C*) Hydrolysis of the oxazolinium ion intermediate leads to protonation of Glu-144 and rotation of Asp-142 to its original position where it shares a proton with Asp-140 (van Aalten et al., 2001).

In addition to the catalytic domain, GH18 chitinases often contain the carbohydrate-binding module (CBM). An S/T-rich linker usually connects the catalytic domain to the CBM. The highly glycosylated linker could protect chitinase from proteolysis (Arakane et al., 2003) (Huang et al., 2012, Tews et al., 1997). The non-catalytic domains (i.e. CBMs) may be involved in recognition of the substrate and binding, while the catalytic domain consist of the active site, where the hydrolysis occurs. The signal peptide is normally the indicator of a secretory protein and demonstrates that GH18 proteins have a signal peptide to guide them

out of cells. The GH18 family can further be divided into enzymatically active chitinases and enzymatically inactive chi-lectins (Henrissat and Davies, 1997). A mutation of the glutamate residue leads to loss of catalytic activity, where proteins with no glutamate residue are termed GH18N and are normally present as chi-lectins. These lack enzymatic activity, but retain active-site chito-oligosaccharide binding ability (Bussink et al., 2007).

1.1.1 Mammalian Chitinase (-like) Members of Familiy 18 Glycosyl Hydrolases

Mammalian chitinases are evolutionarily well-conserved proteins and belong to the GH family 18 based on structural similarity with other bacterial and plant chitinases (Fusetti et al., 2002, Henrissat and Davies, 1997). They include chitinases and chi-lectins (Table 1). The chi-lectins are all members of GH family 18 proteins and consist of five mammalian chi-lectins, which all evolved from subsequent gene duplications of acidic mammalian chitinase (AMCase) and chitotriosidase (Fig. 5).

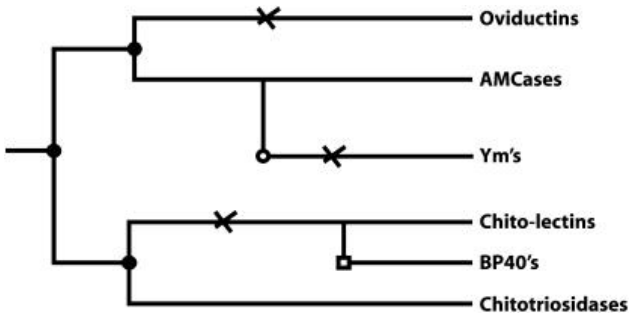

Figure 5 - Overview of the evolution of chitinase (-like) genes. •, the "ancestral" gene duplications; ○, rodent-specific gene duplication; □ signyfies artiodactyle-specific gene duplication; a cross indicates the loss of catalytic activity mutations. "Chito-lectins" are CLPs evolved from the chitotriosidase gene (duplication). Figure from Bussink *et al*, 2007 (Bussink et al., 2007)

Further loss-of-enzymatic-function mutations have led to evolution of a broad spectrum of CPLs in mammals. Chi3l1, Chi3l2 and SI-CLP occur in most mammals, but not in other vertebrates,, while Chi3l2 is also evident in reptiles. Chi3l3 and Chi3l4 in the mammalian family only occur in mice. All of these chi-lectins widely occur in mammals although these organisms lack endogenous chitin (Bussink et al., 2007, Hussain and Wilson, 2013). The evolution of mammalian family 18 chitinase proteins evolved when a gene duplication event occurred, and allowed the specialization of two active chitinases, chitotriosidase (CHT) and acidic mammalian chitinase (AMCase). Duplication of both CHT and AMCase genes

followed by loss-of-enzymatic-function mutation, led to the subsequent evolution of CPLs (Bussink et al., 2007). Human chitotriosidase (HCHT) and human AMCase are the only two active family 18 chitinases produced by humans. Humans also produce the enzymatically inactive enzymes CLPs Chi3l1 and Chi3l2. These enzymes lack the key glutamate residue in the active site that donates a proton required for hydrolysis, but they retain three-dimetional TIM-barrel structure and active-site oligosaccharide binding ability (Houston et al., 2003).

Table 1 - Nomenclature of the mammalian chitinase and chitinase-like proteins

Gene name	Alternative name
ChiA	AMCase,
Chit1	Human chitotriosidase, HCHT
Chi3l1	Human cartilage gp39, YKL-40
Chi3l2	YKL-39
Chi3l3	Ym1, Eosinophil chemotactic factor (ECF-L)
Chi3l4	Ym2

1.1.2 Chitinase-related enzymes in mammalian immune system

Our immune system is an effective and sincere collaboration between all its components and participants. It plays an important role protecting our body against diverse pathogens, microorganisms, fungi, viruses, etc. Our defense against infections is stronger around our openings: mouth, nose, ears, than other areas in the body. The human immune system is capable of recognizing and degrading chitin, an important cell wall component of pathogenic fungi. In the context of host-immune responses to fungal infections, the mechanisms of host chitinase responses may have implications for diagnostic assays as well as novel therapeutic approaches for patients that are at risk of contracting fatal fungal infections.

Macrophages play a crucial role in regulating the initiation, amplification, and resolution of innate immune responses. These cells derive from the granulocyte/macrophage progenitors, which are the precursors of monocytes in the bone marrow. Monocytes coming out from the bone marrow respond to cytokines and chemokines during their recruitment into tissues where they differentiate into resident macrophages (Martinez et al., 2008). Two well-established polarized phenotypes are often referred to as classically activated (M1) and alternatively activated (M2) macrophages. The M1/M2 nomenclature is derived from the cytokines that are associated with each macrophage phenotype, as these cytokines – namely,

interferon-Y (IFNγ), interleukin-4 (IL-4), or interleukin-13 (IL-13) – are linked with T-helper 1 (Th1) and T-helper 2 (Th2) immune responses (Lawrence and Natoli, 2011, Mosser and Edwards, 2008). During Th2-mediated immune responses IL-4 and/or IL-13 can induce macrophage proliferation. M2-macrophages are a critical component of type 2 immunity during helminth infection (Allen and Maizels, 2011) and allergic responses (Palm et al., 2012). Macrophages also adopt an alternatively activated phenotype when activated by IL-4Rα (Van Dyken and Locksley, 2013) and IL-13α (He et al., 2013). M2-macrophages can be derived either from proliferation of tissue resident macrophages, or recruited inflammatory monocytes.

Because the macrophage serves as one of the primary defense mechanisms against invading fungal pathogens (Vazquez-Torres and Balish, 1997), it is possible that chitinases produced by macrophages contribute to the defensive activity of macrophages by degrading fungal chitin (Overdijk et al., 1996, Renkema et al., 1995). This is consistent with the high affinity and specificity of the chitin-binding domain for insoluble chitin (Tjoelker et al., 2000)

1.1.3 Human chitinases

Although humans do not produce chitin, two active mammalian chitinases have been identified: AMCase (Fig. 5; (Boot et al., 2001) and HCHT (Fig. 5; (Hollak et al., 1994, Boot et al., 1995). The two human chitinases share considerable sequence and structural similarity.

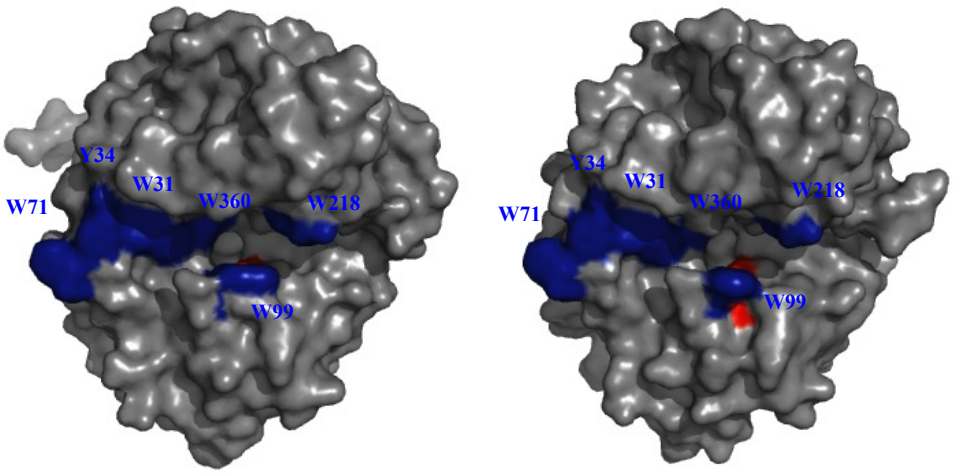

Figure 6 - Aligned crystal structures of HCHT and AMCase. The conserved aromatic residues important for substrate binding are marked in blue, and the catalytic acids are marked in red.

Human chitotriosidase exists in two forms: a 50-kDa protein (HCHT50), and a 39-kDa enzyme (HCHT39) that is produced from HCHT50 by proteolytic processing (van Eijk et al., 2005). A considerable amount of HCHT50 enzyme is routed to the lysosomes and processed into HCHT39 which lacks the C-terminal chitin-binding domain (Fusetti et al., 2002). HCHT and AMCase consist of a C-terminal chitin-binding domain, a hinge region, and a 39-kDa N-terminal domain that has chitinase activity (Renkema et al., 1997). Common to both HCHT isoforms and to AMCase is a catalytic domain with the $(\beta/\alpha)_8$ TIM barrel fold that is characteristic of chitinases belonging to GH family 18 (Fig. 1). HCHT50 and AMCase have an additional proline-rich linker region comprising approximately 29 residues, and followed by a C-terminal carbohydrate-binding module (CBM). Despite these similarities, current findings in the literature indicate several (possible) functional differences and different expression patterns between the enzymes. AMCase has a high acid tolerance that has been ascribed to the presence of His^{208}, His^{269}, and Arg^{145} near the catalytic residues, where HCHT has Asn^{208}, Arg^{269}, and Gln^{145} (Olland et al., 2009, Bussink et al., 2008). In constrast, AMCase only occurs as an exo-enzyme (Chou et al., 2006, Boot et al., 2001). There are also indications that HCHT demonstrates high transglycosylation activity (Aguilera et al., 2003). HCHT shows anomer-specific binding affinities in subsites +2 and +3. Its catalytic domain has six subsites numbered from -3 to +3 (Eide et al., 2013a). These features could support the potential for higher endo-activity and higher transglycosylation potential in HCHT (Eide et al., 2013a). The +3 subsite in HCHT significantly contributes to substrate binding during hydrolysis and degradation (Eide et al., 2013a). This, in turn, corresponds well with the high transglycosylation activity of HCHT, as high affinity of positive subsites for sugar acceptors is known to promote transglycosylation activity in family 18 chitinases (Eide et al., 2013a, Zakariassen et al., 2011, Taira et al., 2010) (Aguilera et al., 2003). AMCase acts as an exo-enzyme (Chou et al., 2006, Boot et al., 2001, Eide et al., 2012). Endo-activity would benefit from an extended substrate-binding cleft with more than two subsites on each site of the catalytic center where cleavage takes place. High oligosaccharide affinity in multiple positive subsites has proven beneficial for transglycosylation (Zakariassen et al., 2011). The AMCase sequence is homologous to those of bacterial chitinases (Bussink et al., 2007). The location of the AMCase gene on human chromosome 1 and the sequence homology and conservation of intron-exon boundaries with HCHT confirms that these genes arise from a duplication event in an ancestor gene (Boot et al., 2001). The slow transglycosylation step in the AMCase

reaction leads to apparent substrate inhibition and may provide feedback regulation of AMCase *in vivo*. AMCase prefers the β-anomer of chitooligosaccharides and mainly functions as an exo-chitobiosidase. AMCase also undergoes elevated chitinolytic activity in a highly-ionic environment (Chou et al., 2006).

AMCase is expressed in the lung epithelial cells, macrophages, and eosinophils of patients with asthma, and its production is driven by the Th2-cytokines IL-4 and IL-13 (Zhu et al., 2004). The chitinase activity of AMCase is induced by IL-13 and triggers allergic inflammation (Boot et al., 2001). (Boot et al., 2001). AMCase co-localizes and physically interacts with epidermal growth factor receptor (EGFR), and secreted AMCase stimulates epithelial chemokine production. EGFR has a role in exocytosis and vesicular trafficking and therefore may also participate in the trafficking of intracellular AMCase (Hartl et al., 2008).

HCHT is a component of innate immunity that may play a role in defense against chitin-containing pathogens. The expression and release of HCHT by human phagocytes is highly regulated (van Eijk et al., 2005). A 24-bp insertion in exon 10 of the HCHT gene that prevents formation of active HCHT occurs in individuals from various ethnic groups (Boot et al., 1998). HCHT is selectively expressed and highly regulated in activated macrophages, and is also present in human neutrophil-specific granules which are released upon stimulation (Boot et al., 1995). The HCHT enzyme was identified in Gaucher disease patients, and is currently used as a biochemical marker of macrophage activation in some lysosomal diseases. HCHT is not an effector molecule in allergic inflammation. Rather, it is regarded as a host-defense mechanism against chitin-containing pathogens (Malaguarnera, 2006). HCHT is also a biomarker for, and a therapeutic target in, scleroderma-associated interstitial lung disease as it is produced by lung-macrophages and epithelial cells. HCHT mediates disease effects in part by interacting with TGF-β1 to augment TGFR1 and TGFR2 expression, and canonical and non-canonical TGF- β1 signaling (Lee et al., 2012).

1.1.4 Chitinase-like Proteins

Chitinase-like proteins (CLPs) lack enzymatic activity, but retain the chitin binding domain and are termed chi-lectins. Chitinase and chitinase-like-protein involvement in inflammation is not necessarily activated by chitin containing organisms, which makes it important to identify the roles of these proteins in inflammation and immunity. Chi-lectins lack apparent GH enzymatic activity, as well as a complete form of the cysteine-rich chitin-binding

domain (Fusetti et al., 2003). However, chi-lectins can identify specific glycan structures in mammalian tissue, and can efficiently interact with chitin fragments via the CBM in its C-terminus (Fusetti et al., 2003).

Chitinase-3-like-1 (Chi3l1) is produced by a wide variety of cells including neutrophils, macrophages, synovial cells, fibroblasts, smooth muscle cells, epithelial cells, and tumour cells (Volck et al., 1998, Mizoguchi, 2006, Johansen, 2006). Expression of the Chi3l1is associated with conditions of increased matrix turnover and tissue remodeling. High levels of this protein have been found in sera and synovial fluids of patients with inflammatory and degenerative arthritis (Ling and Recklies, 2004). Expression of Chi3l1 is also induced specifically during the course of intestinal inflammation. Chi3l1 can enhance the adhesion and invasion of enteric bacteria in/into colonic epithelial cells (CECs), and acts as a pathogenic mediator in acute colitis (Mizoguchi, 2006). Induced expression of Chi3l1 is observed in both LP cells and in CECs from experimental colitis models as well as in human ulcerative colitis (UC) and Crohn's disease. Expression is induced specifically under inflammatory conditions and not while in a healthy state (Mizoguchi, 2006)). CHI3L1 expression is up-regulated in cancer cells and has a significant correlation with macrophage infiltration and micro-vessel density in the tumors of human colorectal cancer patients and in a xenograft mouse model (Kawada et al., 2012). Chi3l1 efficiently activates the NF-κB signaling pathway and subsequently enhances the secretion of pro-inflammatory cytokines such as IL-8 and TNF-α. In addition, Chi3l1 actively promotes cellular proliferation and migration in CECs (Chen et al., 2011b).
The carbohydrate-binding domain of Chi3l1 is specifically associated with the Chi3l1-mediated activation of Akt-signaling in CECs. Downstream, Chi3l1 enhanced the secretion of IL-8 and TNFα in a dose-dependent manner (Chen et al., 2011a). Chi3l1 synergistically activates IL6-mediated STAT3 phosphorylation in intestinal epithelial cells in murine models of infectious colitis (Tran et al., 2014).

Although chi-lectins have no complete chitin-binding domain like AMCase and HCHT, they still preserve a carbohydrate-binding motif which enables them to interact with chitin and chito-oligosaccharides (Fusetti et al., 2003). Binding studies have shown that unlike another chi-lectin, chitinase-3-like-3 (Chi3l3), Chi3l1 binds chitin with high affinity and has therefore been proposed to be a chitin-specific lectin (Fusetti et al., 2002, Renkema et al., 1998). Both Chi3l1 and Chi3l3 have all the signs of an inactivated chitinase and show

high degrees of sequence similarity to family 18 chitinases (Sun et al., 2001). The two key active site residues, the equivalents of Asp^{138} and Glu^{140} in HCHT, have been mutated to Ala and Leu, respectively, which gives the chi-lectins no detectable chitinase activity (Fusetti et al., 2002).

Chitinase-3-like-2 (Chi3l2) is closely related in size and sequence to Chi3l1. It was named according to the convention for that homolog, which is based on the three N-terminal amino acid residues: tyrosine (Y), lysine (K) and leucine (L), and an apparent molecular weight of 39 kDa. As a result, Chi3l2 was termed YKL-39 (see Table 1). Chi3l2 is secreted from articular chondrocytes, and its mRNA has been detected in lung, heart, and glioblastoma, but not in brain, spleen, or pancreas (Hu et al., 1996). Chi3l2 mRNA was also detected in macrophages that were strongly stimulated by a combination of IL-4 and TGF-β (Gratchev et al., 2008). Chi3l2 is currently recognized as a specific biomarker for the activation of chondrocytes and for the progress of osteoarthritis (OA) (Knorr et al., 2003, Steck et al., 2002, De Ceuninck et al., 2005). It is also interesting to note that Chi3l2 is part of the AMCase locus in humans, but on the basis of both phylogenetic analyses and protein features, this results from a gene duplication event in the HCHT locus (Bussink et al., 2007).

Chitinase-3-like-3 (Chi3l3; Ym1) and chitinase-3-like-4 (Chi3l4; Ym2) do not exist in humans (Webb et al., 2001, Julia Kzhyshkowska, 2007). However, their domain organization and expression profiles indicate that the functions of Chi3l3 and Chi3l4 in mice can overlap with those of AMCase and SI-CLP in humans (Julia Kzhyshkowska, 2007). Chi3l3 is a secretory protein produced by activated macrophages that share sequence homology with the chitinases (Chang et al., 2001), and is also expressed in both monocyte and tissue-derived macrophages (Gundra et al., 2014). The function of the Chi3l3 effector molecule is questionable. Its role may be to encapsulate chitin bearing pathogens such as yeast, fungi, or nematodes, or to interact with extracellular matrix components, consistent with a role for Th2-driven macrophages in wound healing (Nair et al., 2003)..Chi3l3 is synthesized and secreted by activated macrophages during inflammation triggered by parasitic infections (Chang et al., 2001). Research shows that Fizz1 (a novel, cysteine-rich secreted protein associated with pulmonary inflammation (Holcomb et al., 2000)) and Chi3l3 are strongly induced in *in-vivo*- and *in-vitro*-elicited, alternatively activated

macrophages, as compared with classically activated macrophages. The *in-vivo* induction of FIZZ1 and Chi3l3 in macrophages depends on IL-4, and *in-vitro*, IFN-γ antagonizes the effect of IL-4 on the expression of Fizz1 and Chi3l3 (Raes et al., 2002). One researcher suggests that Chi3l3 is a more sensitive biomarker in angiostrongyliasis than IL-4 and IL-13 (Zhao et al., 2013).

Chi3l4 (Ym2) is a close homolog of Chi3l3 and is a secretory protein from eosinophilic crystals in both the gastric and respiratory lesions of hyalinosis. It is also expressed in mouse lung responding to allergen exposure, suggesting an important role in asthma (Webb et al., 2001).

1.1.5 Processivity of chitinases

Enzymes acting on crystalline substrates may have the ability to remain attached to their substrates in-between subsequent hydrolytic reactions. This is called processive action, meaning that they bind individual polymer chains in long tunnels or deep clefts and hydrolyze a series of glycosidic linkages along the same chain before dissociation (Horn et al., 2006a) (Horn et al., 2006a, Henrissat and Davies, 1997). Processive degradation is thought to improve catalytic efficiency because single polymer chains are prevented from re-associating with the insoluble material between catalytic cycles (Horn et al., 2006a). It also reduces the number of times the enzyme has to carry out the energetically unfavorable process of gaining access to a single chain. The substrate-binding sites in processive chitinases are lined with aromatic residues, in particular tryptophan residues. These residues are thought to facilitate processivity by functioning as flexible and hydrophobic sheets along which the polymer chain can slide during the processive mode of action. There is little experimental data in support of the idea that processivity is important for enzyme efficiency. This might be due to the fact that it is difficult to address this phenomenon experimentally when working with crystalline substrates (Eijsink et al., 2008). Still, recent studies show that it is possible to measure apparent processivity (P^{app}) when degrading β-chitin by the processive *S. marcensces* chitinases ChiA and ChiB, along with the endo-chitinase ChiC, and a ChiB variant (ChiB-W97A) with reduced processivity (Hamre et al., 2014). Processivity was calculated based on the $[(GlcNAc)_2]/[GlcNAc]$ ratio, and suggests that enzymes with a higher initial degree of processivity are more efficient degraders of β-chitin. At higher degrees of chitin degradation, analysis of $[(GlcNAc)_2]/[GlcNAc]$ ratios reveal

differences between the processive enzymes ChiA and ChiB and the less processive endo-acting ChiC and ChiB-W97A. One explanation might be that the substrate becomes more recalcitrant with conversion, demonstrated by a decrease in processivity of processive enzymes. In addition, there is a difference between substrates (Hamre et al., 2014). This makes it important to include both the nature of the substrate that is used, and to control the extent of substrate degradation when reporting the degree of processivity. The length of obstacle-free pathways on the substrate limits the degradation of chitin by processive chitinases (Kurasin and Vaeljamaee, 2011). In chitin (and cellulose), the sugar units are rotated 180° relative to their neighboring residue, so that the smallest structural unit, and the product of processive enzymatic action, is a disaccharide. Family 18 chitinases use a substrate-assisted reaction mechanism for catalyzing hydrolysis. This means that productive binding only occurs when the sugar positioned in the -1 subsite (Davies et al., 1997) of the enzyme has a correctly positioned *N*-acetyl group (van Aalten et al., 2001, Synstad et al., 2004). Measuring product ratios provides indication of the degree of processivity. Note that each productive binding of a processive enzyme to a highly polymeric substrate chain will lead to production of maximally one product with an odd number of sugars regardless if it is an endo- or exo-binding enzyme (Fig. 7), whereas all other products resulting from the same initial productive enzyme-substrate association will be dimers. Trimeric and monomeric products are indicative of initial binding, whereas dimeric products are primarily, but not exclusively, generated by processive hydrolysis. The ratio between these products will therefore provide an indication of the degree of processivity (Horn et al., 2012).

Processivity of family 18 chitinases can also be assessed using the water-soluble polymeric chitin derivative chitosan (Eijsink et al., 2008). If the enzyme is processive, every product resulting from the same initial enzyme-substrate association will be even-numbered, except for the first. During processive degradation of chitosan, nonproductive complexes may emerge. However, nonproductive complexes may not necessarily result from polymer dissociation by other enzymes in the family 18 chitinases; in other enzymes, the processive movement continues, leading to longer, even-numbered oligomers which signify processivity (Sorbotten et al., 2005, Horn et al., 2006a, Zakariassen et al., 2009a) (Horn et al., 2006b, Horn et al., 2006a). If the enzyme is not processive, one will observe no clear dominance of even-numbered products. This method is handy, but one should take into consideration that chitosan is not a natural substrate. The processivity values coming out of this approach might be underestimated because of the chance of full enzyme-substrate

dissociation that occurring is likely to become larger with the length of the sliding pathway (Horn et al., 2012).

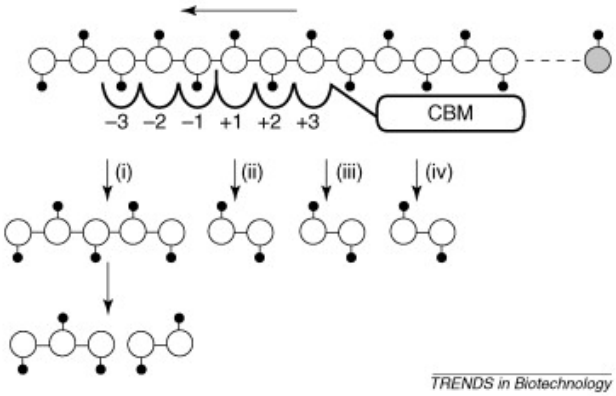

Figure 7 – Processive degradation of chitin. Illustrated for an endo-binding chitinase with a CBM and six subsites (-3 to +3). The numbers (i-iv) indicate the four initial consecutive cuts in the polymer. Figure is from Eijsink *et al*, 2008 (Eijsink et al., 2008).

1.1.6 Transglycosylation activity

Besides hydrolysis of glycosidic bonds, many of the enzymes belonging to the family 18 chitinases can catalyze transglycosylation reactions to form new glycosidic bonds between donor and acceptor saccharides (Eneyskaya et al., 1997, Bardales and Bhavanandan, 1989, Aguilera et al., 2003). In retaining GHs, the transglycosylation reaction occurs via a double displacement mechanism (Ly and Withers, 1999). In the first step, a catalytic acid protonates the glycosidic oxygen, and the anomeric carbon becomes a target for a nucleophilic attack from the catalytic base, leading to cleavage of the glycosidic bond and formation of a glycosyl-enzyme intermediate. In the second step, the intermediate decomposes into one of two different outcomes: either hydrolysis occurs, or transglycosylation occurs if the water molecule is outcompeted by another acceptor. Transglycosylation is a kinetically controlled reaction and requires an enzyme with active site architecture that disfavors correct positioning of the hydrolytic water molecule, and/or favors binding of incoming carbohydrate molecules (Williams and Withers, 2000, Zakariassen et al., 2011). This is of interest because there are numerous potential applications for chito-oligosaccharides, especially in the food, medical, and agriculture fields (Aam et al., 2010b). The bioactivities of chito-oligosaccharides are thought to depend on a combination of oligomer length, degree of acetylation, and acetylation pattern (Aam et

al., 2010a). Chitinases that perform transglycosylation can play a central role in the development of new well-defined mixtures of chito-oligosaccharides with new and improved biological activity by coupling smaller chito-oligosaccharide building blocks to each other or to other functional groups.

1.1.7 Important aromatic residues in the catalytic center of Human Chitotriosidase

Studies on the contribution of aromatic residues to the processivity of ChiA and ChiB reveal that these residues are important for interaction with the substrate (Zakariassen et al., 2009a, Katouno et al., 2004). Mutation of these residues tends to lead to significant reductions of chitin-hydrolysing activity. Studies of ChiA and ChiB have shown that aromatic residues near the catalytic center are crucial in determining both degree and the directionality of processivity (Vaaje-Kolstad et al., 2013). The processive ChiA and ChiB are thought to degrade chitin in opposite directions, even though they have similar catalytic centers with a highly conserved -1 subsite where the catalytically active acetamide group of the substrate binds (van Aalten et al., 2001, van Aalten et al., 2000). ChiA is thought to move toward the non-reducing end, releasing products from its +1 and +2 subsites. Horn *et al* (Horn et al., 2006a, Horn et al., 2006b) showed that mutation of Trp^{97} in the +1 subsite of ChiB reduces processivity and enzyme efficiency toward crystalline chitin while increasing specific activity toward chitosan. The same was seen for Trp^{220} in the +2 subsite. In ChiA, mutations of the same residues did not affect the degree of processivity to any extent. Here, Trp^{167} in the −3 subsite was shown to be important for processivity. Still, all three mutations (aromatic residues, e.g. Trp, to Ala) greatly affected chitin degradation efficiency (Zakariassen et al JBC 2009). Moreover, exposed aromatic residues in both ChiA and ChiB located outside of the active cleft and on CBMs are important for the binding and hydrolysis of crystalline chitin (Watanabe et al., 1990) Katouno, 2004). Furthermore, removal of aromatic residues in subsites +1 and +2 greatly reduces or completely abolishes transglycosylation activity (Zakariassen et al. Biochemistry 2011, Taira et al. 2010), again signifying the importance of these surface exposed aromatic residues. Trp^{99} (+1) and Trp^{218} (+2) in HCHT correspond to Trp^{97} and Trp^{220} in ChiB, and Trp^{275} and Phe^{396} in ChiA, while Trp^{31} (−3) in HCHT corresponds to Trp^{167} in ChiA. Because of this, the knowledge obtained about the bacterial chitinases ChiA and ChiB will serve as a reference when interpreting knowledge obtained on how HCHT acts on soluble and in-soluble substrates.

AIM OF THE STUDY

This study is a part of a long-term project for which the goals are: to characterize chitinases involved in chitin and chitosan degradation, and to provide a detailed understanding of how substrates bind to chitinases.
The main objective of this work was to increase knowledge about the functions and mechanisms of human chitinases and chitinase-like proteins in mammals.

The work involved the following aims:

- To study the enzymatic mechanism of HCHT using methods for in-depth studies of chitinases. To investigate how both HCHT isoforms bind to substrate by exploring the enzymes' action on a polysaccharide (*endo*- or *exo*-), and their degrees of processivity.
- To investigate the importance of the chitin-binding module of HCHT, and how this enzyme depends on the domain when interacting with soluble and non-soluble substrates.
- To study the importance of aromatic residues in positioning the substrate in the HCHT active site by comparing the different mutants to wild-type HCHT.
- To study the role of mammalian chitinases and chitinase-like proteins in a specific case of inflammation to increase knowledge about the roles of these enzymes in the immune system.

RESULTS IN BRIEF

Paper I:

The Effect of the Carbohydrate Binding Module on Substrate Degradation by the Human Chitotriosidase

Substrate degradation of chitosan by both isoforms of human chitotriosidase was performed to measure degree of processivity and efficiency properties of the enzyme's two isoforms. The degree of processivity for HCHT39 was considered low. Even oligomers tended strongly to be more dominant than odd oligomers only when $\alpha < 0.01$. When $\alpha = 0.03$, this tendency is reduced and is not seen at $\alpha = 0.08$. The polymer peak remains present when $\alpha > 0.13$. For chitosan, when α is low, at 0.07, HCHT50 show little tendency for dominance of even numbered oligomers. The degree of processivity may also be measured quantitatively by plotting the total number of reducing ends (α_{tot}) created after chitinase-catalyzed hydrolysis against the amount of polymeric ends as measured by the reduction in viscosity (α_{pol}). Depolymerization of chitosan by HCHT39 and HCHT50, with an F_A value of 0.70, showed 2.0 and 1.4 cuts per formation of an enzyme-substrate complex, respectively, compared to acid-catalyzed chitosan hydrolysis which is a completely random process with the number of cuts per formation set to 1. Processivity was also assessed by degradation of chitin, and during the initial phase of the reaction, degradation of β-chitin yielded a $[(GlcNAc)_2]/[GlcNAc]$ ratio of 11.4 ± 1.3 for HCHT39, and 7.6 ± 0.3 for HCHT50.

In line with observed variations occurring in the relative size of the polymer peaks, and the relative size and distributions of the oligomer peaks, at identical time points in the analyses discussed above, clear variations in α values at identical time points are observed. Both HCHT50 andHCHT39 has a very strong biphasic character, but HCHT50 shows a much faster initial rate compared to HCHT39, followed by a phase that is slower than that of HCHT39. A final α value of 0.28 was determined after 7 days of incubation. Our previous studies with HCHT39 had revealed biphasic depolymerization kinetics with apparent rate constants (k_{cat}^{app}) of 102 s^{-1} and 14 s^{-1} for the initial and later phases of the reaction, respectively, and a maximum α value of 0.33. The chito-oligosaccharide mixtures obtained at maximum degree of scission were analyzed using size exclusion chromatography. The

chromatograms showed that the two HCHT isoforms produce distinctly different oligomer distributions. The efficiency of β-chitin degradation was also monitored. HCHT50 is capable of fully degrading the chitin substrate used in this study, while HCHT39 is only capable of degrading approximately 15 %. The initial apparent catalytic rate constant ($k_{cat,app}$) were found to be 0.55 ± 0.02 s^{-1} for HCHT39 and 0.81 ± 0.10 s^{-1} for HCHT50.

Paper II:

The Importance of Aromatic Residues in the Active Site in Human Chitotriosidase for Substrate Degradation

HCHT is shown to have a rather large area of solvent exposed aromatic residues in the active site. Such residues are common for processive GHs acting on insoluble polysaccharides. HCHT has a Trp-residue on both sides of the catalytic acid; in subsites -3 (Trp^{31}) and +2 (Trp^{218}). We mutated these residues to the nonaromatic residue alanine, to effectively abolish the number of favorable CH-π interactions between the sugar ring of the substrate and the aromatic residue of the enzyme and by this decrease both rate and strength of binding. These mutants showed a slight reduction in specific activity for HCHT50-W31A (91 %) and HCHT50-W218A (75 %) compared to the wild type. We monitored the rates of chitosan degradation for each of two mutants by determining the degree of scission (α) with respect to time, and compared them to rates in the wild-type enzyme. Interestingly, the rate decreases for both mutants compared to what was observed for the wild type. Samples at ~0.05, ~0.08 % and maximum degree of scission was further investigated using size-exclusion chromatography (SEC). Samples of HCHT50-W31A and HCHT50-W218A when α = 0.05 showed no dominance of even-numbered peaks over odd-numbered (results not shown), which is typical for exo-processive chitinases, as observed for the wild-type enzyme (Stockinger et al., 2015). Polymer peaks were present for both mutants and the wild-type enzyme. Interestingly, when α = 0.08, the polymer peak disappears for HCHT50-W31A, but remains for HCHT50 and HCHT50-W218A. Maximum α for both mutants was determined after 7 days of incubation. The maximum degrees of scission we determined were 0.24 and 0.32 for HCHT50-W31A and HCHT50-W218A, respectively.

The efficiency of chitin degradation was also monitored. HCHT50 can fully degrade the chitin substrate used in this study. HCHT50-W31A and HCHT50-W218A were only able to degrade 14 ± 4 and 22 ± 8 %, respectively. Again as a comparison, HCHT39 is able to

degrade 15 % of the same substrate. Apparent processive ability (P^{app}) was determined as $[(GlcNAc)_2]/[GlcNAc]$ ratios. During the initial phase of the reaction, degradation of β-chitin yielded a $[(GlcNAc)_2]/[GlcNAc]$ ratio of 10.6 ± 2.9 for HCHT50-W31A and 4.1 ± 0.7 for HCHT50-W218A, respectively. The initial, apparent, catalytic rate constants ($k_{cat,app}$) k_{cat}^{app} was found to be 0.040 ± 0.006 s^{-1} and 0.086 ± 0.021 s^{-1} for HCHT50-W31A and HCHT50-W218A, respectively.

Paper III:

Screening of Human Chitinases and Chitinase-like Proteins in Inflammatory Disease

Gene-expression of mammalian chitinases and chitinase-like proteins in inflamed colon were investigated. We did not detect AMCase and Chi3l4 mRNA transcripts in normal nor inflamed mouse colon tissue. Chit1 mRNA was expressed in colon tissue, but the expression was not affected by inflammation induced by DSS, nor by feeding the animals a diet containing the bacterial meal Bioprotein (1.2 fold; range (0.58-2.75), 1.05 fold; range (-1.80-1.99 for DSS and Bioprotein, respectively). Expression of mRNA for both Chi3l1 (70 fold; range 42.2-109; $p<0.001$) and Chi3l3 (150 fold; range 96.3-234; $p<0.001$) were significantly increased by DSS treatment, compared to control animals. Feeding DSS-treated animals with the bacterial meal Bioprotein resulted in decreased mRNA expression of both Chi3l1 (2.25 fold; range 1.39-3.63; $p=0.36$) and Chi3l3 (5.5 fold; range 3.5-8.5; $p=0.007$) in colon, compared to DSS-treated animals given the control diet. To verify the findings from real-time data and to localize the chi-lectins in the colon, we used immunohistochemistry on formalin-fixed tissue from the same mice from which we obtained the gene-expression data. We observed data consistent with the quantitative PCR results; Chi3l1 was significantly up-regulated, compared to the healthy mice, both during DSS-induction and when DSS-induced mice were fed BioProtein. As expected, no expression was detectable in healthy mice. Chi3l3 expression was also increased, compared to the healthy mice. To exactly identify the localization of the chi-lectins within the tissue, we double-stained Chi3l1 and Chi3l3 together with markers for neutrophil granulocytes (Mpo) and macrophages (F4/80). Chi3l1 and Chi3l3 were found to co-localize with Mpo in the infiltrating mononuclear cells, which are mainly macrophages and neutrophils. The distribution of infiltrating cells was confirmed as neutrophil granulocytes, and Chi3l1 was

present in almost all of the Mpo-positive cells. The distribution of Chi3l1 in macrophages (F4/80) was less severe than for neutrophil granulocytes (Mpo). There were both positive and negative macrophages for Chi3l1, while almost all cells stained for both Mpo and Chi3l1 were positive.

DISCUSSION

The enzymatic degradation of chitin is carried out by the GH18 family. This process is important for the human immune system, growth of chitin containing organisms, defense mechanisms in lower life forms, and a sustainable bio economy in the biotechnology industry. Humans produce two active GH18 chitinases, as well as chi-lectins which have no enzymatic activity but also bind chitin (Malaguarnera, 2006). Four characteristics are central to describing the hydrolytic activity of chitinases: i) degree of processivity, ii) mode of cleavage, i.e. endo- or exo-, of the polymeric substrate, iii) directionality of chitin degradation, from the reducing or non-reducing end, and iv) kinetic and thermodynamic mechanisms of binding between the substrate and the inhibitor. This thesis considers the first three of these four characteristics. For degradation of crystalline recalcitrant polysaccharides, chitinases must tackle obstacles such as binding to the polymer surface, recognition and capture of a polymer chain, formation of a productive complex, hydrolysis of the polymer chain in a processive or non-processive fashion, and at last, dissociation from the polymer chain. Recent research has shown that substrate association is the rate-limiting step in this hydrolysis (Zakariassen et al., 2010, Kuusk et al., 2015). The general assumption is that catalytic efficiency is improved by keeping the enzyme closely associated with the substrate in between subsequent hydrolytic reactions. For crystalline substrates, the enzyme faces a free energy penalty of 5.6 kcal/mol per chitobiose unit in decrystallization energies. This emphasizes the importance of processive enzymes being capable of keeping once-detached single chains from re-associating with the insoluble material.

To recognize, bind to, and capture of a polymer chain, GHs often have CBMs. In **Paper I,** the following effects of the CBM in HCHT, which belongs to CBM family 14, were investigated: efficiency, processivity, rate and extent of degradation, and oligomer distribution on soluble and insoluble substrates. We also show that the degradation efficiency decreases from 100% to 15% when only the CBM is removed from HCHT. Apparent processivity (P^{app}) was used to calculate actual processivity, in contrast to intrinsic processivity (P^{Intr}) which is the theoretical potential for processive ability. Apparent processivity varies depending on the substrate and the experimental conditions (Horn et al., 2012, Kurasin and Vaeljamaee, 2011). The calculation was performed using the formula

P^{app} = [dimers]/[monomer]. **Paper I** revealed that the CBM14 present in HCHT50 increases its efficiency and rate of hydrolysis, strongly suggesting that the CBM14 is essential for degrading chitin. Interestingly, the CBM slightly reduces processive ability as measured by [$(GlcNAc)_2$]/[GlcNAc] ratios from chitin degradation and N_{cuts}, and the dominant presence of even-numbered oligomers after chitosan degradation. Surprisingly, HCHT is less processive, though still faster and more efficient, than ChiA, which is the most processive GH18 from *S. marcescens* (Igarashi et al., 2014, Hamre et al., 2014). It is normally believed that there is a positive correlation between processive ability and substrate degradation efficiency (Horn et al., 2012, Teeri, 1997). These methods are good for measuring the processive ability of the enzymes, but have several pitfalls and might be misleading. There are no *a priori* reasons to assume that initial binding, be it *endo*- or *exo*-, preferentially yields an odd-numbered product. For further confirmation, it would be interesting to determine P^{intr}. The inherent processivity potential of an enzyme is given as the ratio of k_{cat}/k_{off}, where k_{cat} is the catalytic rate constant and k_{off} is the rate constant for dissociation of the enzyme–substrate complex (Lucius et al., 2003).

We measured processivity with chitosan as a substrate in **Paper I**. Here we prepared chitosan with a random distribution of the acetylated units, F_A = 0.64, using size-exclusion chromatography (SEC). This method is a great tool for qualitative monitoring of processivity. A degraded sample containing chitosan and chitinase with known α (degree of scission) was separated, making it possible to detect the products. HCHT50 reached a lower maximum α than HCHT39 for chitosan with the given degree of acetylation. Transglycosylation is most likely the explanation for these findings. Aguilera et al. previously showed that the 50 kDa isoform exhibits strong transglycosylation activity (Aguilera et al., 2003) and studies on other chitinases demonstrate that strong substrate affinity in positive subsites, which promotes binding of sugar acceptors, promotes transglycosylation (Taira et al., 2010, Zakariassen et al., 2011, Rosengren et al., 2014, Umemoto et al., 2012). The large, 49-residue long CBM14 attaches to the C-terminus of HCHT50 via a 29-residue linker. ChiB exhibits a similar CBM-enzyme attachment (49 and 26 residues in the CBM and linker, respectively) that extends the positive, subsite-binding surface (Fusetti et al., 2002, van Aalten et al., 2000). This suggests that the CBM14 of HCHT50 prolongs the positive subsite binding surface as well compared to HCHT39. In the case of a soluble polymeric substrate such as chitosan, CBM14 is thus likely to promote

transglycosylation, since it facilitates binding to oligomeric, and even polymeric, sugar acceptors. Transglycosylation activity increases the average lengths of the products, generates new substrate molecules that eventually become enriched for unfavorable, i.e. less cleavable, sequences, and thus reduces overall cleavage yields. We observed increased product length and reduced overall cleavage yields as a result of transglycosylation in our study. Interestingly, α_{max} increased (from 0.28 to 0.32) for HCHT50-W218A. This is likely the result of less transglycosylation activity since a strongly binding residue in positive subsites was removed, corroborating observations in ChiA, ChiB (Zakariassen et al., 2011) and a class V chitinase from cycad, (Taira et al., 2010).

Traditionally, the degree of processivity is related to the topology and presence of aromatic amino acids in the active site of glycosyl hydrolases. The active sites of processive GHs are typically lined with aromatic residues responsible for binding individual chains from the polymer crystal (Rouvinen et al., 1990). **Paper II** describes the importance of central aromatic residues in both positive and negative subsites of the active site. Aromatic residues are key for processivity, rate and extent of degradation, and oligomer product distribution when the substrate is soluble. A positive correlation between processive ability and rate of hydrolysis and degradation efficiency was previously identified (Kurasin and Vaeljamaee, 2011, Kostylev et al., 2014, Hamre et al., 2014). For the exo-processive cellulase *Tf*Cel48A that works from the reducing end of the polymer, the residues inside the active tunnel entrance are essential for the enzyme's ability to degrade crystalline substrates. While acting on crystalline bacterial cellulose, processivity, and degradation rate and efficiency were reduced when the tryptophan residue Trp^{315} situated in subsite −4 was mutated. Mutation of Trp^{313} in subsite −5 yielded the same result. Negative subsites *Tf*Cel48A are called substrate-binding subsites because *Tf*Cel48A directionality causes these subsites to remain bound to the polymeric substrate after a processive hydrolysis. Another bacterial GH, ChiA, is also significantly affected when aromatic residues in substrate-binding subsites are mutated. ChiA is also thought to work from the reducing end, releasing products from its positive subsites (van Aalten et al., 2001, van Aalten et al., 2000, Perrakis et al., 1994, Papanikolau et al., 2001). Trp^{167} in ChiA corresponds to Trp^{31} in HCHT. In ChiA, mutation of this residue greatly affected processivity, and rate and efficiency of chitin degradation, while the effects of mutations in the +1- and +2- subsites were clearly less significant. Mutation of Trp^{31} in the -3 subsite of HCHT50 results in a 7-fold reduction in

substrate degradation efficiency (100% to 14%), and a 20-fold reduction in hydrolysis rate (k_{cat}^{app} from 0.81 s^{-1} to 0.040 s^{-1}), consistent with the trends observed for *Tf*Cel48A and ChiA. When Trp^{218} in the +2 subsite is mutated, both efficiency and rate of hydrolysis are affected, but to a slightly lesser extent than for Trp^{31} (5-fold less efficient and 10-fold slower). The results may indicate that negative subsites are substrate-binding subsites in HCHT as well, and that HCHT moves from the reducing end to the non-reducing end of the polymer. This would also concur with the thermodynamic signatures of allosamidin binding to HCHT. Allosamidin is a family 18 chitinase inhibitor that specifically binds subsites -3 to -1. Binding of allosamidin to HCHT and ChiA cause similar enthalpy and solvation entropy changes and make more favorable contributions to free energy change than binding to ChiB, which causes an unfavorable enthalpy change (Eide et al., 2013b). Negative subsites are product release site in ChiB (van Aalten et al., 2001).

An interesting result is the effect of aromatic residue mutations, of both Trp^{31} and Trp^{218}, on the rate of chitosan degradation. When such mutations are performed in ChiA, the rate of chitosan degradation increases ~20-fold compared to the wild-type enzyme (Zakariassen et al., 2009b). The same trend, albeit to a much smaller extent, is observed for the *Tf*Cel48A mutants on phosphoric acid swollen cellulose (PASC), a mostly amorphous form of cellulose that is more accessible than crystalline cellulose (Kostylev et al., 2014). This greatly contradicts the decrease in soluble substrate hydrolysis that is observed for HCHT. A possible explanation is that chitosan hydrolysis by HCHT50 is already very high (k_{cat}^{app} > 102 s^{-1}). This is ~7-fold faster than what was observed for ChiA and ChiB. Moreover, the work of Horn *et al*. shows that processivity comes at a large cost of enzyme speed, and that the mutation of substrate-binding aromatic residues is important for processivity increases enzyme speed when the substrate is readily accessible. HCHT50 wild type appears to have low processive ability, which fits with a potential for fast degradation of the readily available substrate chitosan.

Another interesting result is that after degradation of high molecular weight chitosan by HCHT50-W31A, long polymers disappear at lower α-values, compared to the wild-type enzymes (~8% versus >13% chitosan degradation, respectively). This suggests that the mutant has a higher degree of endo-character than the wild-type enzyme (Horn et al., 2006b). In comparison, disappearance of long polymers takes place at ~5% chitosan degradation for the endo-active ChiC of *S. marcescens*, while the same is observed at 20% chitosan degradation for the exo-processive ChiA and ChiB from the same bacteria.

The processive ability of all mutants, as determined by [$(GlcNAc)_2$]/[GlcNAc] ratios from chitin degradation, and the presence of even-numbered versus odd-numbered oligomers from chitosan degradation, are is quite low; this is similar to what is observed for HCHT50.

Paper II shows that mutation of Trp^{31} in the -3 subsite of HCHT50 greatly reduces chitin degradation efficiency by the enzymes from 100% in HCHT50 to only 14% in the mutated version. Similarly, mutation of Trp^{218} in the +2 subsite of HCHT50 also causes significant reduction in degradation efficiency from 100% in HCHT50 to 22% in the mutant. This finding demonstrates the vital role these residues have in chitin degradation, and shows that these negative-subsite aromatic residues are more valuable than the CBM for degradation efficacy. By combining the results from **Paper I** and **Paper II**, we can conclude that HCHT is a complete chitinolytic machinery by itself. The machine consists of a combination of endo-nonprocessive GHs (open active site), exo-processive GHs (a large area of solvent-exposed aromatic amino acids in the active site), and a somewhat unusual CBM14 (normally designed to interact with oligosaccharides). Moreover, both CBM and surface exposed aromatic residues in the active site are important for both rates of hydrolysis as well as efficiency. Moreover, larger effects are observed on rate of hydrolysis and efficiency on chitin degradation and degree of endo-activity on chitosan for HCHT50-W31A (−3 subsite) compared to HCHT50-W218A (+2 subsite).

Mammalians do not possess chitin, but they have genes encoding the active chitinases chitotriosidase (HCHT/Chit1) and acidic mammalian chitinase (AMCase/ChiA), and chitinase-like proteins (CLPs) without hydrolytic activity. The expression of chitinases and CLPs have been described to be affected in a variety of diseases characterized by chronic inflammation. For **Paper III,** we investigated the expression profile of the mammalian chitinases and chitinase-like proteins in a experimental mouse model for colitis. Both these proteins can bind chitin. Chitin-containing organisms (e.g. parasites) produce chitinases to remodel chitin-containing structures for their morphogenesis (Herrera-Estrella and Chet, 1999). Since chitin is a key structural component of pathogens like fungi, as well as a constituent of the mammalian diet, researchers envisioned a dual function for mammalian chitinases in innate immunity and food digestion (Bussink et al., 2007, Boot et al., 2005). Here we provide new insights on how Chi3l1 and Chi3l3 expression change during inflammation, and on the differences in how they act. HCHT (Chit1) is also taken into consideration, showing different expression patterns compared to chi-lectins.

Murine Chit1 (mChit1) was constitutively expressed in colon tissue from both healthy and DSS-induced mice, and showed no change in expression after adding a bacterial meal to the diet to reduce inflammation. Since human Chit1 (hChit1) expression does not change during inflammation, Chit1 expression might be specific to chitin-containing organisms, so that the expression only occurs if chitin-containing organisms are present during the inflammation. Both murine Chi3l1 (mChi3l1) and murine Chi3l3 (mChi3l3) showed potent increases in gene expression. Based on immunohistochemistry data, mChi3l1 and mChi3l3 co-localize with Mpo positive infiltrating cells and, to some extent, F4/80 positive cells of the submucosa. Gene expression of mChi3l1 and mChi3l3 were significantly down-regulated in mice fed a diet containing Bioprotein, compared to mice fed a normal diet, during DSS-induction of colitis. Mice fed the Bioprotein diet showed a significant decrease in infiltrating neutrophils as reflected by a reduction of Mpo and NOS2 expression. This shows that the increased expression of mChi3l1 and mChi3l3 is mainly caused by increased infiltration of mononuclear cells into the lamina propria.

Chi3l3 and Chi3l4 were the first chi-lectins to be identified as mediators of Th2 inflammation during allergic reactions (Welch et al., 2002, Webb et al., 2001). Numerous publications describe increased expression of mChi3l3 during a wide range of pathologies, but the importance of mChi3l3 participation is often disregarded in chi-lectin biology because of the lack of a true human ortholog of Chi3l3 and/or Chi3l4. Nonetheless, all three mouse chi-lectins are up-regulated in response to Th2-driven inflammation in mouse lungs, and it is believed that studying solely Chi3l1 will diminish abilities to reveal the true functions of this closely-related protein family. We aimed to understand the general biology of chi-lectins by comparing them in parallel. Chi3l1 has previously been studied in colonic epithelial cells (CECs) (Mizoguchi, 2006, Chen et al., 2011a, Kawada et al., 2008). Others demonstrate that some potentially pathogenic bacteria that are strongly associated with the development of IBD interact with Chi3l1 molecules on CECs (Kawada et al., 2008, Mizoguchi, 2006). We reported that mChi3l1 or mChi3l3 is present in CECs, but both are expressed more prominently in neutrophils and macrophages. Thus, it is possible that Chi3l1 and Chi3l3 may play distinctly different roles depending on cell type under inflammatory conditions. This study shows that changes in expression of both mChi3l1 and mChi3l3 are caused by reduced numbers of neutrophil cells in the colons of mice fed DSS and BioProtein, compared to mice fed DSS only. Because chi-lectins are up-regulated in the context of both helminth and fungal infections (Sutherland et al., 2009, Chen et al., 2007)

this study may explain why one will find the chi-lectins in such different circumstances, and demonstrates that chi-lectin expression profiles depend on the type of infection.

CONCLUDING REMARKS

The main objectives of this thesis were to increase knowledge about human chitinases, their carbohydrate-binding domains, key aromatic residues in the HCHT active site, and the role of human chitinases in inflammation. To this end, the work produced the following outcomes:

- HCHT is less processive than ChiA, which is the most efficient and processive GH18 from S. *marcescens*, but HCHT is still faster and more efficient than ChiA.
- Removal of the aromatic residues in the HCHT active site dramatically decreases its initial rate of chitosan degradation.
- Subsite -3 is more important than subsite +2 for initial degradation rate, and it also extends degradation.
- HCHT is constitutively expressed in colon, regardless of inflammation.
- Increased expression of Chi3l1 and Chi3l3 is mainly caused by increased infiltration of mononuclear cells into the lamina propria.
- Chi3l3 is both higher up regulated in acute inflammation compared to Chi3l1, and shows the highest increase for infiltrating neutrophil cells
- A Bioprotein diet reduces inflammation and neutrophil influx at the inflammation site. Chi3l1 and Chi3l3 follow that expression profile, which makes them good markers of different stages of inflammation.
- Chi3l3 has a more distinct expression in macrophages compared to Chi3l1 and might be a better marker for macrophages than Chi3l1.

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Paper I

The Effect of the Carbohydrate Binding Module on Substrate Degradation by the Human Chitotriosidase.

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Abstract

Human chitotriosidase (HCHT) is one of two active glycoside hydrolase family 18 chitinases produced by humans. The enzyme is associated with several diseases and is thought to play a role in the anti-parasite responses of the innate immune system. HCHT occurs in two isoforms, one 50 kDa (HCHT50) and one 39 kDa variant (HCHT39). Common for both isoforms is a catalytic domain with the $(\beta/\alpha)_8$ TIM barrel fold. HCHT50 has an additional linker-region, followed by a C-terminal carbohydrate-binding module (CBM) classified as CBM family 14 in the CAZy database. To gain further insight into enzyme functionality and especially the effect of the CBM, we expressed both isoforms and compared their catalytic properties on chitin and high molecular weight chitosans. HCHT50 degrades chitin faster than HCHT39 and much more efficiently. Interestingly, both HCHT50 and HCHT39 show biphasic kinetics on chitosan degradation where HCHT50 is faster initially and HCHT39 is faster in the second phase. Moreover, HCHT50 produces distinctly different oligomer distributions than HCHT39. This is likely due to increased transglycosylation activity for HCHT50 due the CBM extending the positive subsites binding surface and therefore promoting transglycosylation. Finally, studies with both chitin and chitosan showed that both isoforms have a similarly low degree of processivity. Combining functional and structural features of the two isoforms, it seems that HCHT combines features of exo-processive and endo-nonprocessive chitinases with the somewhat unusual CBM14 to reach a high degree of efficiency, in line with its alleged physiological task of being a "complete" chitinolytic machinery by itself.

Keywords: Glycoside hydrolases; recalcitrant polysaccharides; human chitotriosidase; carbohydrate binding modules; transglycosylation.

Abbreviations: HCHT, human chitotriosidase; HCHT50, the 50 kDa variant of HCHT; HCHT39, the 39 kDa variant of HCHT; AMCase, acidic mammalian chitinase; GH, glycoside hydrolase; GlcNAc (A), 2-acetamido-2-deoxy-β-D-glucopyranose; GlcN (D), 2-amino-2-deoxy-β-D-glucopyranose, CBM, carbohydrate-binding module; 4-MU, 4-methylumbelliferyl; DP, degree of polymerization; MBTH, 3-methyl-2- benzothiazolinone hydrazine; α, the degree of scission, F_a, fraction of acetylated sugar moieties, N_{cuts}, number of catalytic events before substrate dissociation

1. Introduction

Chitin is an essential structural component in the exoskeleton of crustaceans, arthropods, and insects, and is also found in the cell walls of certain fungi, algae, and in parasitic nematodes [1]. This insoluble polymer is composed of β(1-4)-linked units of 2-acetamido-2-deoxy-*β*-D-glucopyranose (GlcNAc; **A**-unit). Chitosans are a family of water-soluble, linear and binary heteropolysaccharides composed of β(1-4)-linked **A**-units and 2-amino-2-deoxy-*β*-D-glucopyranose (GlcN, **D**-unit), which can be prepared from chitin by chemical or enzymatic means, yielding varying extents of deacetylation. The metabolism of chitin in nature is controlled by enzymatic systems that produce and break down chitin, primarily chitin synthases and chitinases, respectively.

Humans do not possess chitin. Still they express two active human chitinases (EC 3.2.1.14) that are classified as family 18 glycosyl hydrolases (GH) in the CAZY database (www.cazy.org, [2]), human chitotriosidase (HCHT) and acidic mammalian chitinase (AMCase). Both chitinases are thought to play a role in anti-parasite responses of the innate human immune system [3, 4] and they are associated with several diseases. AMCase is expressed in exaggerated quantities in human asthma [5], while HCHT is a biochemical marker of macrophage activation in some lysosomal diseases like Gaucher disease [6]. Furthermore, there are indications that both human chitinases play a role in the response to fungal infections. For example, elevated levels of mammalian chitinases have been reported in guinea pig blood following systemic infection with *Aspergillus fumigatus* [7]. A recombinant form of AMCase has been shown to inhibit fungal growth *in vitro* [8]. Engraftment of microcapsules containing cells transducted with chitotriosidase gene has the potential to combat infections caused by chitinous pathogens through the prolonged delivery of recombinant chitotriosidase [9].

HCHT is known to exist in two isoforms, one 50 kDa and one 39 kDa variant, hereafter referred to as HCHT50 and HCHT39. HCHT50 is synthesized and secreted in human macrophages. A portion of the produced enzyme is further routed to lysosomes where it is converted to HCHT39 isoform by carboxyl-terminal proteolytic processing [10]. Common for both isoforms is a catalytic domain with the $(\beta/\alpha)_8$ TIM barrel fold that is characteristics for chitinases belonging to the GH18 family (Fig. 1). HCHT50 has an additional proline-rich linker region, comprising approximately 29 residues, followed by a C-terminal carbohydrate-binding module (CBM) [10]. This CBM, consisting of 49 amino acids, belongs to the CBM14 family according to the CAZy database (www.cazy.org, [2, 11]). At the time being only two NMR structures are solved (pdb codes 1dqc, Fig. 1, and 2mfk) and little is known about this particular CBM. The CBM has been shown to both interact with chitin, i.e. fungal cell walls, as well as chito-oligosaccharides [12, 13]. A typical feature for this CBM is the presence of 6 conserved cysteine residues that are able to form three disulfide bonds [14]. It also appears that the residues of Cys, Pro, and Gly, all which have significant influence in the structural constructions, are well conserved in both tachycitin and HCHT50 [15].

The catalytic domain of HCHT belongs to family 18 of the glycoside hydrolases. A common feature for this family is that it employs a substrate-assisted catalytic mechanism that involves the *N*-acetyl group of the sugar moiety bound in the −1 subsite. The *N*-acetyl group acts as the catalytic nucleophile and its attack on the anomeric carbon results in the formation of an oxazolinium ion reaction intermediate [16-20]. A nucleophilic attack of a water molecule on the oxazolinium ion reaction intermediate completes the hydrolytic reaction in what is referred to as the deglycosylation step. In principle, a chitin fragment could replace the water molecule in the deglycosylation step and the outcome of the reaction is a tranglycosylation, rather than

hydrolysis. GH18 chtinases vary in terms of their tendency to catalyze transglycosylation, a variation that has been attributed to variation in both negative and positive subsites, as well as variation in the catalytic machinery [21-23]. Notably, due to the substrate-assisted catalytic mechanism, substrate binding to family 18 chitinases is only productive if subsite −1 is occupied by an acetylated sugar.

GHs can cleave polymeric substrates at the chain ends (exo-action) or at random positions (endo-action) [24]. Each of these modes of action can also be combined with processivity. Processive GHs tend to have long and deep substrate binding clefts or even tunnels lined with aromatic amino acids [25-29]. The general idea is that processivity improves catalytic efficiency by keeping the enzyme closely associated to the substrate in between subsequent hydrolytic reactions [29].

Although HCHT seems to play a role in several diseases [30], relatively little is known about its physiological role, functional properties, and the effect the CBM14 has on substrate degradation. To gain further insight into enzyme functionality, we have expressed both forms of the chitotriosidase and compared their catalytic properties, including the degradation rates and conversion efficiencies on both soluble and non-soluble substrates and the degree of processivity.

2. EXPERIMENTAL

2.1 Protein expression and purification of the two isoforms of human chitotriosidase

For production in HEK293-6E cells two vectors were constructed designated pHCHT50 and pHCHT39 expressing HCHT including its native signal peptide and with and without the C-

terminal chitin binding domain, respectively. The genes were synthesized (Genescript) as BamHI-XbaI fragments and ligated into pTT5V5H8Q (NRC Biotechnology Research Institute) resulting in a C-terminal His-tag on the recombinant proteins produced (8xHis). All cloning steps were performed in *E. coli* DH5α. pHCHT50 and pHCHT39 were transfected into HEK293-6E cells grown in F17 medium (Invitrogen) supplemented with Kolliphor P188 (Sigma) and L-glutamate (Sigma) to final concentrations of 0.1% and 4 mM, respectively. Cells were cultivated in 90 ml medium in disposable 500 ml flasks with gentle shaking (70 rpm, at 37^{o}C, with 5% CO_2 and 80% humidity). Transfection was performed with PEIpro (Polyplus) when the cell density in the cultures was 1.7x10^{6} cells/ml. 48 hours after transfection, tryptone N1 feeding medium (TekniScience) was added to a final concentration of 0.5%, and harvesting of the protein containing culture supernatant was performed 96 h after transfection. The cell density at harvesting was 2.2x10^{6} cells/ml. Recombinant HCHT was purified using a HisTrap HP column (GE Healthcare) according to the instructions given by the manufacturer. Enzyme purity was verified by SDS-PAGE and estimated to be >95% for both proteins. Protein concentrations were determined by using the Bradford-method from BioRad. Chitinolytic activity was determined using the analogue 4-MU-$(GlcNAc)_2$ (4-methylumbelliferyl-β-D-N,N`-diacetylchitobiose). This is a trimer-analogue containing two sugar moieties bound to a fluorescent group (4-methylumbelliferyl)[31].

2.2 Degradation of chitin for determination of enzyme processivity

Squid pen β-chitin, 180 μm, from France Chitine (Marseille, France) was dissolved to a final concentration of 2.0 mg/ml in 50 mM sodium acetate buffer pH 6.1. The reaction tubes

were further sonicated for 20 minutes in a sonication bath (Transsonic, Elma). This to increase the surface of the substrate and thereby increase substrate availability for the enzymes [32]. A total concentration of 2.5 µM enzyme was added. To avoid settling of chitin particles the samples were incubated at 37 °C in an Eppendorf thermo mixer at 800 rpm. Aliquots of 75 µl were withdrawn at regular time intervals from 0 to 144 hours, and the enzymes were inactivated by adding 75 µl 20 mM H_2SO_4. Prior to HPLC analysis of soluble products, samples were filtrated through a 0.45 µm Duapore membrane (Millipore) to remove denatured protein and chitin particles. All reactions were run in multiple duplicates, and all samples were stored at –20 °C until HPLC analysis. The degree of degradation is defined by the percentage of number of moles solubilized GlcNAc-units with respect to number of moles GlcNAc-units in solid form (chitin) used in the experiments.

2.3 Degradation of chitin for determination of apparent k_{cat}

Squid pen β-chitin, 180 µm, from France Chitine (Marseille, France) was dissolved to a final concentration of 2.0 mg/ml in 50 mM sodium acetate buffer pH 6.1. The reaction mixtures were preincubated in a 37°C heatingblock (Transsonic, Elma) without mixing for 10 min. The samples were further incubated with a total concentration of 170 nM enzyme. Samples were mixed randomly by hand to mimic nature. Aliquots of 75 µl were withdrawn at regular time intervals between 0-16 min. The enzymes were inactivated by adding 75 µl 20 mM H_2SO_4. Prior to HPLC analysis all samples were filtrated through a 0.45 µm Duapore membrane (Millipore) to remove denatured protein and chitin particles. All reactions were run in duplicate, and all samples were stored at –20 °C until HPLC analysis. The substrate concentration was high (20

mg/mL chitin corresponds to a dimer concentration in the order of 25 mM) to create substrate saturating conditions. Reactions carried out at even higher chitin concentrations (30 to 50 mg/mL) gave similar initial rates, confirming that substrate concentrations indeed were saturating.

2.4 High performance liquid chromatography

Concentrations of monomer and dimer were determined using a Dionex Ultimate 3000 HPLC system equipped with a Rezex Fast fruit H^+ column (100 x7.8mm) (Phenomonex). An 8 µl sample was injected on the column, and the mono and oligosaccharides were eluted isocratically at 1 ml/min with 5 mM H_2SO_4 at 85 °C. The chito-oligosaccharides were monitored by measuring absorbance at 210 nm, and the amounts were quantified by measuring peak areas that were compared with peak areas obtained with standard samples with known concentrations of mono- and disaccharides.

2.5 Degradation of High-Molecular Mass Chitosan with F_A Values of 0.63, 0.49, and 0.35.

Chitosans with different fractions of N-acetylated units (F_A) were prepared by homogeneous de-N-acetylation of chitin [33]. The characteristics of the chitosans in this study are listed in Table 1.

Chitosan with an acetylation degree (F_A) of 63% was dissolved in 80 mM sodium acetate buffer, pH 5.5, and dH_2O (1:1) to a final concentration of 10 mg/mL. Degradation reactions with chitosan contained 10 mg/ml chitosan, 0.1 mg/mL BSA, and 0.075 µg of HCHT/mg of chitosan.

Samples were withdrawn at regular time points between 30 seconds and 9 days and the chitinase was inactivated by adjusting the pH to 2.5 by adding 1 M HCl, followed by two minutes of boiling. Reactions with chitosans with F_A values of 0.49 and 0.35 were carried out in the same manner.

The degree of scission, α, indicates the fraction of glycosidic linkages that have been cleaved by the enzyme and can be determined by monitoring the amount of reducing end resonances relative to the amount of resonances from internal protons in a ^{1}H NMR spectrum, as described previously [34]. The degree of scission 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.

2.6 Separation of chitosan degradation products

Oligomeric products resulting from the enzymatic depolymerization of chitosan were separated by size-exclusion chromatography using three Superdex 30 columns from GE Healthcare coupled in series (overall dimension 2.60 cm × 180 cm), in 0.15 M ammonium acetate pH 4.5. The flow rate was 0.8 ml/min and products were monitored using a RI detector. By using this method, oligomers were separated by degree of polymerization, i.e. number of sugar units, (DP) only, except for oligomers with low DPs (<5). At low DP there is also some separation according to sugar composition.

2.7 Proton NMR

Samples from enzymatically depolymerized chitosan were lyophilized and dissolved in D_2O, after which the pD was adjusted to 4.2 using DCl. The ^{1}H NMR spectra were recorded at

85 °C and 300 MHz (Oxford NMR300, Varian) [35, 36]. 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 average degree of polymerization (DP_n) was determined using the anomer (H-1) resonances as follows: DP_n = (area of H-1 resonances of internal and reducing end sugars) / (area of H-1 resonances of reducing end sugars) [34]. The degree of scission was calculated as $\alpha = 1/DP_n$.

2.8 Experiments with the simultaneous determination of relative viscosity and reducing ends

For determination of the relative viscosity and reducing ends of solutions, chitosan with an F_A of 0.63 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 [37] were performed as described by Sikorski et al [38].

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}). The inverse of the slopes of the lines represents the number of cuts (N_{cuts}) per formation of an enzyme− substrate complex. The observed number of cuts is expressed as a relative number, where N_{cuts} observed for acid hydrolysis is set to 1.

3. RESULTS

3.1 Degree of processivity

Studies with high molecular weight chitosan can give valuable insight into the degree of processivity [34, 38]. Processivity in family 18 chitinases leads to a diagnostic product profile dominated by even-numbered products early in the reaction with chitosan along with a slow disappearance polymers (DP > 40). Previous experiments have shown that HCHT39 degrades chitosan primarily via an endoprocessive mechanism as would be expected on the basis of the structural features of its substrate-binding cleft [39]. Still, the degree of processivity was considered to be low. Only at α below 0.01 there is a stronger tendency for dominance of even oligomers compared to odd. Even at α = 0.03, this tendency is reduced and is not seen at α = 0.08 (Fig. 2). Nonetheless, the polymer peak remains present at α above 0.13. When degrading the same chitosan under identical conditions with HCHT50, several interesting observations were made. Firstly, variations occur in the relative size of the polymer peaks and the relative size and distributions of the oligomer peaks at identical time points in the analyses. Aguilera *et al*. have previously shown that HCHT50 exhibits strong transglycosylation activity [23]. Considering the size of the substrate with an average molecular weight of 280 000 (Table 1), which corresponds to an average DP of ~1500 sugar moieties, initial endo-attack coupled with hydrolysis as well as transglycosylation will create an overabundance of new molecules likely to vary in size distributions for each experimental parallel. Secondly at low α of 0.07, HCHT50 show little tendency for dominance of even numbered oligomers (Fig. 2). Still, HCHT50 also show a slow disappearance of the polymer peak (α > 0.13) as observed for HCHT39. It is natural to compare the obtained results to those of the chitinases from *Serratia marcescens*, an efficient degrader of chitin, with a small yet complete chitinolytic machinery that has been used as a model system for enzymatic chitin degradation [40, 41]. For the endo-nonprocessive ChiC, there are no even-numbered oligomer domination at any stage of the reaction, and the polymer peak disappears

when $\alpha \approx 0.05$ (Fig. 7 in Horn *et al*. [42]). For the processive ChiA and ChiB, the polymer peak disappears when $\alpha \approx 0.20$ where also the even-numbered oligomer domination stops (Fig. 5 and 6 in Horn *et al*. [42]).

The degree of processivity may also be measured quantitatively by plotting the total amount of reducing ends (α_{tot}) created after chitinase-catalyzed hydrolysis against the amount of polymeric ends as measured by the reduction in viscosity (α_{pol}) [38]. HCHT39 and HCHT50 depolymerization of chitosan with an F_A value of 0.70 showed 2.0 and 1.4 cuts per formation of an enzyme-substrate complex, respectively, compared to acid catalyzed chitosan hydrolysis, which is completely random and set to be 1 [38]. The nonprocessive ChiC also show an N_{cuts} = 1.0. The same numbers are 9.1 and 3.4 cuts per formation of the enzyme−substrate complex for ChiA and ChiB, respectively, for a chitosan substrate with F_A of 0.65 [38]. It is conceivable that the analysis of processivity for the HCHT isoforms to some extent is disturbed by transglycosylation. 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.

Moreover, the degree of processivity may be assessed by degradation of chitin. Sugar units in chitin are rotated 180° relative to their neighboring residues making the smallest structural unit a disaccharide. Upon formation of a productive enzyme-substrate complex, be it an exo-binding or an endo-binding complex, the first cleavage may result in an odd or even numbered oligosaccharide, but all subsequent processive cleavages will result in the release of disaccharides. Odd numbered products will eventually always yield a single monomer. Thus, apparent enzyme processivity (P^{app}) can be assessed on the basis of the $(GlcNAc)_2$/GlcNAc product ratio [43-45]. This approach has several pitfalls, like the assumption of the exclusive

formation of odd numbered oligosaccharides from the first cleavage which may not hold as different enzymes may have different preferences for the orientation of the chain end relative to the polymer surface or different probability of endo-mode initiation. Moreover, P^{app} tends to decrease as the substrate is consumed, most likely because the substrate becomes enriched for more recalcitrant parts where there are less obstacle-free paths for processive enzymes [44, 46, 47]. It is thus important to assess processivity during the early stages of the reaction.

During the initial phase of the reaction, degradation of β-chitin yielded a $[(GlcNAc)_2]/[GlcNAc]$ ratio of 11.4 ± 1.3 for HCHT39 and 7.6 ± 0.3 for HCHT50, respectively (Fig. 3). For ChiA, the exo-processive chitinase of *S. marcescens* acting from the reducing end of the polymer, a $[(GlcNAc)_2]/[GlcNAc]$ ratio of 30.1 ± 1.5 has been reported [44], whereas this value is 24.3 ± 2.0 for ChiB, the exo-processive chitinase of *S. marcescens* acting from the non-reducing end of the polymer [41]. For the nonprocessive ChiC, the ratio is 14.3 ± 1.4. Even though determination of P^{app} through $[(GlcNAc)_2]/[GlcNAc]$ ratio has been confirmed by the use of high speed atomic force microscopy (HS AFM) for ChiA and ChiB [48], the lower $[(GlcNAc)_2]/[GlcNAc]$ ratios for HCHT50 and HCHT39 vs. ChiC suggest that these ratios should not be used as absolute values for the degree of processivity. Still, the results obtained from degradation of chitosan suggest that both isoforms do have an inherent degree of processivity, albeit likely very small, and that the results from both degradation of chitosan and chitin show that HCHT50 is likely less processive than HCHT39.

3.2 Rate and extent of substrate degradation.

The degree of scission (α) was also monitored with respect to time for HCHT50 against a chitosan with an F_A of 0.62. Our previous studies with HCHT39 had revealed biphasic

depolymerization kinetics with apparent rate constants (k_{cat}^{app}) of 102 s^{-1} and 14 s^{-1}, for the initial and later phases of the reaction, respectively, and a maximum α of 0.33. The fast phase of the reaction likely reflects cleavage of optimal sequences containing at least three acetylated units in a row (**AA-A** stretch), whereas the slower phase reflects cleavage of less optimal sequences (the enzyme can bind productively to any sequence as long as there is an **A** bound in subsite −1, but has preferences for **A** in subsites −2 and +1; [39]). Two experimental parallels for degradation of chitosan by HCHT50 with respect α with under identical conditions is shown in Fig. 4. Firstly, in line with observed variations occurring in the relative size of the polymer peaks and the relative size and distributions of the oligomer peaks at identical time points in the analyses discussed above, clear variations in α values at identical time points are observed. Secondly, HCHT50 catalyzed degradation also has a very strong biphasic character, with a much faster initial rate compared to HCHT39, followed by a phase that is slower than that of HCHT39. Again, the large variations are likely due to the creation of an overabundance of new molecules by HCHT50. The complexity of the obtained datasets precludes the determinations of apparent rate constants for the two different phases. Final α of 0.28 was determined after 7 days of incubation (Fig. 5). Remarkably, this value is 0.33 for HCHT39. The chito-oligosaccharide mixtures obtained at maximum degree of scission were analyzed using size exclusion chromatography (Fig. 6). It revealed that the two HCHT isoforms produce distinctly different oligomer distributions. While the HCHT39 almost exclusively produces shorter oligomers with a DP up to 10 and a strongly dominating dimer peak [39], HCHT50 produces longer oligomers and a high level of trimers.

To further assess the differences between HCHT39 and HCHT50, the degradation of chitosans with lower F_A values (0.49 and 0.32) to a maximal degree of scission was also studied. As expected, the distribution of the depolymerization products shifted toward higher oligomer

lengths and a lower maximum α. In all cases, HCHT50 reached a lower maximum α than HCHT39 (Fig. 5).

The efficiency of chitin degradation was also monitored (Fig. 2). Due to the recalcitrance of polysaccharides such as chitin and cellulose, it is difficult for a single GH to degrade such a substrate completely [49]. Often, a combination of GHs with complementary activities, e.g. exo-acting from opposite ends and endo-acting, is required, [44, 45, 50] and recent works show that additional oxidizing enzymes called lytic polysaccharide monooxygenases also are important [41, 51]. It is interesting to note that HCHT50 is capable of fully degrading the chitin substrate used in this study, while HCHT39 is only capable of degrading approximately 15 %. In comparison, all three GH18s of *S. marcescens* (ChiA, ChiB, and ChiC) are needed to fully degrade the same substrate under identical conditions [44]. ChiA alone was able to convert 74 %, while ChiB and ChiC reached 45 % and 28 %, respectively. It is a slight paradox that the apparent, less processive HCHT50 is more efficient than the more processive ChiA as it is, as stated above, both suggested and observed that processivity normally improves catalytic efficiency of recalcitrant polysaccharide degradation. The initial apparent catalytic rate constants ($k_{cat,app}$) were 0.55 ± 0.02 s^{-1} for HCHT39 and 0.81 ± 0.10 s^{-1} for HCHT50. In comparison, k_{cat}^{app} ChiA acting on the same substrate under identical conditions is 0.54 s^{-1} [52].

4. DISCUSSION

The data detailed above clearly show that the CBM14 present in HCHT50 has a major impact on enzyme functionality. Compared to HCHT39, HCHT50 shows higher initial hydrolysis rates on chitin and chitosan, and a seemingly slightly lower degree of processivity. The CBM14 is essential for degrading the chitinous material used in this study, and its strong

role in substrate binding is illustrated by the somewhat paradoxal results obtained in chitosan degaradtion where HCHT50 reaches a lower maximum α than HCHT39 for the chitosans tested. The most likely explanation for this finding lies in the transglycosylation activity of HCHT. As stated above, Aguilera *et al*. have previously shown that the 50 kDa isoform exhibits strong transglycosylation activity [23] and from studies on other chitinases, it is known that strong substrate affinity in positive subsites, which promotes binding of sugar acceptors, promotes transglycosylation [53-56]. The 49 amino acid residue large CBM14 of HCHT50 is attached through its 29 residue linker to the protein C-terminus similar to what is observed in ChiB (49 and 26 amino acid residues in the CBM and linker, respectively) where it extends the positive subsite binding surface [57, 58]. This suggests that the CBM14 of HCHT50 prolongs the positive subsite binding surface as well compared to HCHT39. In the case of a soluble polymeric substrate such as chitosan, the presence of the CBM14 is thus likely to promote transglycosylation, since it will promote binding to oligomeric and even polymeric sugar acceptors. Transglycosylation activity will increase the average lengths of the products, as indeed observed (Fig. 3) and generates new substrate molecules that eventually will become enriched for unfavorable, i.e. less cleavable sequences, thus reducing overall cleavage yields, as is also observed (Fig. 4).

CBMs have two general roles with respect to function for its associated catalytic modules: i) a proximity effect and ii) a targeting function [11]. In addition, it has been suggest that CBMs can have a disruptive function as well [59]. To better understand the function of CBMs, two other classifications have been suggested in addition to division of CBMs into families based on their sequence similarity in CAZY: i) fold family where the CBMs can be grouped based on the conservation of protein fold, and ii) type surface binding family where the

CBMs are grouped based on structural and functional similarities [11]. There are seven Fold families and three Types for relationship between structure and function. The CBM of HCHT, CAZY family 14, is placed fold family 7, unique; containing hevein-like fold. Hevein folds comprises predominantly coil, but also have a small region of helix. Such CBMs bind optimally to tetrasaccharides [11]. The three structure function families are Type A surface binding CBMs, Type B glycan chain-binding CBMs, and Type C small-sugar binding CBMs. Type C is a unique class with lectin-like properties that optimally bind to mono-, di-, or tri-saccharides and lacks the extended binding-site groove of Type B CBMs. Recently, a refinement of Types B and C have been proposed where Type B CBMs are redefined as those that bind internally on glycan chains (endo-type), CBMs that bind to the termini of glycan chains are defined as Type C modules (exo-type) [60]. The CBM14 family is classified as a Type C CBM. The oligomer binding ability for the CBM of HCHT strengthens the idea of an extended binding surface resulting in increased transglycosylation activity of HCHT50 compared to HCHT39.

Type A surface binding CBMs are normally associated with degradation of crystalline cellulose or chitin [11]. Such CBMs typically have a planar platform of surface exposed aromatic amino acids. ChiB and ChiC both have Type A CBMs (CBM5 and CBM12, respectively, [11, 41] while ChiA have a flat fibronectin III like module containing exposed aromatic residues that are important to substrate binding [61]. Still, HCHT50 is more efficient in the degradation of the same chitin substrate. There are two significant differences besides the nature of the CBM between HCHT and the most efficient chitinase of *S. marcescens*; ChiA. Firstly, the area of solvent-exposed aromatic amino acids in the active site cleft in both enzymes is significantly larger for HCHT than ChiA where former has a Trp-Tyr-Trp-Trp-Trp-Trp while ChiA has Phe-Tyr-Trp-Trp-Trp-Phe (Fig. 1). This may increase the number of favorable CH-π interactions

between the sugar rings of the substrate and the aromatic residues of the enzyme and by this increasing both rate and strength of binding [62].

Secondly, HCHT has a more open active site than ChiA [57, 63]. A consequence of this is that more water is displaced upon binding as observed by changes in solvation entropy from HCHT ($-T\Delta S_{solv}^{\circ} = -41.8$ kJ/mol) compared to ChiA ($-T\Delta S_{solv}^{\circ} = -20.4$ kJ/mol) upon allosamidin binding, a well-known family18 chitinase inhibitor binding from −3 to −1, [64]. Interestingly, the observed solvation entropy change of HCHT is equal to that of the nonprocessive endochitinase ChiC ($-T\Delta S_{solv}^{\circ} = -39.3$ kJ/mol, [65]) of *Serratia marcescens* that do have a shallow and open active site [66]. ChiC, though, have fewer aromatic residues in the active site compared to HCHT and ChiA and are completely lacking those in subsites +1 and +2. It thus appears as if HCHT combines features of exo-processive (i.e. ChiA), endo-nonprocessive (i.e. ChiC) chitinases and a somewhat unusual CBM14 to reach a high degree of efficiency, in line with its alleged physiological task of being a "complete" chitinolytic machinery by itself.

Acknowledgments

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Table 1. Characterization of chitosans[a]

chitosan (F_a)	[η] (mL/g)	MW
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

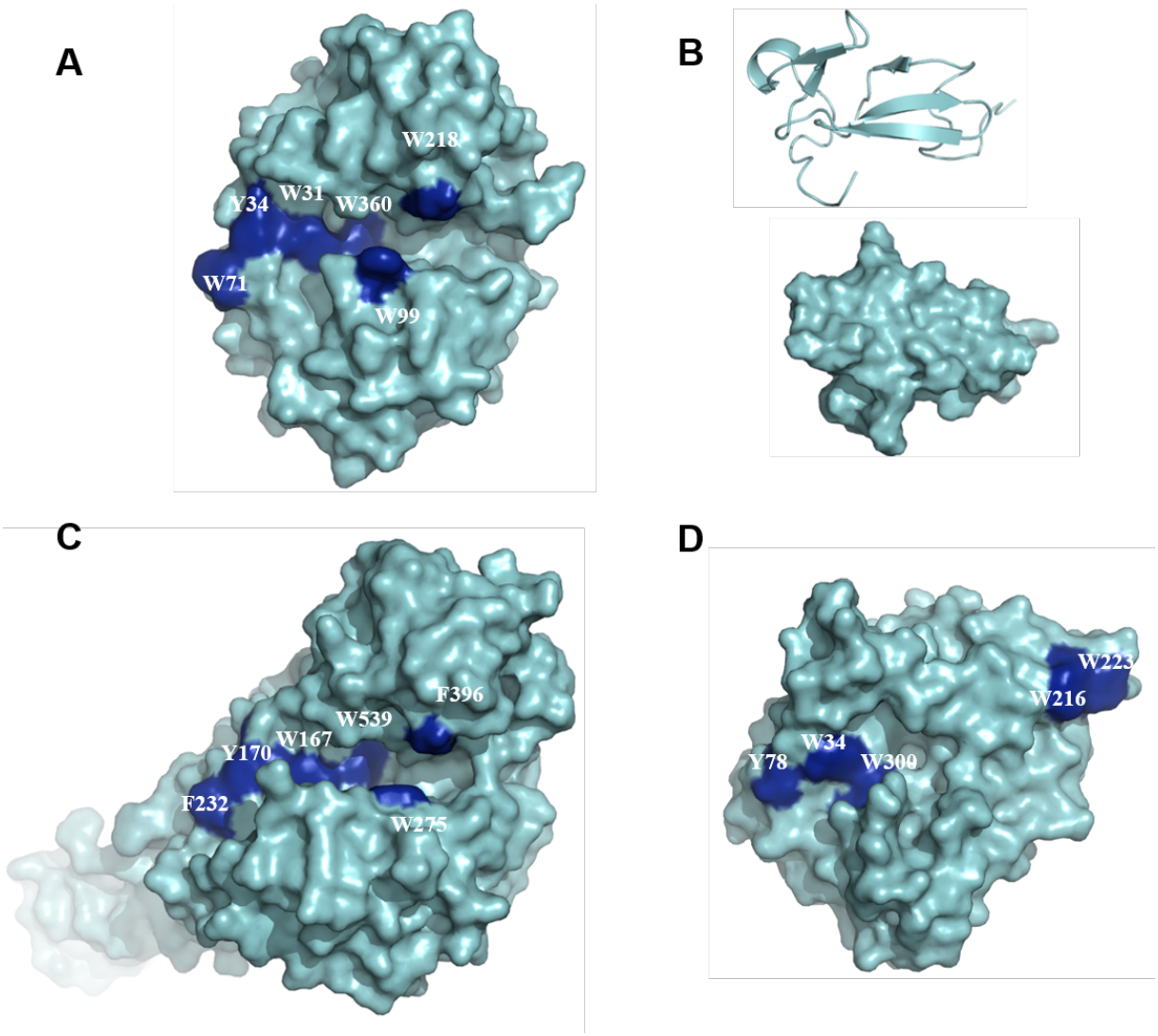

Fig. 1. Structures of HCHT39 (A, [57], pdb code 1guv), tachycitin, an example of CBM family 14 (one of only two available structures of CBM14) (B, [15], pdb code 1dqc), ChiA (C, [63], pdb code 1ctn), and ChiC (D, [66], pdb code 4axn). Both HCHT and ChiA have solvent-exposed aromatic amino acid motif in the active site cleft, which are highlighted in blue. The active site openness of HCHT is intermediate to that of ChiA and the nonprocessive endo active ChiC.

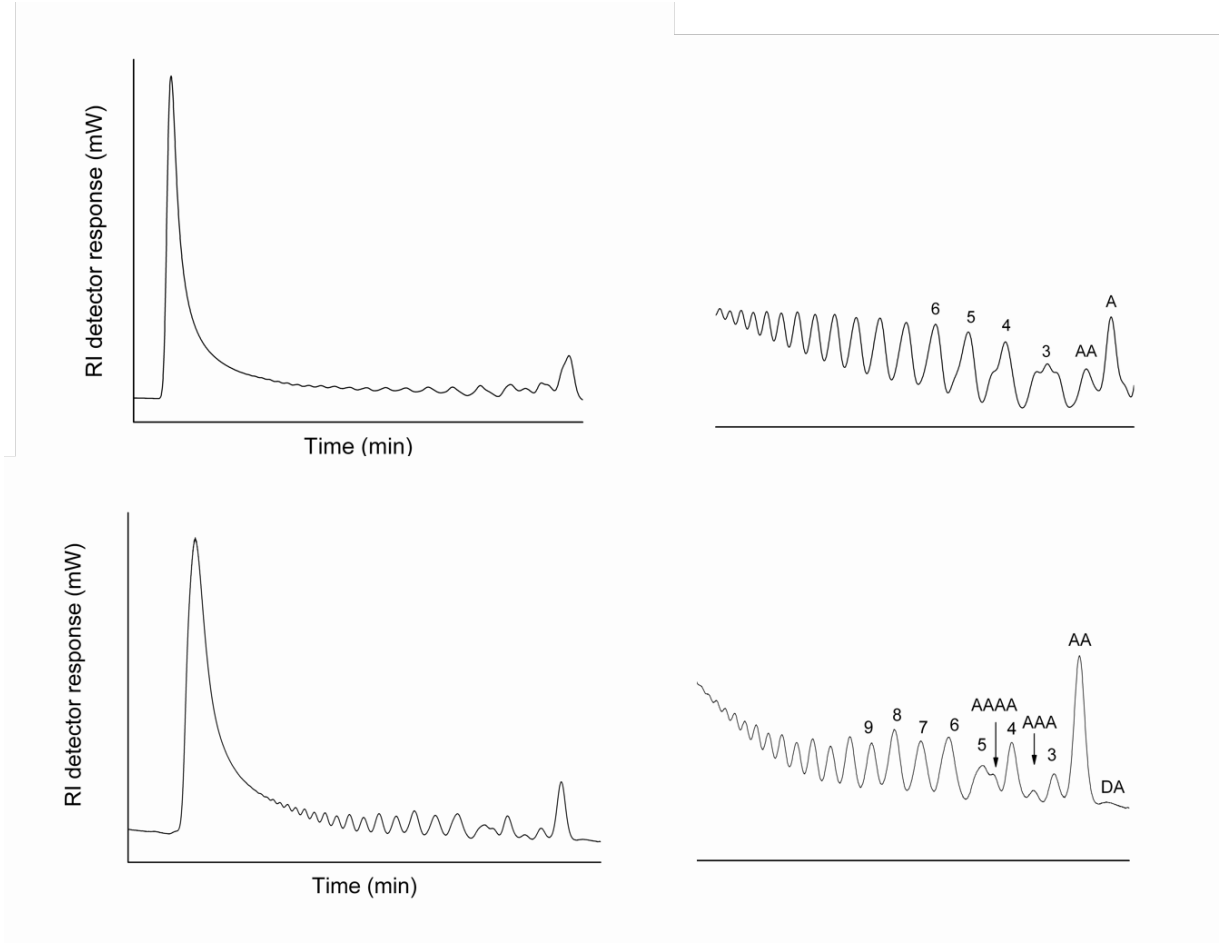

Fig. 2. SEC chromatograms for the degradation of F_A = 0.62 chitosan with HCHT50 (top) and HCHT39 (bottom) at ~7 % degradation. At this level of degradation, no tendency for dominance of even oligomers are observed that are typical for processive degradation. Still, the polymer peak is clearly present, which is not the case for nonprocessive endo-action.

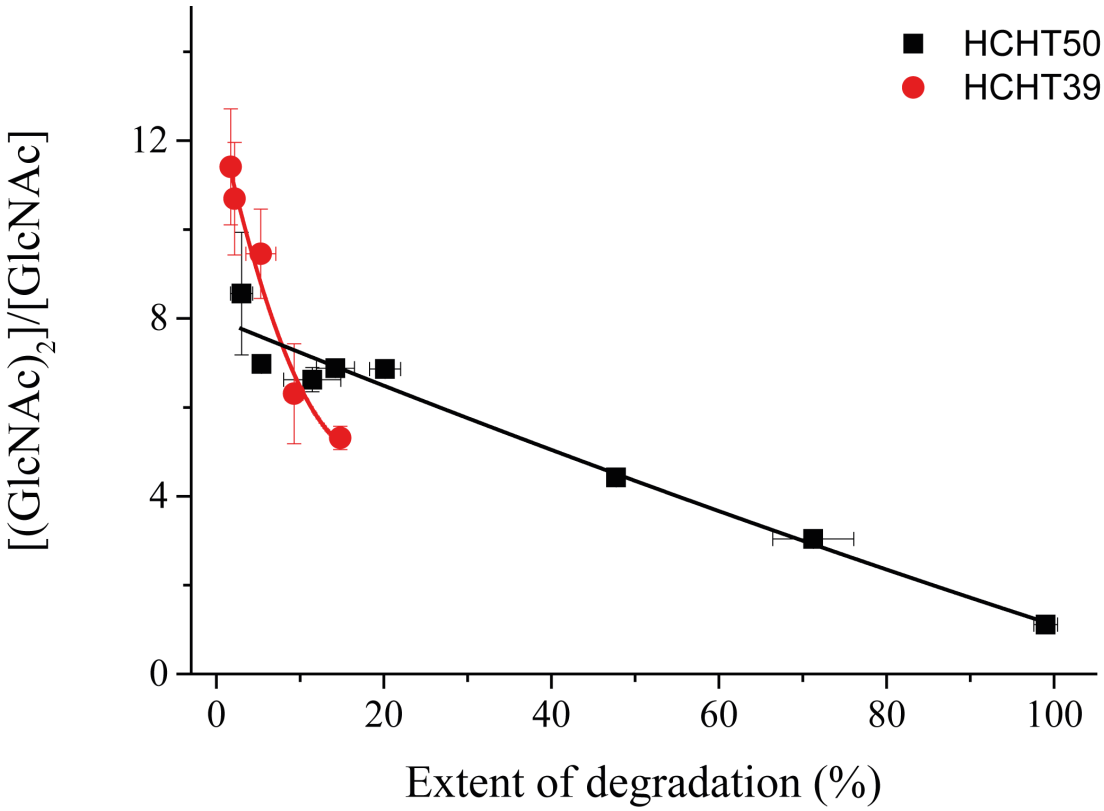

Fig. 3. Comparison of the $[(GlcNAc)_2]/[GlcNAc]$ ratio against extent of degradation for HCHT50 and HCHT39.

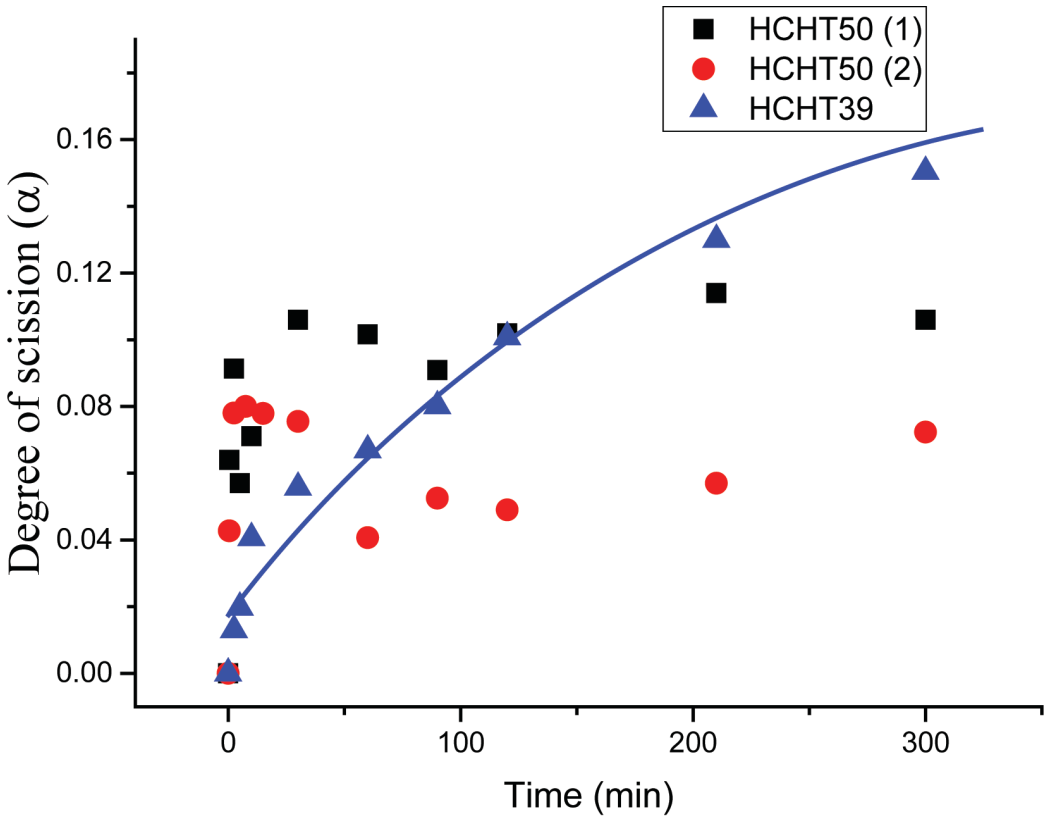

Fig. 4. Time course for degradation of F_A = 0.62 chitosan with HCHT39 (▲) and two independent experimental parallels for HCHT50, parallel 1(■) and parallel 2 (●). The graph shows the degree of scission, α, as a function of time. The data shown from HCHT39 are from Eide *et al*., 2012. Progress curves were recorded up to seven days and the results are described in the main text.

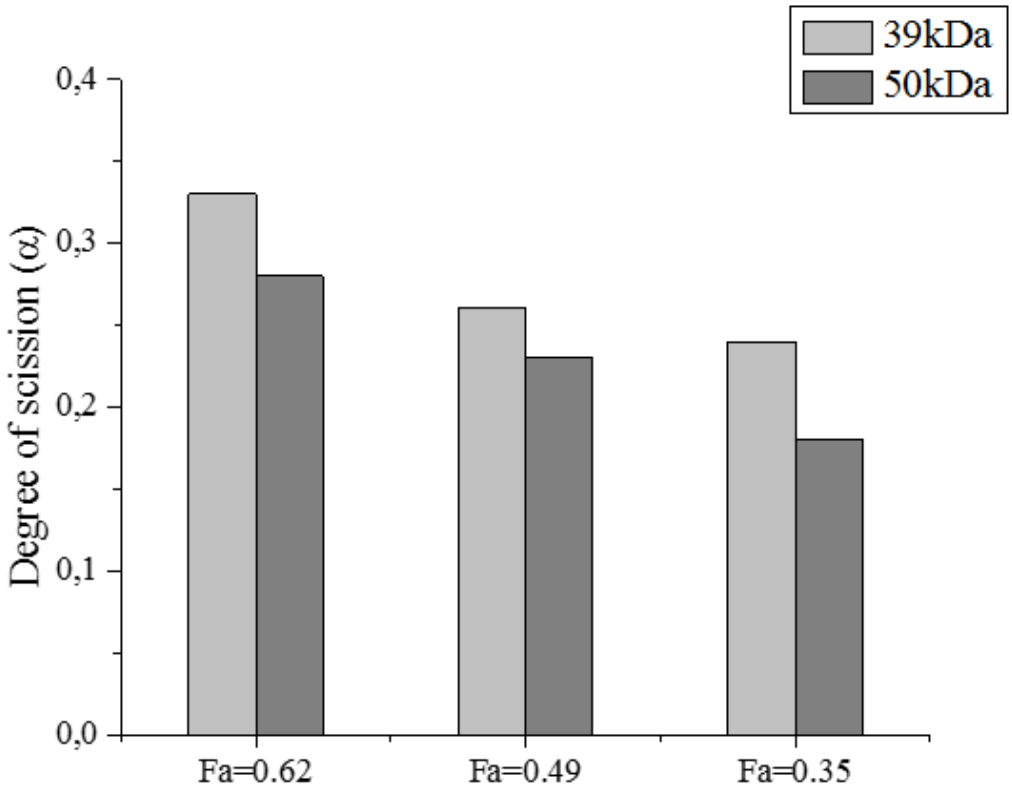

Fig. 5. Degradation of chitosans with varying F_A values to maximum degree of scission. To ensure that maximum α values were reached, samples were collected and analyzed after it had been established that addition of fresh enzyme to the reaction mixtures did not produce a further increase in α. Light grey bars represent HCHT39, while dark grey bars represent HCHT50. Data for HCHT39 are from Eide *et al*., 2012.

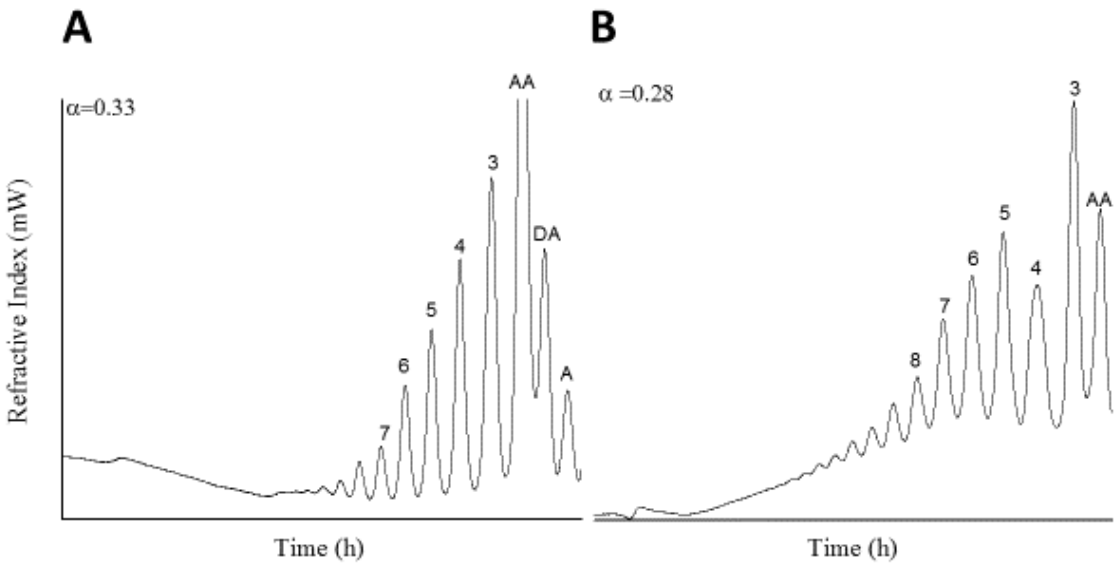

Fig. 6. Size exclusion chromatograms of oligomers obtained after degradation of F_A = 0.62 chitosan to maximal degrees of scission (α) by HCHT39 (A) and HCHT50 (B). Peaks are labeled with DP values or sequence. Clear differences are that longer oligomers are observed for HCHT50 and that the amount of **AA** is significantly higher for HCHT39 (monomers, **A**, are masked by the presence of salt in the chromatogram for HCHT50 and not shown in the Figure). The data for HCHT39 are from Eide *et al*., 2012.

Paper II

The Role of Active Site Aromatic Residues in Substrate Degradation by the Human Chitotriosidase.

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Abstract

Human chitotriosidase (HCHT) is a glycoside hydrolase family 18 chitinase synthesized and secreted in human macrophages. The enzyme is thought to be an innate part of the human immune system. It consists of a catalytic domain with the $(\beta/\alpha)_8$ TIM barrel fold having a large area of solvent-exposed aromatic amino acids in the active site. In addition, it has a family 14 carbohydrate-binding module. To gain further insight into enzyme functionality and especially the effect of the active site aromatic residues, we expressed two variants with mutations in subsites on either side of the catalytic acid, subsite −3 (W31A) and +2 (W218A), respectively. We compared their catalytic properties on both chitin and high molecular weight chitosans. Exchange of Trp to Ala in subsite −3 results in a 12-fold reduction in efficiency and a 20-fold reduction in k_{cat}^{app}, while the values are 5-fold and 10-fold for subsite +2. Moreover, aromatic residue mutation resulted in a decrease of the rate of chitosan degradation contrasting previous observations for bacterial family 18 chitinases. Interestingly, the presence of product polymers of 40 sugar moieties and higher starts to disappear already at 8 % degradation for HCHT50-W31A. Such behavior contrasts that of the wild type and HCHT-W218A and resembles the action of endo-nonprocessive chitinases. The combined results may indicate that the enzyme moves from the reducing end towards the non-reducing end of the polymer during degradation.

Keywords: Glycoside hydrolases; recalcitrant polysaccharides; human chitotriosidase; aromatic residues; processivity.

Abbreviations: HCHT, human chitotriosidase; HCHT50, the 50 kDa variant of HCHT; HCHT39, the 39 kDa variant of HCHT; AMCase, acidic mammalian chitinase; GH, glycoside hydrolase; GlcNAc (A), 2-acetamido-2-deoxy-*β*-D-glucopyranose; GlcN (D), 2-amino-2-deoxy-*β*-D-glucopyranose, CBM, carbohydrate-binding module; 4-MU, 4-methylumbelliferyl; DP, degree of polymerization; MBTH, 3-methyl-2- benzothiazolinone hydrazine; α, the degree of scission, F_a, fraction of acetylated sugar moieties.

1. Introduction

Chitin is the second most abundant biopolymer in nature after cellulose and an essential structural component found in a number of biological systems such as exoskeleton of crustaceans, arthropods, and insects, in the cell walls of certain fungi, algae, and in parasitic nematodes (Palli and Retnakaran, 1999). Chitin is composed of β(1-4)-linked units of 2-acetamido-2-deoxy-*β*-D-glucopyranose (GlcNAc; **A**-unit) rotated 180° relative to each other. The metabolism of chitin in nature is controlled by enzymatic systems that produce and break down chitin, primarily chitin synthases, and chitinases, respectively.

The human genome codes for eight family 18 glycoside hydrolases (GHs). This despite that mammals neither synthesize nor use chitin as nurture. Still, only two of the eight chitinases have shown chitinolytic activity, namely human chitotriosidase (HCHT) and acidic mammalian chitinase (AMCase) (EC 3.2.1.14). Both chitinases are associated with several diseases and are thought to play a role in anti-parasite responses of the innate human immune system (van Eijk et al., 2007, Elias et al., 2005).

HCHT exist in two isoforms, one two-domain protein of 50 kDa (HCHT50) and one single-domain protein of 39 kDa (HCHT39). Common for both isoforms are a catalytic domain characteristic for family 18 GHs, often referred to as a $(\beta/\alpha)_8$ TIM barrel. In addition, HCHT50 has a hinge region of 29 amino acids and a C-terminal carbohydrate-binding module (CBM) (Renkema et al., 1997). This particular CBM, consisting of 49 amino acids, is by the CAZy database classified in CBM family 14 (www.cazy.org) (Lombard et al., 2014, Boraston et al., 2004).

GHs are the general designation of enzymes that catalyze the hydrolysis of glycosidic bonds between different carbohydrate moieties. The GHs can either cleave

polymeric substrates randomly on the polymer chain or they can act from a chain end. This is often referred to as endo- or exo activity (Davies and Henrissat, 1995). Exo active enzymes often have a preference for either the reducing or the non-reducing chain end of the substrate. The above mentioned properties can furthermore be combined with a processive or non-processive mode of action. A processive enzyme has the ability to remain attached to the substrate in between subsequent hydrolytic reactions (Davies and Henrissat, 1995, Rouvinen et al., 1990). A typical feature for processive enzymes is a path consisting of aromatic residues near the catalytic center. Aromatic residues have shown to be important for interactions with the substrate, both regarding to hydrolysis, degradation efficiency and rate, and mediating processivity (Zakariassen et al., 2009, Kostylev et al., 2014, Horn et al., 2006a, Varrot et al., 2003).

CBMs have two general roles with respect to function for its associated catalytic modules: i) a proximity effect and ii) a targeting function (Boraston, 2004). In addition, it has been suggest that CBMs may have a disruptive function (Din et al., 1994). To better understand the function of CBMs, two other classifications have been suggested in addition to division of CBMs into families based on sequence similarity. The two other classifications being: i) fold family where the CBMs can be grouped based on the conservation of protein fold, and ii) type surface binding family where the CBMs are grouped based on structural and functional similarities (Boraston et al., 2004). There are a total of seven fold families and three types of structure function families. The different structure function families are characterized as Type A, Type B, and Type C CBMs. Type A is a surface binding CBM associated with degradation of crystalline chitin or cellulose, while Type B is a glycan-chain binding CBM (Boraston et al., 2004). Type C is defined as a small-sugar binding CBM.

Lately it has been proposed that Type B CBMs bind internally on glycan chains (endo-active), while Type C CBMs bind to the termini of glycan chains (exo-active) (Gilbert et al., 2013). The CBM of HCHT is classified as structure function family Type C.

HCHT is shown to have a rather large area of solvent exposed aromatic residues in the active site. These aromatic residues leads to an increased number of favorable CH-π interactions with the substrate (Nishio et al., 1998). Further, the topology of the active site of HCHT is more open compared to other known chitinases (Perrakis et al., 1994, Fusetti et al., 2002). These two features, combined with a rather unusual CBM, originally optimized to bind oligosaccharides, has been shown to reach a high degree of efficiency on crystalline substrate (Stockinger et al., 2015). This is in line with the idea of HCHT being a "complete" chitinolytic machinery by itself.

In this work, we have compared the catalytic properties of full-length HCHT and two variants with mutations in subsite −3 (W31A) and +2 (W218A), for both soluble polymeric substrate and non-soluble substrate. By use of size exclusion chromatography chito-oligomers were separate after partial and complete degradation of chitosan. The composition of the oligomer fractions was further to compare the degree of processivity. Hydrolytic activities were determined using crystalline β-chitin and chitosan as substrates.

2. EXPERIMENTAL

2.1Protein expression and purification of the mutants of human chitotriosidase

For production in HEK293-6E cells two vectors were constructed designated pHCHT50 and pHCHT39 expressing HCHT including its native signal peptide and

with and without the C-terminal chitin binding domain, respectively. The genes were synthesized (Genescript) as BamHI-XbaI fragments and ligated into pTT5V5H8Q (NRC Biotechnology Research Institute) resulting in a C-terminal His-tag on the recombinant proteins produced (8xHis). All cloning steps were performed in *E. coli* DH5α. pHCHT50 and pHCHT39 were transfected into HEK293-6E cells grown in F17 medium (Invitrogen) supplemented with Kolliphor P188 (Sigma) and L-glutamate (Sigma) to final concentrations of 0.1% and 4 mM, respectively. Cells were cultivated in 90 ml medium in disposable 500 ml flasks with gentle shaking (70 rpm, at 37^{o}C, with 5% CO_2 and 80% humidity). Transfection was performed with PEIpro (Polyplus) when the cell density in the cultures was 1.7x10^{6} cells/ml. 48 hours after transfection, tryptone N1 feeding medium (TekniScience) was added to a final concentration of 0.5%, and harvesting of the protein containing culture supernatant was performed 96 h after transfection. The cell density at harvesting was 2.2x10^{6} cells/ml. Proteins were purified by using Ni-NTA Agarose (Qiagen, Venlo, Netherlands) matrix. The column was pre-equilibrated in buffer A (20 mM Tris-HCl, 20 mM imidazole,500 mM NaCl, pH 8.0) before the periplasmic and cytoplasmic extracts were applied. After washing with buffer B (20 mM Tris-HCl, 500 mM NaCl, pH 8.0), fractions containing the enzyme were eluted with buffer C (20 mM Tris-HCl, 250 mM imidazole, 500 mM NaCl, pH 8.0). A flow rate of 2.5 ml / min was used at all times. Enzyme purity was verified by SDS-PAGE and estimated to be >95% for all proteins. Protein concentrations were determined by using the Bradford-method from BioRad. Chitinolytic activity was determined using the analogue 4-methylumbelliferyl-β-D-N,N`-diacetylchitobiose ((GlcNAc)$_2$-4-MU). This is a trimer-analogue containing two sugar moieties bound to a fluorescent group (4-methylumbelliferyl) (Brurberg et al., 1996) .

2.2 Degradation of chitin for determination of enzyme processivity

Squid pen β-chtin, 180 μm, from France Chitine (Marseille, France) was dissolved to a final concentration of 2.0 mg/ml in 50 mM sodium acetate buffer pH 6.1. The reaction tubes were further sonicated for 20 minutes in a sonication bath (Transsonic, Elma). This to increase the surface of the substrate and thereby increase substrate availability for the enzymes (Fan et al., 2008). A total concentration of 2.5 μM enzyme was added. To avoid settling of chitin particles the samples were incubated at 37 °C in an Eppendorf thermo mixer at 800 rpm. Aliquots of 75 μl were withdrawn at regular time intervals from 0 to 144 hours, and the enzymes were inactivated by adding 75 μl 20 mM H_2SO_4. Prior to HPLC analysis of soluble products, samples were filtrated through a 0.45 μm Duapore membrane (Millipore) to remove denatured protein and chitin particles. All reactions were run in multiple duplicates, and all samples were stored at –20 °C until HPLC analysis. The degree of degradation is defined by the percentage of number of moles solubilized GlcNAc-units with respect to number of moles GlcNAc-units in solid form (chitin) used in the experiments.

2.3 Degradation of chitin for determination of apparent k_{cat}

Squid pen β-chtin, 180 μm, from France Chitine (Marseille, France) was dissolved to a final concentration of 20 mg/ml in 50 mM sodium acetate buffer pH 6.1. The reaction mixtures were preincubated in a 37°C heatingblock (Transsonic, Elma) without mixing for 10 min. The samples were further incubated with a total

concentration of 170 nM enzyme. Samples were mixed randomly by hand to mimic nature. Aliquots of 75 µl were withdrawn at regular time intervals between 0-16 min. The enzymes were inactivated by adding 75 µl 20 mM H_2SO_4. Prior to HPLC analysis all samples were filtrated through a 0.45 µm Duapore membrane (Millipore) to remove denatured protein and chitin particles. All reactions were run in triplicates, and all samples were stored at –20 °C until HPLC analysis. The substrate concentration was high (20 mg/mL chitin corresponds to a dimer concentration in the order of 25 mM) to create substrate saturating conditions. Reactions carried out at even higher chitin concentrations (30 to 50 mg/mL) gave similar initial rates, confirming that substrate concentrations indeed were saturating.

2.4 High performance liquid chromatography

Concentrations of monomer and dimer were determined using a Dionex Ultimate 3000 HPLC system equipped with a Rezex Fast fruit H^+ column (100 x7.8mm) (Phenomonex). An 8 µl sample was injected on the column, and the mono and oligosaccharides were eluted isocratically at 1 ml/min with 5 mM H_2SO_4 at 85 °C. The chitooligosaccharides were monitored by measuring absorbance at 210 nm, and the amounts were quantified by measuring peak areas that were compared with peak areas obtained with standard samples with known concentrations of mono- and disaccharides.

2.5 Degradation of High-Molecular Mass Chitosan with F_A Value of 0.62

Chitosans, a water-soluble deacetylated polymeric chitin analogue, with N-acetylated units (F_A) fraction of 0.62 were prepared by homogeneous de-N-acetylation of chitin (Sannan et al., 1976).

Chitosan with an acetylation degree (F_A) of 62% was dissolved in 80 mM sodium acetate buffer, pH 5.5, and dH_2O (1:1) to a final concentration of 10 mg/mL. Degradation reactions with chitosan contained 10 mg/ml chitosan, 0.1 mg/mL BSA, and 0.075 μg of HCHT/mg of chitosan. Samples were withdrawn at regular time points between 30 seconds and 7 days and the chitinase was inactivated by adjusting the pH to 2.5 by adding 1 M HCl, followed by two minutes of boiling.

The degree of scission, α, indicates the fraction of glycosidic linkages that have been cleaved by the enzyme and can be determined by monitoring the amount of reducing end resonances relative to the amount of resonances from internal protons in a ^1H NMR spectrum, as described previously (Sørbotten et al., 2005). The degree of scission 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.

2.6 Separation of chitosan degradation products

Oligomeric products resulting from the enzymatic depolymerization of chitosan were separated by size-exclusion chromatography using three Superdex 30 columns from GE Healthcare coupled in series (overall dimension 2.60 cm × 180 cm), in 0.15 M Ammonium acetate pH 4.5. The flow rate was 0.8 ml/min and products were monitored using a RI detector. By using this method, oligomers were separated

by degree of polymerization, i.e. number of sugar units, (DP) only, except for oligomers with low DPs (<5). At low DP there is also some separation according to sugar composition.

2.7 Proton NMR

Samples from enzymatically depolymerized chitosan were lyophilized and dissolved in D_2O, after which the pD was adjusted to 4.2 using DCl. The ^1H NMR spectra were recorded at 85 °C and 300 MHz (Oxford NMR300, Varian) (Vårum et al., 1996, Vårum et al., 1991a). 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 degree of polymerization (DP_n) was determined using the anomer (H-1) resonances as follows: DP_n = (area of H-1 resonances of internal and reducing end sugars) / (area of H-1 resonances of reducing end sugars)(Sørbotten et al., 2005). The degree of scission was calculated as $\alpha = 1/DP_n$.

3. RESULTS

3.1 Mutant design and initial mutant characterization

As previously mentioned, HCHT is shown to have a rather large area of solvent exposed aromatic residues in the active site (Figure 1). Such residues are common for processive GHs acting on insoluble polysaccharides (Rouvinen et al., 1990). According to nomenclature for sugar-binding subsites in GHs where hydrolysis occurring between subsite −1 and +1, as for all GHs (Davies et al., 1997), HCHT has a Trp-residue on both sides of the catalytic acid; in subsites -3 (Trp^{31}) and +2 (Trp^{218}). Mutating these residues to the nonaromatic residue alanine effectively

abolishes the number of favorable CH-π interactions between the sugar ring of the substrate and the aromatic residue of the enzyme and by this decrease both rate and strength of binding (Nishio et al., 1998).

To initially assess activity of the constructed mutants, specific activities of the mutants were determined using $(GlcNAc)_2$-4-MU at pH 6.3 and compared to the value of the wild type. The results show a slight reduction in specific activity for HCHT50-W31A (91 %) and HCHT50-W218A (75 %) compared to the wild type. It is expected that a mutation in subsite −3 (Trp^{31}) would be less affected than +2 (Trp^{218}) as (GlcNAc)-4-MU productively binds from −2 to +2 (Krokeide et al., 2007).

3.2 Degradation of chitin

Previously, the initial apparent catalytic rate constant (k_{cat}^{app}) for chitin degradation has been determine to be 0.81 ± 0.10 s^{-1} for HCHT50 (Table 1) (Stockinger et al., 2015). Using the same substrate and conditions, k_{cat}^{app} was found to be 0.040 ± 0.006 s^{-1} and 0.086 ± 0.021 s^{-1} for HCHT50-W31A and HCHT50-W218A, respectively (Table 1). As comparison, the same value is 0.55 ± 0.02 s^{-1} for HCHT39. Moreover, both mutants were clearly less efficient in degrading the substrate. While the wild type is able to degrade completely the insoluble substrate, HCHT50-W31A and HCHT50-W218A were only able to degrade 8 ± 1 and 20 ± 4 %, respectively. Again as a comparison, HCHT39 is able to degrade 15 % of the same substrate. Finally, apparent processive ability (P^{app}) was determined as $[(GlcNAc)_2]/[GlcNAc]$ ratios (Hamre et al., 2014). During the initial phase of the reaction, degradation of β-chitin yielded a $[(GlcNAc)_2]/[GlcNAc]$ ratio of 8.2 ± 0.2 for HCHT50-W31A and 3.5 ± 0.4 for HCHT50-W218A, respectively. The same value is 7.6 ± 0.3 for HCHT50. This approach has several pitfalls, like the assumption

of the exclusive formation of odd numbered oligosaccharides from the first cleavage which may not hold as different enzymes may have different preferences for the orientation of the chain end relative to the polymer surface or different probability of endo-mode initiation (Hamre et al., 2014). These ratios should not be used as absolute values for processive ability. Still, all values are low and suggest a low degree of processive ability.

3.2 Degradation of chitosan

Chitosans are a family of water-soluble, linear and binary heteropolysaccharides composed of β(1-4)-linked **A**-units and 2-amino-2-deoxy-*β*-D-glucopyranose (GlcN, **D**-unit). It can be prepared from chitin by chemical or enzymatic means, yielding varying extents and pattern of deacetylation (Vårum et al., 1991a, Vårum et al., 1991b). Depolymerization of high molecular chitosan has shown to give valuable insight into processive as well as degree of endo-activity (Sikorski et al., 2006, Sørbotten et al., 2005). Processivity in family 18 chitinases leads to a diagnostic product profile dominated by even-numbered products early in the reaction with chitosan along with a slow disappearance of the polymer peak (DP > 40). Moreover, it is has also been observed that mutation of aromatic residues in the active site of processive family 18 chitinases greatly increases the speed (~20-fold) of chitosan degradation (Horn et al., 2006a, Zakariassen et al., 2009).

Firstly, we monitored the rate of chitosan degradation by determining the degree of scission (α) with respect to time for the two mutants and compared this to the wild type (Figure 2). Interestingly, the rate decreases for both mutants compared to what was observed for the wild type. Samples at ~0.05, ~0.08, and maximum

degree of scission was further investigated using size-exclusion chromatography (SEC). Samples of $\alpha = 0.05$ for HCHT50-W31A and HCHT50-W218A showed no dominance of even-numbered peaks vs. odd-numbered (results not shown), which would be typical for exo-processive chitinases, in line with what was observed for the wild type (Stockinger et al., 2015). Polymer peaks were present for both mutants and the wild type. Interestingly at $\alpha = 0.08$, the polymer peak disappears for HCHT50-W31A while it remains for HCHT50 and HCHT-W218A (Figure 3).

Further, maximum α for all mutants was determined after 7 days of incubation (Table 1 and Figure 4). Final α have previously been determined to 0.28 for HCHT50 (Stockinger et al., 2015). While, maximum degree of scission were determined to be 0.24 and 0.32 for HCHT50-W31A and HCHT50-W218A, respectively. For all full-length enzymes, trimers are the dominant product contrasting a strongly dominating dimer peak for HCHT39 (Eide et al., 2012). The mutation of Trp^{31} makes HCHT less efficient against chitosan while it is opposite for Trp^{218}.

4. DISCUSSION

Enzymatic degradation of recalcitrant polysaccharides is thought to occur through the synergistic action of GHs that have complementary activities (Henrissat et al., 1985, Merino and Cherry, 2007). Endo-acting GHs make random scissions on the polysaccharide chains, whereas exo-acting GHs mainly target single reducing and non-reducing chain ends often coupled with processivity. Moreover, lytic polysaccharide monooxygenases (LPMO), a newly discovered class of enzymes, targets crystalline regions using an activated dioxygen to cleave glycosidic bonds, creating new chain ends for exo-acting GHs and by this greatly enhances rate of depolymerization (Quinlan et al., 2011, Vaaje-Kolstad et al., 2010, Hamre et al.,

2015). In human macrophages, a single chitinase, HCHT, has to cover all these roles. It does so by combining features of endo-nonprocessive GHs (open active site), exo-processive GHs (a large area of solvent-exposed aromatic amino acids in the active site), and a somewhat unusual CBM14 (normally designed to interact with oligosaccharides). Previously, we have shown that the CBM is highly important for both rate and efficiency of insoluble substrate degradation with a 7-fold reduction in efficiency and a 1.5-fold reduction in k_{cat}^{app} (Stockinger et al., 2015). Interestingly while exchanging surface exposed aromatic residues in the active site of HCHT50 yield similar effects in loss of efficiency as the loss of the CBM. The rate of hydrolysis severely affected. Exchange of Trp to Ala in subsite −3 results in a 12-fold reduction in efficiency and a 20-fold reduction in k_{cat}^{app} and while the values are 5-fold and 10-fold for subsite +2.

Furthermore, especially efficiency of chitosan degradation and product formation is affected by the mutations in the −3 and +2 subsites. HCHT50-W31A shows a slightly lower α_{max} (0.24 vs. 0.28). The mutation of the analogue residue in ChiA (Trp^{167}) clearly affects the positioning of the substrate for the recognizing and orienting the N-acetyl groups before the –1 subsite (Norberg et al., 2011). Trp^{31} may have the same role in HCHT and by this making HCHC50-W31A less efficient than the wild type. Interestingly, α_{max} increases (0.28 to 0.32) for HCHT50-W218A. HCHT50 have an inherent high degree of transglycosylation activity (Aguilera et al., 2003, Stockinger et al., 2015). Transglycosylation activity will increase the average lengths of the products and generate new substrate molecules that eventually will become enriched for unfavorable, i.e. less cleavable sequences, thus reducing overall cleavage yields. The increase in α_{max} for HCHT50-W218A is likely the result of less transglycosylation activity as a strong binding residue in positive subsites have been

removed in line with what has been observed for both ChiA and ChiB (Zakariassen et al., 2011) and a class V chitinase from cycad (Taira et al., 2010).

Apparent processive ability (P^{app}) as determined by $[(GlcNAc)_2]/[GlcNAc]$ ratios for HCHT50-W31A and HCHT50-W218A was observed to be low, in line with observed ratios for HCHT50 and HCHT39 (Stockinger et al., 2015). Interestingly while the ratios are equal for HCHT50 and HCHT50-W31A, it is significantly smaller for HCHT50-W218A (7.6 vs. 4.5 wild type vs. mutant). Previous experiments have shown that HCHT39 degrades chitosan primarily via an endo-processive mechanism (Eide et al., 2012). Still, the degree of processivity was considered to be low. Only at α below 0.01 there is a stronger tendency for dominance of even oligomers compared to odd. Even at α = 0.03, this tendency is reduced and is not seen at α = 0.08. Nonetheless, the polymer peak remains present at α above 0.13. All full-length enzymes showed no preference for even-numbered oligomers in line with the low $[(GlcNAc)_2]/[GlcNAc]$ ratios, but retained a polymer peak at α = 0.05. Interestingly, the polymer peak starts to disappear already at α = 0.08 for HCHT50-W31A. Such behavior resembles that of the endo-nonprocessive ChiC of *S. marcescens* where polymer peak disappears at α ~0.05 (Horn et al., 2006b), suggesting that this mutant have a higher degree of endo-character than the wild type enzyme. In comparison, disappearance of long polymers takes place at α ~ 0.20 for the exo-processive ChiA and ChiB from *S. marcescens*. Moreover, ChiA and ChiB are exo-processive attacking the insoluble polymer from opposite ends; ChiA from the reducing end and ChiB from the non-reducing end (Hult et al., 2005). In line with this, negative subsites in ChiA and positive subsites in ChiB are also called substrate-binding subsites in that these remains bound to the polymeric substrate after a processive hydrolysis. When mutating Trp^{220} in the substrate-binding subsite +2 (Trp^{218} in HCHT) in ChiB,

dominance of even-numbered oligomers and the presence of long polymer chains disappears early upon chitosan degradation (Horn et al., 2006a). The same is observed when Trp^{167} in the substrate-binding subsite −3 (Trp^{31} in HCHT) is mutated (Zakariassen et al., 2009). When Phe^{396} in the product-binding subsite +2 in ChiA (Trp^{218} in HCHT) is mutated, the dominance of even-numbered peaks and the presence of long polymer chains remain intact. Furthermore, both efficiency and rate of chitin degradation is more affected (i.e. poorer and slower) when Trp^{167} is mutated compared to Phe^{396}, in line with what we observe for HCHT50-W31A vs. HCHT50-W218A. The results may indicate that negative subsites are substrate-binding subsites in HCHT as well and that HCHT moves from the reducing end towards the non-reducing end of the polymer. This would also be in line with the thermodynamic signatures of allosamidin binding to HCHT. Allosamidin is a family 18 chitinase inhibitor that specifically binds from subsite −3 to −1. Binding of allosamidin to HCHT and ChiA are similar with enthalpy and solvation entropy changes making the most favorable contributions to the free energy change, while binding to ChiB takes place with an unfavorable enthalpy change (Eide et al., 2013). A complicating factor is that $[(GlcNAc)_2]/[GlcNAc]$ ratio for HCHT50-W218A is lower than for both the wild type as well as HCHT50-W31A, which would be consistent with +2 being a substrate-binding subsite. Then again, as stated previously, caution must taken when interpreting such ratios.

A final interesting result is the effect aromatic residue mutation, both Trp^{31} and Trp^{218}, has on the rate of chitosan degradation. When such mutations are performed in ChiA and ChiB, the rate of chitosan degradation increases ~20-fold compared to the wild type enzyme (Zakariassen et al., 2009, Horn et al., 2006a). The same trend, albeit to a much smaller extent, is observed for the exo-processive

cellulase *Tf*Cel48A from *Thermobifia fusca* on phosphoric acid swollen cellulose (PASC), a mostly amorphous form of cellulose and more accessible compared to crystalline cellulose (Kostylev et al., 2014). *Tf*Cel48A works from the reducing end of the polymer, and mutations of aromatic residues inside the active tunnel entrance (subsites −5 and −4, substrate-binding subsites) resulted in an increase in hydrolysis rate (efficiency, processive ability, and hydrolysis rate on crystalline cellulose decreased consistent with what is observed for ChiA). This contrasts what we observe for HCHT where the rate of soluble substrate hydrolysis decreases. A possible explanation is that chitosan hydrolysis by HCHT50 is already very high compared to what is observed for ChiA and ChiB. The creation of an overabundance of new molecules by HCHT50 results in a high degree of complexity in obtained datasets when monitoring α vs. time and this precludes the determination of initial apparent rate constant (Stockinger et al., 2015). Still, HCHT50 initial rate is faster than that of HCHT that has an initial apparent rate constant of 102 s^{-1}. This is ~7-fold faster than what was observed for ChiA and ChiB. Moreover, the work of Horn *et al*. shows that processivity comes at a large cost of enzyme speed, and that the mutation of substrate-binding aromatic residues important for processivity increases enzyme speed when the substrate is readily accessible (Horn et al., 2006a). HCHT50 wild type appears to have low processive ability, which fits with a potential for fast degradation of the readily available substrate chitosan.

In conclusion, we show that surface exposed aromatic residues in the active site are vital for substrate degradation and important determinants for the mode of action of HCHT. Even though classical thinking is that processive ability and efficiency are linked in GH catalyzed polysaccharide degradation, it appears as if the design of HCHT makes it an intrinsically fast and efficient enzyme without being

very processive.

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Table 1. Chitin and chitosan degradation by HCHT wild type and mutants.

	HCHT50	**HCHT50-W31A**	**HCHT50-W218A**	**HCHT39**
$k_{cat}^{app, a}$	0.81 ± 0.10	0.040 ± 0.006	0.086 ± 0.021	0.55 ± 0.02
Efficiency [b]	100	8 ± 1	20 ± 4	15
$P^{app, c}$	7.6 ± 0.3	8.2 ± 0.2	3.5 ± 0.4	11.4 ± 1.3
α_{max}	0.28	0.24	0.32	0.33

[a] (s^{-1}); [b] as measured by percentage degradation of the substrate, [c] as determined by the $[(GlcNAc)_2]/[(GlcNAc)]$ ratio.

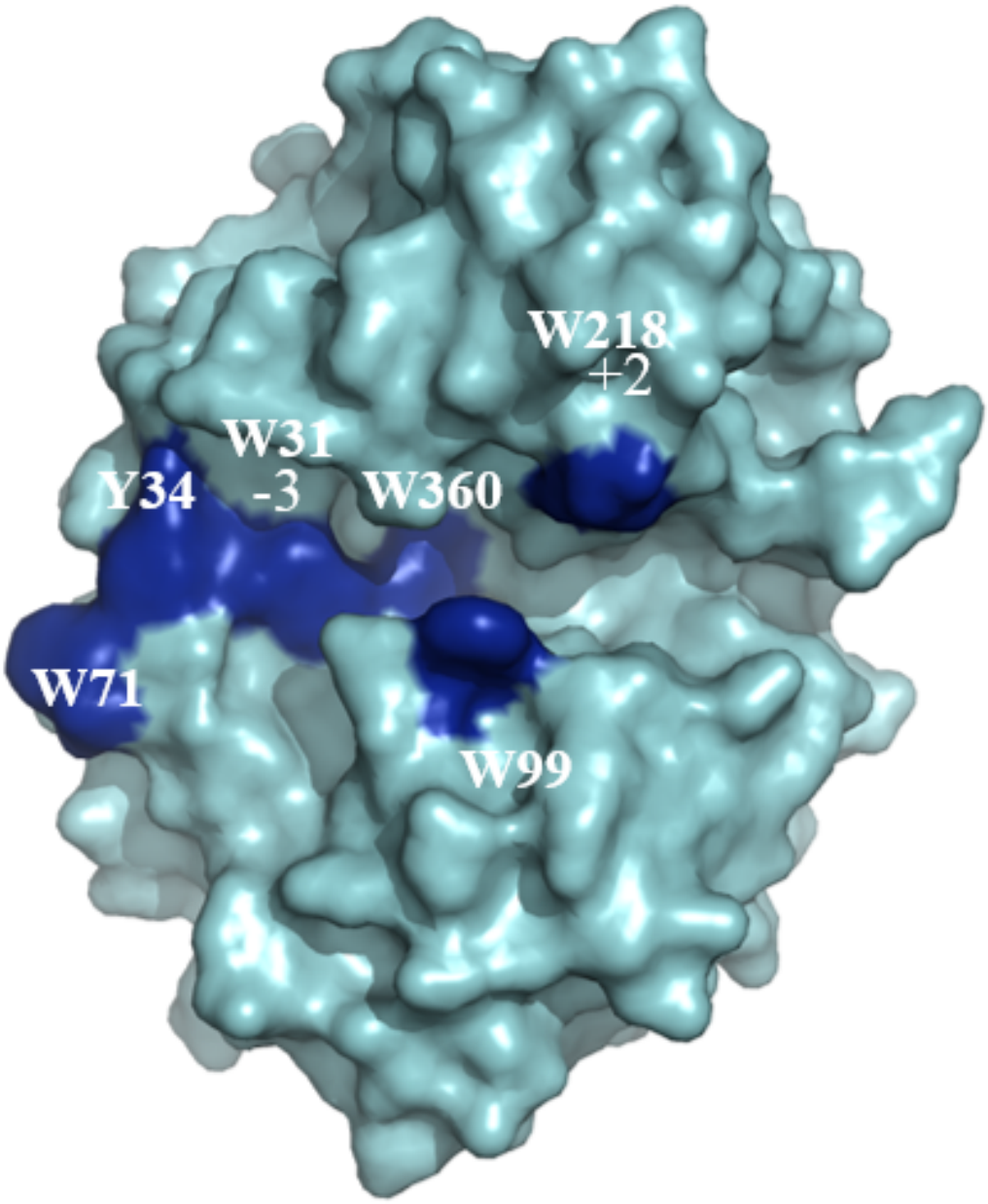

Figure 1. Structure of HCHT (the 39 kDa isoform, (Fusetti et al., 2002), pdb code 1 guv). HCHT has solvent-exposed aromatic amino acid motif in the active site cleft, which are highlighted in blue. Trp^{31} and ^{Trp218} are situated in subsite −3 and +2, respectively.

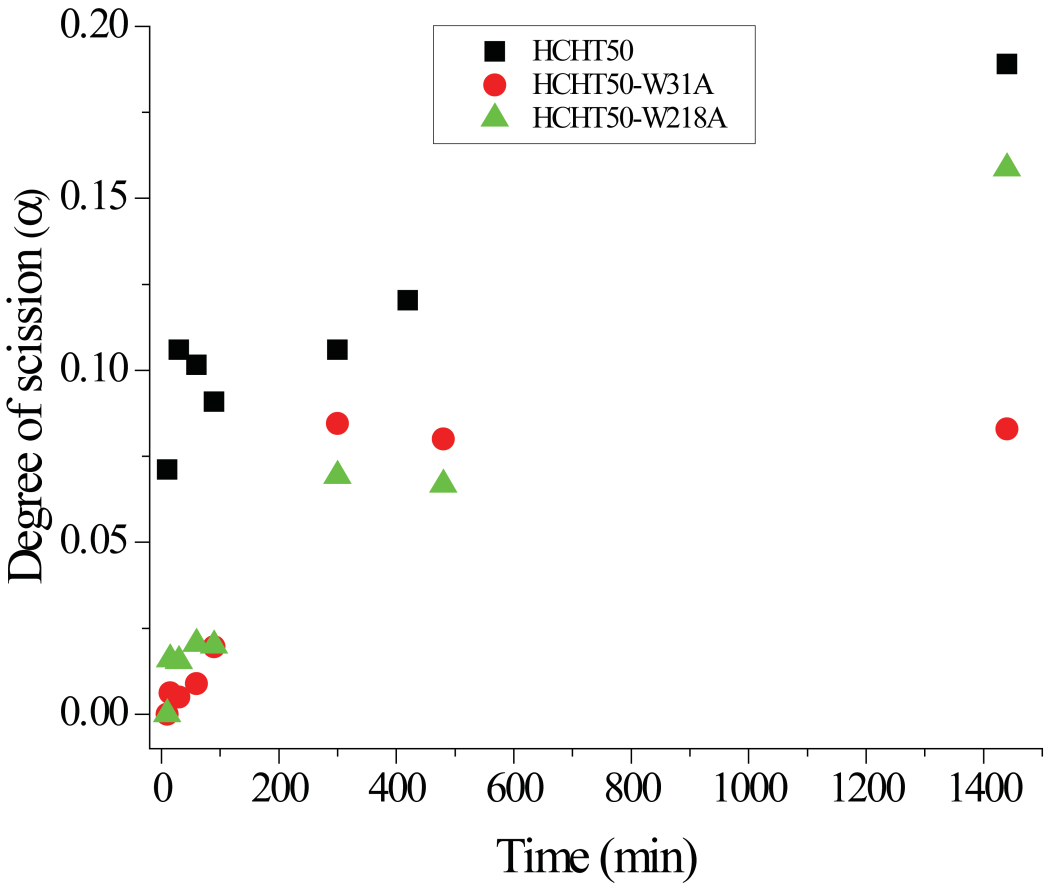

Figure 2. Time course for degradation of F_A = 0.62 chitosan with HCHT50 (■), HCHT50-W31A (●), and HCHT50-W218A (▲). The graph shows the degree of scission, α, as a function of time.

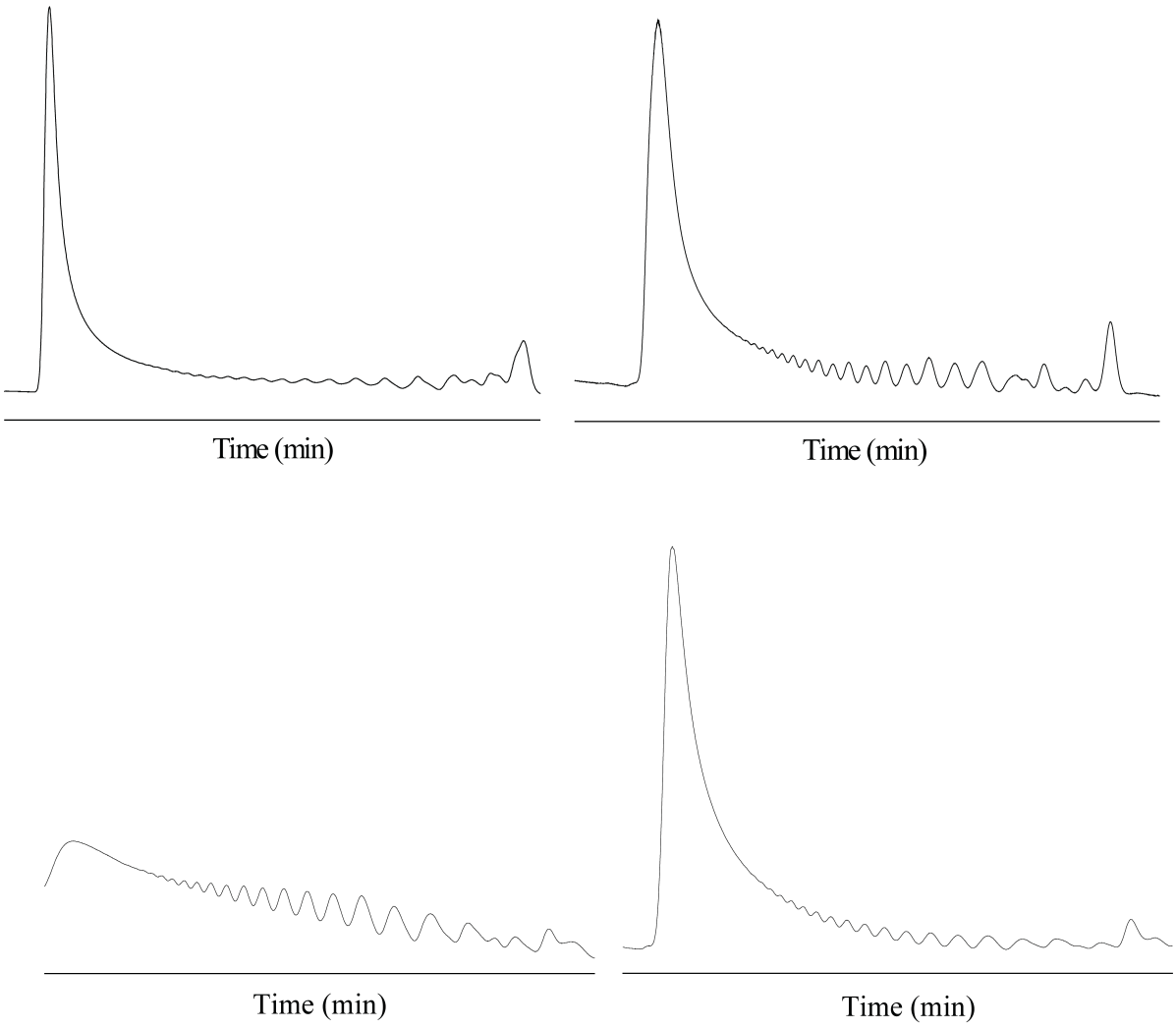

Figure 3. SEC chromatograms for the degradation of F_A = 0.62 chitosan with HCHT50 (top left), HCHT50-W31A (bottom left), and HCHT50-W218A (bottom right) and HCHT39 (bottom) at ~8 % degradation. At this level of degradation, no tendency for dominance of even oligomers are observed. Moreover, the polymer peak for HCHT50-W31A starts to disappear, which is typical for nonprocessive endo-action (Horn et al., 2006b, Sikorski et al., 2006). HCHT39 (top right) is shown for comparison.

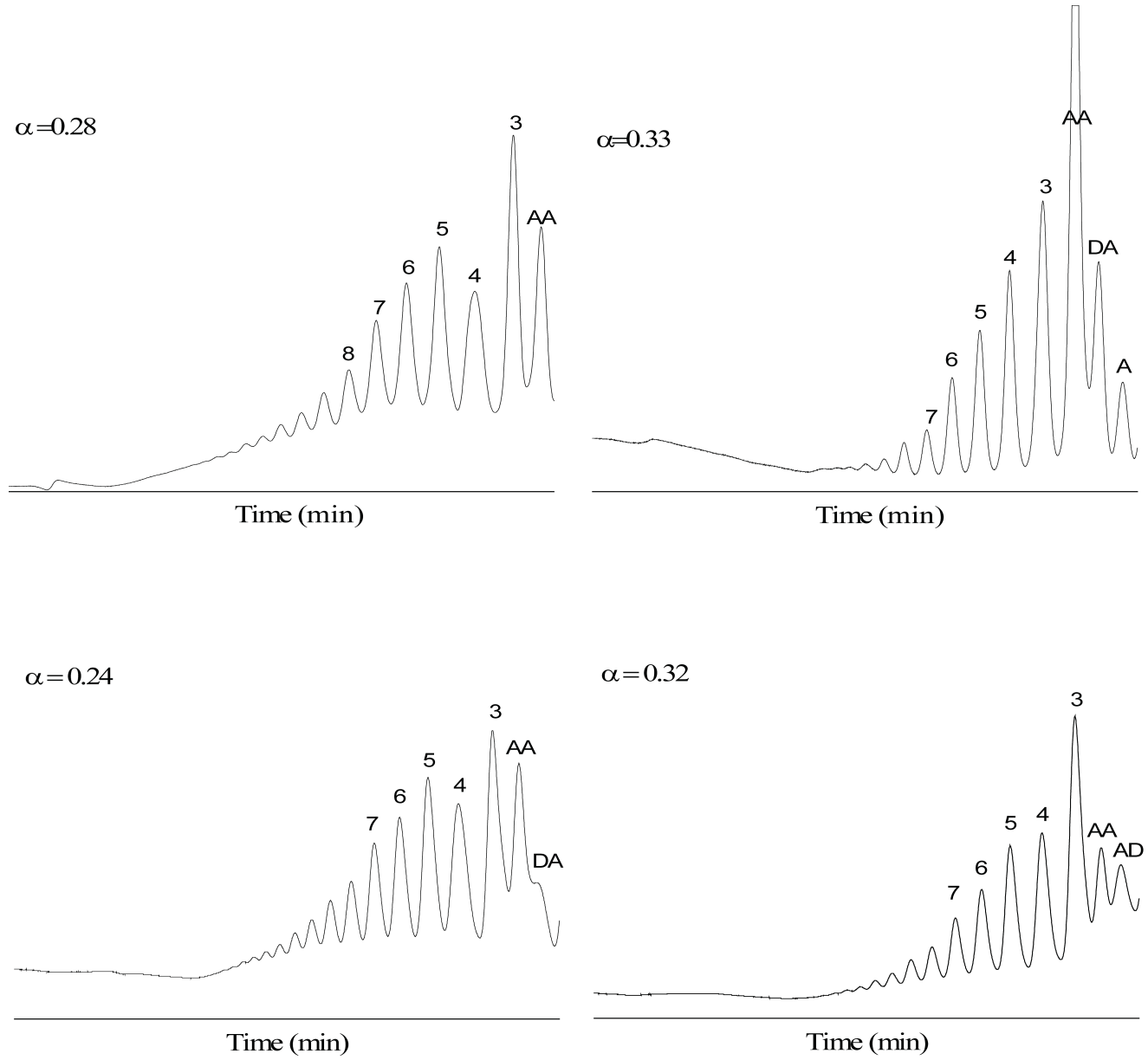

Figure 4. SEC chromatograms for the maxiumum degradation of F_A = 0.62 chitosan with HCHT50 (top left), HCHT50-W31A (bottom left), and HCHT50-W218A (bottom right). HCHT39 (top right) is shown for comparison.

Paper III

Screening of Human Chitinases and Chitinase-like Proteins in Inflammatory disease

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Abstract

Chitinases and chitin-like-proteins (CLPs) have been described as dysregulated in a variety of diseases characterized by chronic inflammation, however, their function remains poorly understood. Chitinases catalyse hydrolysis of chitin, and have been identified in a variety of organisms ranging from bacteria to eukaryotes. Humans do not possess chitin, but we do have genes encoding the active chitinases chitotriosidase (HCHT/Chit1) and acidic mammalian chitinase (AMCase/ChiA), (1) and chitinase-like proteins (CLPs) without hydrolytic activity. The expression of chitinases and CLPs have been described to be affected in a variety of diseases characterized by chronic inflammation

We have here investigated the profile for expression of mammalian chitinases and CLPs in a mouse model for acute intestinal inflammation. mRNA and protein for two of the CLPs, Chi3l1 and Chi3l3, were found to be up-regulated in mouse colon after dextran-sodium-sulphate (DSS)-induced colitis. It was shown by immunohistochemistry that the increased mChi3l3 expression mainly was localized in infiltrating neutrophils and macrophages, while mChi3l1 was expressed by infiltrating neutrophils and to some extent epithelial cells of the colon. AMCase and the CLP mChi3l4 mRNA was not expressed in mouse colon tissue neither in normal nor inflamed tissue, whilst the expression of Chit1 is not affected by colon inflammation.

Keywords: Human chitinases and chitinase-like-proteins; inflammation; expression profile; immunohistochemistry

Abbreviations: HCHT/hChit1, human chitotriosidase, AMCase, acidic mammalian chitinase; CLP, chitinase-like proteins; GH, glycoside hydrolase; DSS, dextran sodium sulphate; IL-13, interleukin-13; IL-4, interleukin-4; UC**,** ulecerus colitis; IBD, inflammatory bowels disease; LP, lamina propria; Mpo, myeloperoxidase; NOS2, nitric oxide synthase 2; aaMΦ, alternatively activated macrophages; caMΦ, classical activated macrophages; CEC, colonic epithelial cells;

Introduction

Chitin is an essential structural component of the exoskeleton of crustaceans, arthropods and insects, the cell wall of certain fungi and algae, and the microfilarial sheath of parasitic nematodes (2-5). The metabolism of chitin in nature is controlled by enzymatic systems that produce and break down chitin, primarily chitin synthases, and chitinases, respectively. (6). Mammalian chitinases are evolutionarily well-conserved proteins that belong to the glycosyl hydrolase 18 (GH18) family based on their high structural similarities with bacterial and plant chitinases. The major cell types expressing mammalian chitinases and chitinase-like proteins are neutrophils (7), macrophages (8), tumor cells, epithelial cells (9), chondrocytes and synovial cells (10). A common feature for the GH18 family is that it employs a substrate-assisted catalytic mechanism that induces a conformational change that is critical in the mechanism of family 18 chitinases (11).

Chitinases have mostly been studied in lower life forms, wherein they are produced in significant quantities as a host defense against infections with chitin-containing organisms. This is considered to be part of the innate immune response against chitin-containing pathogens. Chitinases may function directly as a chemotactic agents or indirectly by inducing other chemokines that attract eosinophils and T cells to sites of parasitic infection, or modulate tissue inflammation, immunity and/or remodeling (12). Recent research has also shown indications that chitinases have fungicidal properties (1).

Interestingly, while chitin has not been described in mammals, they express two active chitinases with catalytic activity which are classified as GH18 family in the CAZY database (www.cazy.org; EC 3.2.1.14): acidic mammalian chitinase

(AMCase) (13) and human chitotriosidase (from now termed hChit1) (14). Both chitinases are believed to play a role in anti-parasite responses of the human innate immune system (15, 16), and are involved in several diseases (e.g. Gaucher diseases, Niemann-Pick syndrome, fungal and bacterial infections, chronic inflammation, liver diseases and neurodegenerative diseases (17, 18). The transcription for AMCase is driven by the Th2-cytokines interleukin (IL)-4 and IL-13 which are sufficient for induction in the mouse lung (12). AMCase is stimulated in an IL-13 dependent, Th2-specific fashion in the murine lung where it plays an important role in Th2 inflammation and IL-13 effector pathway activation. In addition it is expressed in pulmonary epithelial cells, macrophages, and eosinophils in patients with asthma (19).

hChit1 is expressed and highly regulated in activated macrophages, and is also present in human neutrophil-specific granules which is released upon cytokine stimulation (20). During the last decade, a substantial progress has been made in the study of the physiological roles of hChit1, but its functions remain to be fully understood.

Chitinase-like proteins (CLPs) (also called chitinase-like lectins (chi-lectins)) also belong to the GH18 family. These proteins lack chitinase activity, but have retained the C-terminal carbohydrate-binding motif (21), which has been shown to constitute a critical region in interaction with chitin (22, 23). Gene duplications of both AMCase and chitotriosidase, followed by loss-of-enzymatic-function mutations, led to the subsequent evolution of chi-lectins (1). The CLPs include oviductin (Ovgp1) and chitinase 3–like 1 (Chi3l1) (also known as YKL-40 or GCP-39), both found in mice and humans, Chi3l3 in humans, and Chi3l3 and Chi3l4 in mice (alternatively termed Ym1 and Ym2) (1, 24). Additionally, two poorly characterized murine CLPs are known, named basic Ym (bYM) and brain chitinase-like protein 2 (Bclp2) (1, 24). An

overview over the nomenclature of human chitinases and chitinase-like proteins are given in Table S1, supplementary information. Chi3l1 is produced by a wide variety of cells including neutrophils, macrophages, synovial cells, fibroblasts, smooth muscle cells, epithelial cells and tumour cells (7, 9, 25). Expression of the Chi31 is associated with conditions of increased matrix turnover and tissue remodeling (25). High levels of this protein have been described in the sera and synovial fluids of patients with inflammatory and degenerative arthritis (26). Chi31 expression is reported to be specifically induced during the course of intestinal inflammation like human ulcerative colitis (UC), Crohns disease and inflammatory bowel disease (IBD) (9). Chi31 has been observed in both lamina propria (LP) cells and in colonic epithelial cells in mice experimental colitis models (27) and can also acts as a pathogenic mediator in acute colitis, despite lacking enzymatic activity (9).

Chi3l3 and Chi3l4 are solely found in rodents and have no human genomic or protein orthologues (28, 29). Chi3l3 is predominantly produced by macrophages and is synthesized and secreted by activated macrophages during inflammation elicited by parasitic infections (28). In murine models of Th2-type parasite infections, expression of both Chi3l4 and AMCase depend on the activity of the IL-21 receptor, which has a structural homology to IL-4Rα chain, and responds to the Th2 cytokine IL-21 (30). Chi3l4 is a close homologue of Ym1 and is a secretory protein from eosinophilic crystals in both the gastric and respiratory lesions of hyalinosis (31). Also, it is expressed in lungs of allergic mice, thus possibly indicating an important role in asthma (31). Asthma is a complex inflammatory disease, and although it is clear that Th2 cells are pivotal in this process, the precise molecular links between immune mediators and the expression of allergic disease are not clearly defined. The human

chitinases and chitinase-like proteins are well-known enzymes in the world of inflammatory diseases, but their function and mechanisms are not fully understood.

In this work, we have used a dextran sodium sulfate (DSS) mouse model to investigate the gene expression pattern for mammalian chitinases and chi-lectins during acute colonic inflammation. Moreover, we have exploited the potential of a bacterial meal named BioProtein, which has been shown to modulate positively inflammatory processes in IBD (32), in combination with DSS induction to observer effect on chitinase and chi-lectin regulation as a control experiment. BioProtein is a bacterial meal obtained by aerobic fermentation of natural gas by *Methylococcus capsulatus* (Bath), an obligate methanotroph (33), together with the heterogenic bacteria *Ralstonia* sp., *Brevibacillus agri*, and *Aneurinibacillus* sp., representing minor fractions of the preparation. Clinical evaluation of DSS-exposed mice fed a standard diet including 25% bacterial meal revealed that the mice had a strikingly enhanced well-being compared to DSS-exposed mice fed the standard diet. (32).

Materials and methods

Animals

Female C57Bl/6NTac mice (Taconic, Denmark), age 5-6 weeks, weight 15-18 g with conventional microbiological status were used. The animals were divided into four experimental groups of six mice per group. The mice were feed *ad libitum* with either a control diet based on AIN-93G (SD) or an experimental diet where 25% of the casein content (200 g/kg body weight) and corn starch (54 g/kg body weight) were substituted with 254 g/kg Bioprotein (Norferm AS, Stavanger, Norway). Two groups of animals were feed with the control diet and the other two with the experimental diet for 2 weeks. The animals were acclimatized on the diet 7 days prior to induction of colitis. At day 8, colitis was induced by 3.5% dextran sodium sulphate (DSS) (TdB Consultancy AB, Uppsala, Sweeden) *ad libitum* in the drinking water for 6 days. The mice were sacrificed 6 days after DSS induction. Animal experiments were performed by trained personnel certified for conducting animal experiments, and were carried out in accordance with national regulations.

Tissue collection

The colon from the cecum to the rectum was dissected and the content of the colon was washed out with phosphate-buffered saline before it was opened along the length and prepared in a Swiss roll format. The Swiss roll was immediately snap-frozen in liquid nitrogen and then stored at -80 °C until further use.

RNA extraction and cDNA synthesis

20-30 mg of colon tissue and 600µl RLT lysis buffer (Qiagen, Germantown, MD) with mercaptoethanol were placed in a M-tube (Miltenyi GmBH, Bergisch Gladbach,

Germany). The tissue was then dissociated by using a gentleMACS Dissociator program RNA_02. TotalRNA was extracted using the Qiagen RNeasy Kit (Qiagen, Germantown, MD) in accordance with the manufacturers protocol. DNase treatment was included in the protocol to avoid any interference of remnant genomic DNA. The concentration of each totalRNA sample was quantified from A_{260} measurements using the NanoDrop 2000 (Thermo Fischer Scientific Inc.). The RNA integrity number (RIN) was measured using the Agilent 2100 Bioanalyzer (Agilent Technologies) and ranged from 6.5-8. cDNA synthesis was performed on 2.3 µg totalRNA/50 µl using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, UK) according to the manufacturer's recommendations. cDNA synthesis program: 25 ºC 10 min, 42 ºC 120 min, 85 ºC 5 min, 4 ºC forever (MJ Thermocycler, Watertown, MA). The cDNA samples were stored at -20 ºC until downstream applications.

To assess inflammatory activity after DSS treatment, gene expression of two well-known diagnostic markers of intestinal inflammation, S100a9 (calprotectin) and serum amyloid A3 (Saa3) was determined in the colon (34, 35).

Real time-PCR analysis

Mouse *Chit1* (Assay ID: Mm01291359_m1)*, CHIA* (Assay ID: Mm00458221_m1), *Chi3l1* (Assay ID: Mm00801477_m1)*, Chi3l3* (Assay ID: Mm00657889_mH), *Chi3l4* (Assay ID: Mm00840870_m1), *S109A* (Assay ID: Mm00656925)*, Saa3* (Assay ID: Mm00441203_ml) and *Rpl32* (Assay ID: Mm02528467) cDNA were quantified by real-time PCR using TaqMan® Gene Expression Master Mix reaction (Applied Biosystems, Carlsbad, CA) in a Rotor Gene 6000 Real-Time PCR Machine (Qiagene, Germany) according to the manufacturer's recommendations. 25 ng of cDNA template was used in all reactions. The following PCR program was used: 50

ºC 2 minutes, 95 ºC 5 minutes, 95 ºC 15 seconds, 60 ºC 1 minute (40 cycles of two last steps). All primer/probe sets were TaqMan® Gene Expression assays, (Applied Biosystems, Carlsbad). *Rpl32* was identified as the most stable reference gene, using mouse geNorm software. Relative quantification ($2^{-\Delta\Delta Ct}$) was calculated from the data. The samples were also run on a 2 % agarose gel to verify amplification of correct products (data not shown).

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens were cut in 8 µm sections and placed on polysine-coated microscope slides (LSL, Rochdale, UK). The sections were de-waxed by incubation with xylene for 3 × 5 min, rehydrated in a series of alcohol, washed in PBS, and subjected to antigen retrieval in IHC-Tek™ Epitope Retrieval Solution (IHCWorld) or Citric acid, pH 6.0, at 100°C in a steamer for 40 min. The slides were left to cool in the buffer for 20–30 min and incubated with serum (Sigma) directed to the host of the secondary antibody for 60 min. Primary antibodies for CHI3L1 (R&D Systems), CHI3L3 (R&D Systems), Arginase1 (ProteinTech group) and iNos (Abcam®) were incubated over night at 4°C in a humid chamber.

Slides were washed in TBS-T between incubations and secondary antibody conjugated with FITC or Alexa 647 (Jackson ImmunoResearch) were incubated for 60 min at RT in a humid chamber and 10 min with Hoechst nuclear dye for 10 min. For Biotin—Avidin stainings, biotinylated IgG (1:100) was incubated for 30 min after primary antibody, followed by ABC-reagent after manufacturers protocol. A substrate (AEC or DAB) was incubated for several minutes (time estimated through microscope). The slides were dried and mounted with Glycergel and a coverslide. Tissue sections were analysed with a Leica SP5 confocal microscope (Leica

microsystems, GE). In sections where two fluorochromes where used, the images were taken sequentially to avoid bleed-through.

The double-stainings for mChi3l1 and F4/80, mChi3l1 and Mpo, and for mChi3l3 were performed with formalin-fixed, paraffin embedded specimens as previously described. The sections were de-waxed in a heat cabinet for 45 min: xylene for 2 x 10 min, absolute alcohol 2 x 3 min, 80 % and 50 % alcohol for 3 min and finally in sterile water for 5 min.

Antigen retrieval was performed in citrate buffer pH 6.0 and boiled for 15 min. The staining was performed with TBS-T (TBS + 0.5% Tween) for 5 min, 30 min of 3.5% BSA in PBS, before the first primary antibody (Chi3l1, 1:600, sheep anti mouse) or (mChi3l3 1:1000, goat anti mouse) was diluted in PBS and 1% BSA and incubated over night at 4°C in a humid chamber. The slides were washed in-between all incubation for 10 min in TBS-T. The first secondary antibody (FITC rabbit anti sheep or Texas Red Donkey anti Goat) was diluted in PBS and incubated for 30 min in RT, then washed and the second primary antibody (F4/80 (1:600 Alexa Fluor® 594: Donkey anti Rat) or Mpo (myeloperoxidase) (1:50 Alexa Fluor® 647: Goat anti Rabbit) was incubated for 60 min at RT, then washed again. The final step of the second secondary antibody was diluted in PBS (FITC rabbit anti Rat or FITC Goat anti Rabbit) and incubated for 30 min at RT. From the step with the first secondary antibody was incubated, all work was performed in the dark, and all incubation were performed in a humid chamber.

Statistics

Data were analyzed using one-way ANOVA or t-tests. Tukey's post hoc test was included when appropriate. Analyses were performed using the open-source statistical

language and environment, R (www.r-project.org). Differences between means were considered significant if the P value was < 0.05.

Results

Assessment of DSS-induced disease activity

The mRNA transcription levels of both S100a9 and Saa3 were found to be up regulated (Figure 1) after treatment with DSS in mice fed a standard control diet (1350 fold (478-3822); $p < 0.0001$ for S100a9 and 64- fold (28-147); $p < 0.001$, for S100a9 and Saa3, respectively), indicating inflammatory activity in the colon. The bacterial meal Bioprotein has been reported to reduce intestinal inflammation in both mice and salmon (32) (36). Moreover, DSS-induced gene expression of S100a9 was reduced 84-fold ($p < 0.001$) in mice fed a diet containing the bacterial meal compared to mice fed control diet. A reduction in the expression of Saa3 was identified in mice fed a diet containing the bacterial meal compared to mice fed a control diet, although not significant, (15-fold, $p > 0,05$) (Figure 1).

Gene-expression profile of chitinases and chithinase-like proteins in inflamed colon

Gene-expression pattern of mammalian chitinases and chitinase-like proteins in colon were investigated. AMCase and mChi3l4 mRNA transcripts were not detected in mouse colon tissue neither in normal nor inflamed tissue. Chit1 mRNA was expressed in colon tissue, but the expression was not affected by inflammation induced by DSS or by feeding the animals a diet containing the bacterial meal Bioprotein (1.2 fold; range (0.6-2.8), 1.0 fold; range (−1.8-2.0) for DSS and Bioprotein respectively (Figure 2). Both Chi3l1 (68 fold; range 42-109; $p<0.001$) and Chi3l3 (150 fold; range

96-234; p<0.001) mRNA expression were significantly increased by the DSS treatment compared to the control animals. Feeding DSS treated animals with the bacterial meal Bioprotein resulted in decreased expression of both Chi3l1 mRNA expression in colon (2.3 fold; range 1.4-3.6; p = 0,36) and Chi3l3 (5.5 fold; range 3.5-8.5; p = 0.007) compared to DSS treated animals given the control diet.

Immunhistochemical staining of Chi3l1 and Chi3l3

The protein expression of the up-regulated chi-lectins in inflamed colon was investigated by enzymatic immunohistochemistry. The expression profile observed is consistent with the above quantitative PCR results; mChi3l1 expression is increased in colon both in mice after DSS-induction and DSS-induced mice fed BioProtein meal, compared to the healthy mice. mChi3l1 expression is mainly localized to infiltrating cells of lamina propria and to some extent in epithelial cells. mChi3l3 also showed increased expression compared to the healthy mice (Figure 4). The mChi3l3 is expressed in infiltrating cells of lamina propria. Expression of mChi3l1 and mChi3l3 were not detectable in colon of healthy mice.

Neutrophil infiltration

DSS-induced colitis is characterized by infiltration of immune cells in the mucosa. The expression of Mpo has been found to be proportional with the number of neutrophils in inflamed tissue (37). We show that the mRNA level for Mpo in the colon was significantly increased in both DSS-treated groups compared to that in the control group ((5-fold [range, 3.2- to 6.5-fold; $P < 0.001$] and 2-fold [range, 1.5- to 3.0-fold; $P < 0.001$] for mice fed the standard diet and bacterial meal, respectively). However, Mpo mRNA expression decreased by more than 50% in DSS-treated mice

receiving the bacterial meal compared to that in the group receiving the standard diet, although the difference was not statistically significant (Figure 1). Mpo

activity has been strongly associated with colonic tissue injury resulting from DSS administration in inducible *NOS2*$^{-/-}$ mice, suggesting that NOS2 is important for the signaling process in neutrophils (38). After DSS treatment, there was a significant increase in NOS2 mRNA transcription in the colons both of mice fed the standard diet and mice fed the bacterial meal (79-fold [range, 51- to 119-fold; $P < 0.001$] and 23-fold [range, 16- to 32-fold; $P < 0.001$], respectively). The NOS2 mRNA transcript level was, however, approximately 70% lower in the colons of mice exposed to DSS receiving bacterial meal than the colons of mice fed the standard diet (3.5-fold; range, 2.3- to 5.3-fold; $P < 0.01$) (Figure 1).

Immunohistochemical characterization of Chi3l1 and Chi3l3 in inflamed colon.

Chi3l1 was earlier reported to be a marker for neutrophils (7), macrophages (8), inflammation associated epithelial cells (39) and a potential marker of disease activity in IBD (40). Chi3l3 is reported to be expressed in macrophages (41). To further be able to determine the localization of the chi-lectins, we double-stained mChi3l1 and mChi3l3 together with both a marker for neutrophil granulocytes (Mpo) and macrophages (f4/80). mChi3l1 and mChi3l3 (Figure 5 and 6) were found to co-localize with MPpo in the infiltrating mononuclear cells, which mainly are macrophages and neutrophils. The distribution of infiltrating cells was confirmed as neutrophil granulocytes, and mChi3l1 was present in almost all of the Mpo-positive cells.

The distribution of mChi3l1 (Figure 5) in macrophages (f4/80) was less severe than for neutrophil granulocytes (Mpo), there were both positive and negative

macrophages for mChi3l1, while almost all cells stained for both Mpo and mChi3l1 were positive.

Discussion

The present study investigated the expression profile of the mammalian chitinases and chitinase-like proteins in a mouse experimental colitis model. Common for all these enzymes is that they interact with chitin. Chitin-containing organisms (e.g. parasites) produce chitinases to remodel chitin-containing structures for their morphogenesis (42). Chitinases are also produced by bacteria and plants for their defence against chitin-containing pathogens and for maintenance the ecological balance in nature (42). Therefore, chitinases have been considered to play a crucial role in innate immune responses in lower life forms to control the infection with chitin-containing pathogens (43, 44). Since chitin is an important structural component of pathogens like fungi and as well as constituent of the mammalian diet, a dual function for mammalian chitinases in innate immunity and food digestion has been envisioned (1, 45). Despite the detailed knowledge about structure, insights to the exact physiological function of the chitinases and chi-lectins are limited. Here we provide new insights into how these proteins are regulated during an acute colon inflammation.

hChit1 was constitutively expressed in colon from both healthy and DSS-induced mice, and showed no increase or decrease in expression when a bacterial meal was added to the diet to reduce inflammation. In humans, hChit1 is produced in macrophages and neutrophils, and it has been suggested that chitinases in organisms that do not produce chitin play a role in the degradation of chitin-containing

pathogens. Since the expression of hChit1 did not change during inflammation, this expression pattern might be specific for chitin-containing organisms, so that the expression only occurs if the chitin-containing organisms are present during the inflammation. Both mChi3l3 and mChi3l1 showed a potent increase in gene expression. mChi3l1 was found to be expressed by both lamina propria cells (mainly neutrophils and macrophages) in the colon of mice with DSS-induced colitis, while it is more or less undetectable in colon tissue of healthy mice. It is well accepted that in many inflammatory disorders of the intestine, the combination of epithelial injury, disease activity, and symptoms parallel neutrophil infiltration of the mucosa (32) and also is the model used here we identify a significant increase in Mpo gene expression in the colon mucosa of mice with DSS-induced colitis compared to normal mice. mChi3l1 and mChi3l3 were by immunohistochemistry found to co-localize with Mpo positive infiltrating cells and to some extent F4/80 positive cells of the submucosa. The gene expression of mChi3l1 and mChi3l3 were significantly down regulated in mice fed a diet containing Bioprotein compared to mice fed a normal diet, during DSS-induction of colitis. Mice fed a diet containing Bioprotein showed a significant decrease in infiltrating neutrophils by a reduction of Mpo and NOS2 expression. This shows that the increased expression of mChi3l1 and mChi3l3 is mainly caused by an increase of infiltrating mononuclear cells in the lamina propria.

mChi3l3 and mChi3l4 were the first chi-lectins to be identified as mediators of Th2 inflammation in allergy (31, 46). There have been numerous publications describing increased expression of mChi3l3 during a wide range of pathologies, but mChi3l3 is often disregarded as an important participant in chi-lectin biology because of the lack of a true human ortholog of Chi3l3 and/or Chi3l4. However, all three mouse chi-lectins are up regulated in response to Th2-driven inflammation in the lungs of mice,

and it is believed that studying solely Chi3l1 will diminish the ability to reveal the true functions of this closely related protein family. We aimed to understand the general biology of chi-lectins by comparing them in parallel. Chi3l3 has consistently been associated with acute injury, and our data show that mChi3l3 has a quicker response to inflammation and is higher up regulated than mChi3l1. This was seen in both at protein levels and by gene-expression.

mChi3l3 (Figure 6) has a more distinct expression in macrophages compared to mChi3l1 (Figure 5), and might be a better marker for macrophages than mChi3l1. These data also shows the distribution of macrophages during inflammation and among the infiltrating cells, there are mainly neutrophil granulocytes and less macrophages. In a healthy tissue, the infiltrating cells are, as expected, of total absence, and hence also the chi-lectins. When we distinguish between alternatively activated macrophages (aaMΦ) and classical activated macrophages (caMΦ), mChi3l3 reported (29, 41) markers for the alternatively activated macrophages through an IL-4-dependent manner. Findings from Webb et al (31) was in agreement with our findings, as well as that IL-13 induces the expression of both mChi3l3 and mChi3l4. The sequence of Chi3l3 shows 92% identity with Chi3l4, and Raes *et al* (41) does not exclude the possibility that Chi3l4 also is up regulated in aaMΦ. In contrast, our data shows no expression of Chi3l4 during inflammation, neither in healthy tissue.

mChi3l3 is both higher up regulated in acute inflammation compared to mChi3l1, and shows the highest increase for infiltrating neutrophil cells, although it is reported as a marker for macrophages and then specifically aaMΦ(47). Chi3l1 has also been reported in colonic epithelial cells (CECs) (9, 48, 49) and it is also demonstrated that

some potentially pathogenic bacteria are strongly associated with the development of IBD by interacting with Chi3l1 molecules on CECs (9, 49). Since we have reported that either mChi3l1 or mChi3l3 are present in CECs, but highly expressed in neutrophils and macrophages, it is possible that Chi3l1 may play distinctly different roles depending on cell types under inflammatory conditions. This study shows that the change of expression for both mChi3l1 and mChi3l3 is caused by reduction of neutrophil cells in colon when looking at the DSS/BioProtein fed mice compared to the DSS-fed mice. Because chi-lectins are upregulated in the context of both helminth and fungal infections (50, 51), this study may explain why one will find the chi-lectins in such different manners, and that the expression profile of the chi-lectin will depend on the type of infection.

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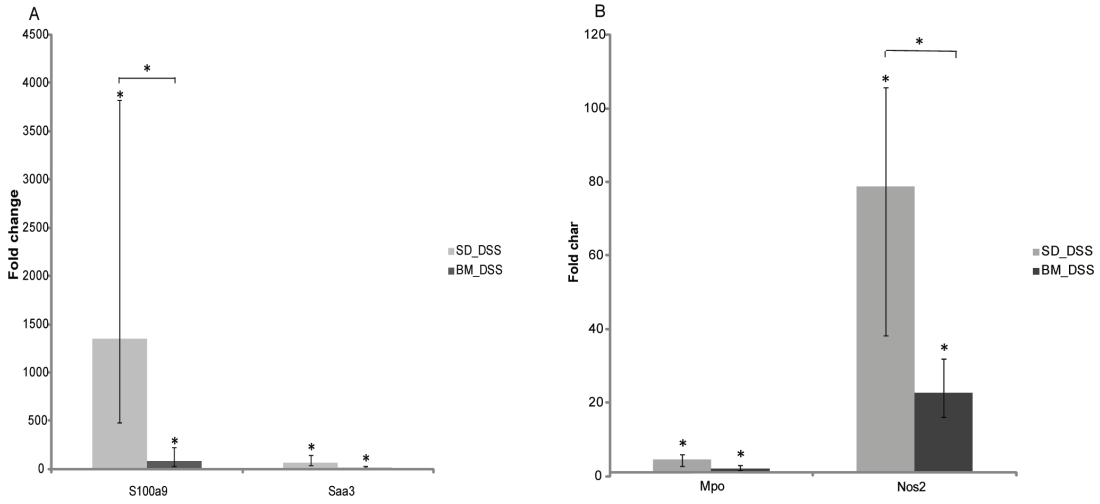


Figure 1: A) mRNA levels for S100a9 (calprotectin) and Saa in DSS-treated mice given standard or bacterial meal diet relative to DSS-untreated mice given standard diet. mRNA levels were determined by real-time PCR. B) mRNA levels for Mpo and Nos2 in DSS-treated mice given standard or bacterial meal diet relative to DSS-untreated mice given standard diet. mRNA levels were determined by real-time PCR. Data are presented as mean fold change ±SD. Difference between means was tested by one-way ANOVA, $p<0.05$ was considered statistically significant (*).

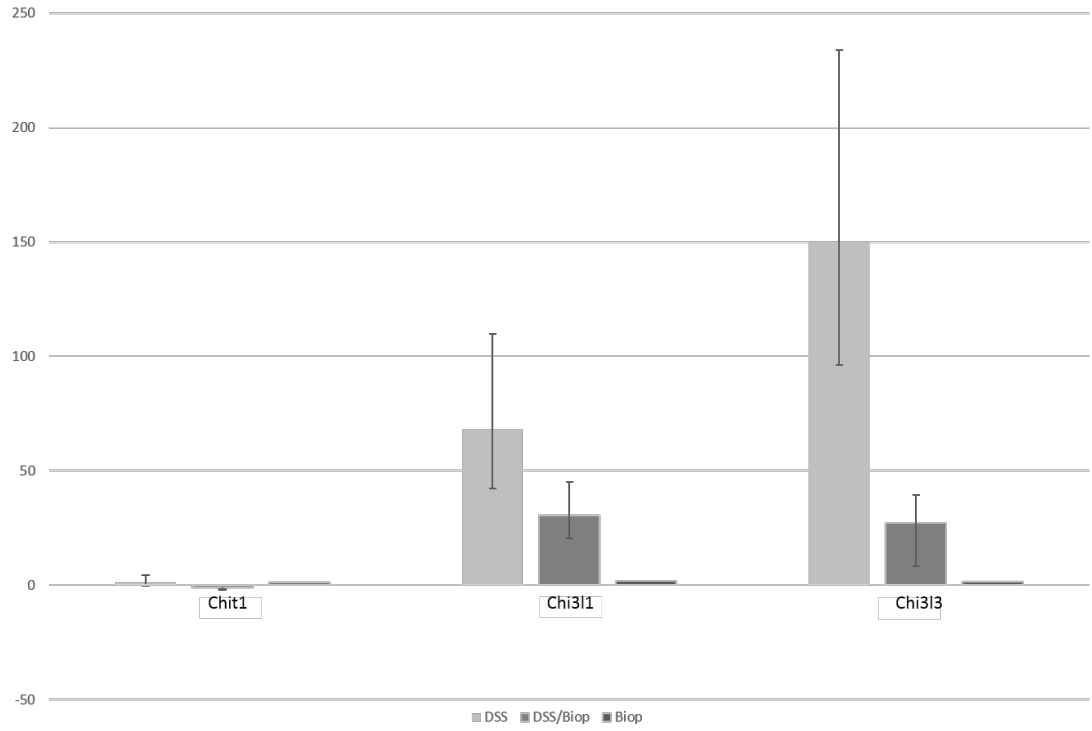


Figure 2: Relative changes in mRNA expression of chitotriosidase (*Chit1*), chitinase-3-like-1 (*Chi3l1*) and chitinase-3-like-3 (*Chi3l3)* in the intestine of mice fed with a normal diet and dextran sulphate sodium (DSS), Bioprotein and Bioprotein and DSS. Mice fed with a normal diet were used as an untreated reference group. Data represents the fold change in gene expression normalized to the reference gene *Rpl32* and relative to the untreated reference group (y-axis) and the respective diets are shown on the x-axis.

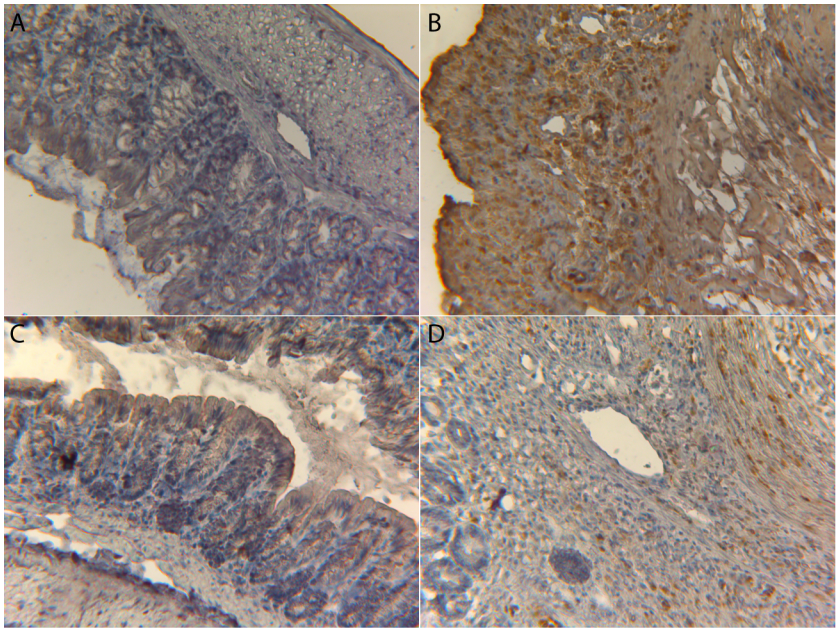


Figure 3: Immunohistochemical staining with antibodies against Chi3l1 in Formalin-fixed, paraffin-embedded samples of the intestine from mice fed with dextrat-sulphate-sodium (DSS), BioProtein, and BioProtein and DSS, where immunostained with antibody against chitinase-3-like-1 (Chi3l1). The untreated reference group received normal diet. A) Mice fed with normal diet, used as an untreated reference control B) DSS-treated + normal diet C) Received BioProtein diet and D) received BioProtein diet + DSS treatment.

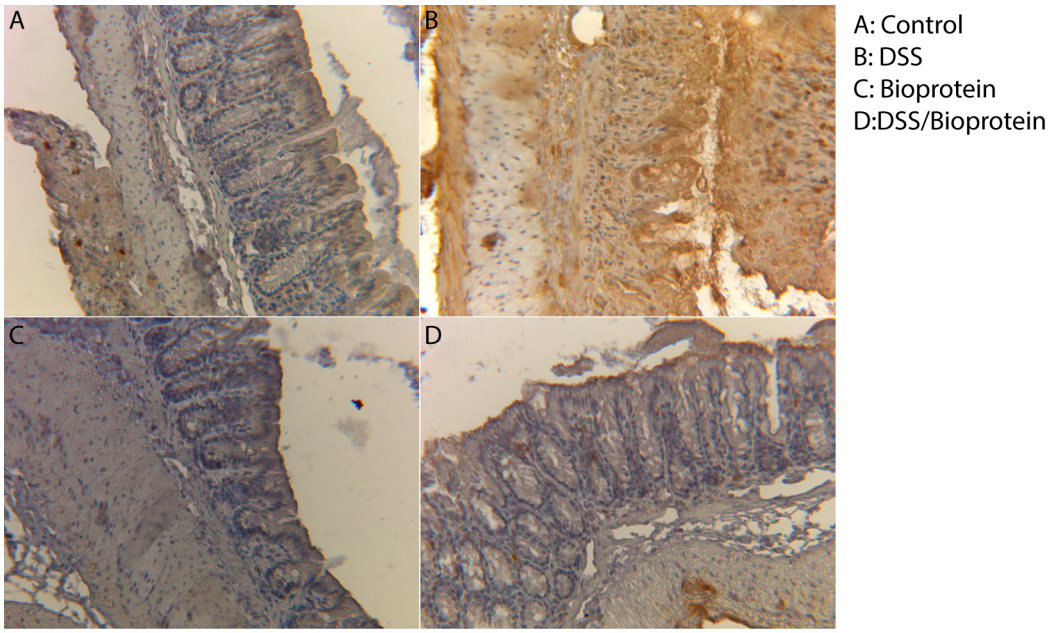
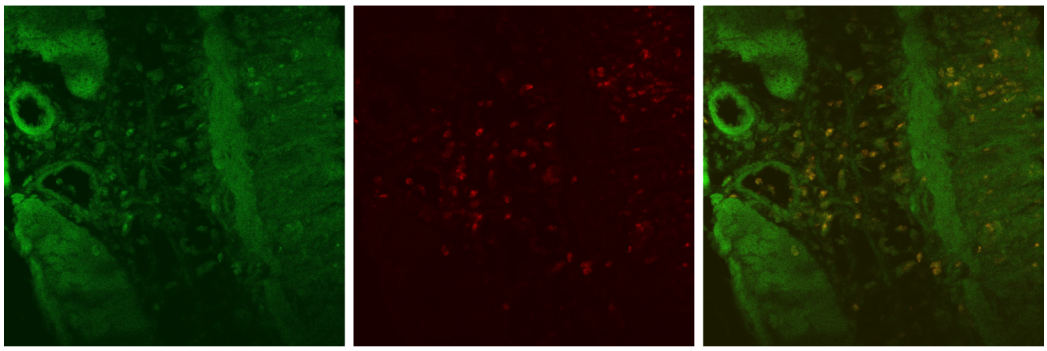


Figure 4: Immunohistochemical staining with antibodies against Chi3l3 in Formalin-fixed, paraffin-embedded samples of the intestine from mice fed with dextrat-sulphate-sodium (DSS), BioProtein, and BioProtein and DSS, where immunostained with antibody against chitinase-3-like-3 (Chi3l3). The untreated reference group received normal diet. A) Mice fed with normal diet, used as an untreated reference control B) DSS-treated + normal diet C) Received BioProtein diet and D) received BioProtein diet + DSS treatment.

Treatment: DSS

Green: Chi3l1
Red: Mpo



Green: F4/80
Red: Chi3l1

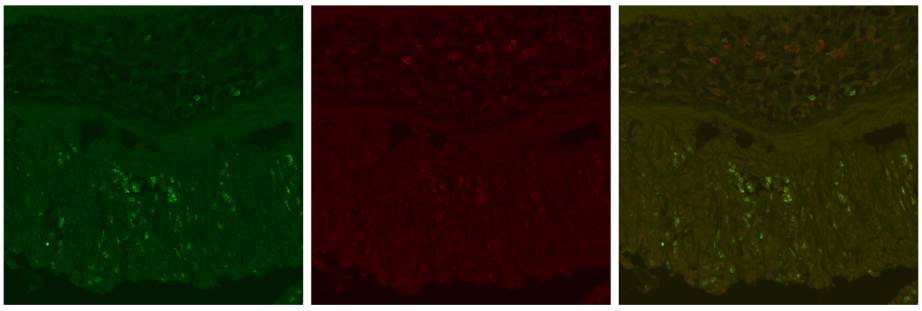
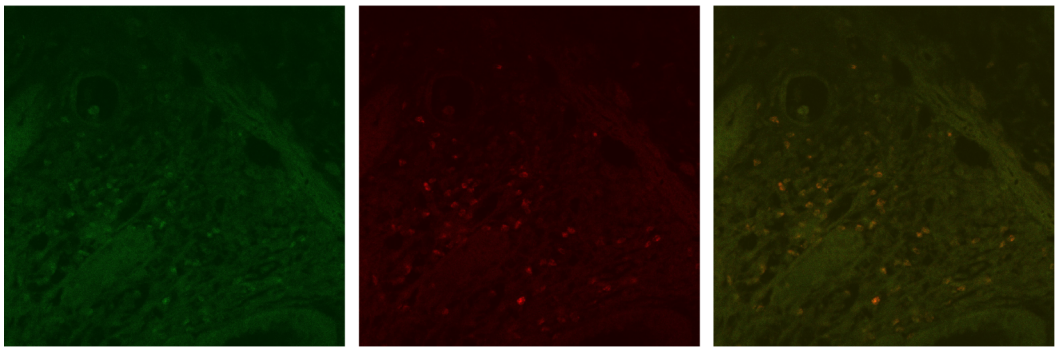


Figure 5: Immunohistochemical double-stainings with antibodies against Chi3l1 and Mpo or f4/80 with fluorescent secondary antibodies on Formalin-fixed, paraffin-embedded samples of the intestine from mice fed with dextrat-sulphate-sodium (DSS). In the upper panel the green channel shows the Mpo and the red channel shows the Chi3l1 staining, while the lower panel shows f4/80 in the green channel and Chi3l1-staining in the red channel. The last picture in both panels, we merged them together.

Treatment DSS

Green: Mpo
Red: Chi3l3



Green: Chi3l3
Red:F4/80

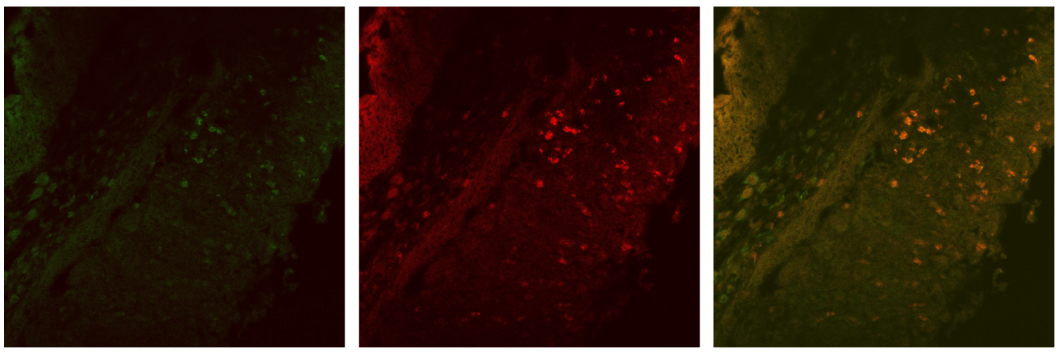


Figure 6: Immunohistochemical double-stainings with antibodies against Chi3l3 and Mpo or f4/80 with fluorescent secondary antibodies on Formalin-fixed, paraffin-embedded samples of the intestine from mice fed with dextrat-sulphate-sodium (DSS). In the upper panel the green channel shows the Mpo and the red channel shows the Chi3l3 staining, while the lower panel shows f4/80 in the red channel and Chi3l3-staining in the green channel. The last picture on both panels, we merged them together.

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Supplementary Information:

Table 1- Nomenclature of the mammalian chitinase and chitinase-like proteins

Gene name	**Alternativly named**
Chia	AMCase,
Chit1	Human Chitotriosidase, HCHT
Chi3l1	Human Cartilage gp39, YKL-40
Chi3l2	YKL-39
Chi3l3	Ym1, Eosinophil chemotactic factor (ECF-L),
Chi3l4	Ym2
Chid2	SI-CLP