Elsevier Editorial System(tm) for FEBS Letters
Manuscript Draft

Manuscript Number: FEBSLETTERS-D-15-00034R1

Title: The directionality of processive enzymes acting on recalcitrant polysaccharides is reflected in the kinetic signatures of oligomer degradation

Article Type: Research Letter

Section/Category: Enzymology

Keywords: Processivity; kinetics; glycoside hydrolases; recalcitrant polysaccharides

Corresponding Author: Prof. Morten Sorlie,

Corresponding Author's Institution:

First Author: Anne Grethe Hamre

Order of Authors: Anne Grethe Hamre; Daniel Schaupp; Vincent G Eijsink; Morten Sorlie

Manuscript Region of Origin: NORWAY

Abstract: The enzymatic degradation of the closely related insoluble polysaccharides cellulose (β(1-4)-linked glucose) by cellulases and chitin (β(1-4)-linked N-acetylglucosamine) by chitinases is of large biological and economical importance. Processive enzymes with different inherent directionalities, i.e. attacking the polysaccharide chains from opposite ends, are crucial for the efficiency of this degradation process. While processive cellulases with complementary functions differ in structure and catalytic mechanism, processive chitinases belong to one single protein family with similar active site architectures. Using the unique model system of Serratia marcescens with two processive chitinases attacking opposite ends of the substrate, we here show that different directionalities of processivity are correlated to distinct differences in the kinetic signatures for hydrolysis of oligomeric tetra-N-acetyl chitotetraose.

Cover Letter

Dear Editor,

Please find enclosed our manuscript "The directionality of processive enzymes acting on recalcitrant polysaccharides is reflected in the kinetic signatures of oligomer degradation", which we would like to be considered for publication in *FEBS Letters*.

Polysaccharide depolymerization in nature is accomplished by processive glycosyl hydrolases (GHs) with different inherent directionalities, i.e. attacking the polysaccharide chains from opposite ends. Our manuscript show that different directionalities of processivity are correlated to distinct differences in the kinetic signatures for hydrolysis of oligomeric substrate. We clearly believe our findings are of importance and will be of value to the research field on GHs.

On behalf of all authors, I declare that neither this nor any manuscripts describing the contents of this paper in any related form have been submitted for publication in another journal or published as conference proceedings.

We hope that you will find our manuscript acceptable for publication and we are looking forward to your reply.

Yours sincerely,

Morten Sørlie

Dear Editor,

We are grateful for the thorough and positive review of our manuscript “The directionality of processive enzymes acting on recalcitrant polysaccharides is reflected in the kinetic signatures of oligomer degradation”. We have taken the very constructive comments provided by the Reviewers and improved the quality of our manuscript as detailed below. Hopefully, it is now acceptable for publication in FEBS Letters.

Sincerely,

Morten Sørlie

Reviewers' comments:

Reviewer #1: Hamre and coworkers demonstrated kinetic experiment of two processive chitinases from chitinolytic bacteria Serratia marcescens in this manuscript. Although the experimental approach itself is interesting to explain processivity of these enzymes using soluble oligosaccharide (N-acetyl chitotetraose in this study), there is too less experimental result shown as one manuscript. I judged it is too early to be single manuscript even letter type. Actually, the manuscript contain only one graph for actual data and one table calculated from the graph, and the other figures are just decorations. Please also answer following questions.

Q1 The authors set typical Michaelis-Menten type equation with substrate inhibition. How do they decide this model is suitable for the analysis of the processive chitinases? These enzymes have long subsite, where more than two substrates can bind. How do they guarantee the suitability of the model to the enzyme?

Q2 Similarly, if there are several possibilities to accommodate the substrate, how do they distinguish productive and non-productive binding of the substrate?

Response: We cannot of course guarantee the suitability of the model. Still, our data are obtained at initial rates (always between 0 to 20 % of the substrate being consumed) so it is likely that we are observing substrate inhibition and not i.e. product inhibition (K_i = 0.45 mM and K_d = 0.4 mM for $(GlcNAc)_2$ to ChiA, Kuusk *et al.* J. Biol. Chem. 2015). Moreover, all results show that hydrolysis of $(GlcNAc)_4$ always yielded two $(GlcNAc)_2$ molecules showing that productive binding exclusively takes place at −2 to +2 subsites. Inhibition should therefore arise from non-productive binding on either side (one or the other or both) of the catalytic acid. It is unlikely that exchanging Trp^{275} to Ala allows for other types of inhibitions. We have measured k_{cat} and K_M for at least 15 ChiA mutants in addition to the wild type and ChiA-W275A is the only one showing any type of inhibition likely for the reasons explained in the text. We agree that we can be more "compelling" in our arguments have made the following changes to address the concern of the Reviewer

"In the substrate concentration range studied, all enzymes with the exception of ChiA-W275A showed straightforward Michaelis-Menten kinetics, and the experimental data were therefore fitted to the standard Michaelis-Menten equation (Eq. 1; Fig. 2)."

has been changed to:

"Hydrolysis of $(GlcNAc)_4$ always yielded two $(GlcNAc)_2$ molecules showing that productive binding exclusively takes place at −2 to +2 subsites. Moreover, in the substrate concentration range studied, all enzymes with the exception of ChiA-W275A showed straightforward Michaelis-Menten kinetics, and the experimental data were therefore fitted to the standard Michaelis-Menten equation (Eq. 1; Fig. 2).

and

"Notably, ChiA has an extended substrate-binding cleft and surface, displaying considerable substrate affinities in many subsites [34-36]. Fitting of the data to Eq. 2 yielded a substrate inhibition constant, K_i, of 25 μM."

has been changed to:

“Notably, ChiA has an extended substrate-binding cleft and surface, displaying considerable substrate affinities in many subsites [35-37]. MacDonald *et al*. observed from a crystal structure the binding of two chitotriose thiazolines in ChiA, one molecule binding in the −3 to −1 subsites (as expected), and a second molecule in the “leaving-group subsites” +1 and +2 (with the third moiety of the ligand disordered in solvent) [36]. It may therefore be that for a fraction of available ChiA-W275A two molecules of $(GlcNAc)_4$ bind of either side of the catalytic acid in the same manner as the chitotriose thiazolines causing the substrate inhibition. Other substrate inhibition binding modes cannot of course be completely ruled out. It is unlikely that we observe product inhibition since our data are obtained at initial rates (always between 0 to 20 % of the substrate being consumed), and that product inhibition by and binding of $(GlcNAc)_2$ to ChiA has been found to be 0.45 mM (K_i) and 0.4 mM (K_d). respectively [38]. Fitting of the data to Eq. 2 yielded a substrate inhibition constant, K_i, of 25 μM.”

Reviewer #2: Hamre and co-workers clearly and succinctly describe and interpret the impacts of subsite mutagenensis on the processive action of chitinases that migrate to either the reducing or non-reducing end of chitin (or chito-oligosaccharides). Though a rather straightforward study in itself, the data interpretation were refreshingly well-written and insightful. The authors are asked to simply correct the few typographical errors remaining in the text, and to indicate as a footnote to Table 1, the number of replicates included in each data point.

Response: A footnote has been added to Table 1 indicating that both the k_{cat} and the K_m values are the average of three measurements.

Reviewer #3: This paper examines the kinetics of two structurally related chitinase enzymes that process along chitin in different directions (i.e, towards the reducing end or nonreducing end). The authors find that mutations to aromatic residues involved in product (ChiA) or substrate (ChiB) binding affect the kinetics of catalysis differently for the two enzymes: W275A and W97A mutations decrease the intrinsic rate of catalysis (k2) for both, by they increase the rate of product release (k3) for ChiB only. While this study is interesting, many of the interpretations are speculative (i.e., the aforementioned conclusion); the authors need to take much greater care in drawing out their analysis. With revision, I think that this paper is publishable.

We thank this reviewer for insightful comments. Before responding to specific comments we would like to point out that we acknowledge that there is an element of speculation in the interpretation of the data. The important novel fact is, however, that similar mutations have very different effects of ChiA and ChiB. The interpretation of the experimental observations is an important part of our paper, which we believe has its own value, even in the unlikely event that an assumption would be partly incorrect. One of the other reviewers characterizes our interpretation data interpretation as “refreshingly well-written and insightful”. Still, we agree that we could have taken greater care in drawing out our analysis and we hope that the changes outlined below are satisfactory.

Major points:
1. Page 7, "Kinetic data have previously been obtained...(Table 1) [31]." The entire analysis relies on Table 1. Were these data collected in a previous paper? If so, what new data was collected in this paper? This should be clarified.

Response: We apologize for not making it clear enough that we have collected data in our paper as well. To address this, the following has been changed:

“To address the connection between directionality, structural features of the +1 and +2 subsites (Fig. 1), and chitinase kinetics, the mutants ChiA-W275A (subsite +1) and ChiA-F396A (subsite +2) were characterized, allowing comparative analyses.”

to

“In this work, we have obtained kinetic data for ChiA-W275A (subsite +1) and ChiA-F396A (subsite +2) allowing comparative analyses of effects on the same mutations in ChiB to address the connection between directionality and structural features of the +1 and +2 subsites (Fig. 1).”

2. Page 9: "Considering the high similiarty of the catlytic centers of ChiA and ChiB, including the high similarity in stacking interaction of the Trp in subsite +1, we assume that mutaitonal effects on k2 are similar for the two enzymes." There are two major issues with this statement: (i) it does not follow from the discussion in the paragraph in which it sits, and (ii) it is the basis for the paper's analysis. This assumption must be backed up by a much more careful discussion.

Response: The text quoted refers to our most important, and very plausible, assumption and should have been crystal clear. We have made several minor adjustments in this paragraph that, hopefully, improve clarity.

Old text:

“It is likely that mutations in the +1 and +2 subsites affect the catalytic reaction itself (k_2). Productive binding of $(GlcNAc)_4$ involves that the sugar moiety in the −1 subsite undergoes a conformational change from a chair ($^4C_1$) to a skewed boat ($^{1,4}B$) [22, 40], with a free energy penalty of 8 kcal/mol [41]. Upon weakening the +1 and +2 subsites, less binding energy is available to overcome the free energy penalty of this conformational change, while the changed binding interactions also may affect the stereochemistry of the enzyme-substrate complex. Considering the high similarity of the catalytic centers of ChiA and ChiB, including high similarity in stacking interaction of the Trp in subsite +1 (Trp97/Trp275) [22, 40], we assume that mutational effects on k_2 are similar for the two enzymes.”

New text

“It is likely that mutations in the +1 and +2 subsites affect the rate constant for product formation, k_2. Productive binding of $(GlcNAc)_4$ involves that the sugar moiety in the −1 subsite undergoes a conformational change with a considerable free energy penalty [42]. Classical work of lysozyme (REFs) has shown that in such cases, catalysis depends on this energy penalty being compensated by binding energy resulting from interactions between other sugar moieties and the enzyme. Weakening enzyme-substrate interactions in the +1 and +2 subsites is likely to reduce this binding energy (carbohydrate-aromate stacking interactions in GHs typically yield ~2 kcal/mol in favorable free energy change [35,44]). In addition to affecting binding energies, changes in the +1 and +2 subsites may affect the stereochemistry of the enzyme-substrate complex, with possible additional consequences on k_2. Importantly, ChiA and ChiB have very similar catalytic centers, including a fully conserved -1 subsite. Structural data for their Michaelis complexes [22,45] show almost identical enzyme-substrate interactions in subsites near the catalytic center, including similar stacking interactions of the Trp (Trp97/Trp275) in subsite +1 and the aromate

(Trp220/Phe396) in subsite 2 (Fig. 1). Thus, it seems reasonable to assume that the effects of the mutations on k_2 are similar for the two enzymes."

3. Page 9, paragraph 2: "Removal of aromatic....k3 increases." If k1 was reduced for both enzymes (which would make sense as we would expect the substrate on rate to be reduced), k2 could be increased more for ChiB than for ChiA, leading to the same results. I agree that this is probably the less likely of the two scenarios, but as the analysis relies on a speculative dissection of kcat and Km, there is no experimentally justified reason to distinguish between this possibility and that stated in the paper. This paragraph (page 9, paragraph 2) is the heart of the paper, and at the moment, it seems unsupported.

Response: The key experimental finding is that removal of aromatic residues in the +1 and +2 subsites increases k_{cat} for ChiB and reduces k_{cat} for ChiA (a quite remarkable finding indeed). So, in general terms, we would argue that there is no basis for claiming that this paragraph is "unsupported". For reasons explained in the now revised preceding paragraph, we believe it is unlikely that the effects of the mutations on k_2 would differ between ChiA and ChiB. It is even more unlikely that such effects on k_2 would be positive upon removal of a major enzyme-substrate interaction in the +1 subsite. Thus, in our view, the experimental fact that k_{cat} increases provides an "experimentally justified reason" to distinguish between the possibilities. We have made a few changes in the text to clarify this issue

Old text:

Removal of aromatic side chains in the +1 and +2 subsites is likely to reduce the substrate association rate, k_1, increase the substrate dissociation rate, k_{-1}, and increase the rate of product release, k_3. All these possible effects lead to an increase in K_m, as is indeed observed. The key difference between the two enzymes lies in the effect of the mutations on k_{cat}, which, considering Eq. 5 and the assumption that changes in k_2 are similar for both enzymes, must imply a difference in the effect on k_3 and/or in the overall importance of k_3. In ChiA, the mutations reduce k_{cat}, which is due to a negative effect on k_2 that, apparently, is not compensated by an increase in k_3. In ChiB, however, the presumed similar effect on k_2 is more than compensated for by a positive effect of k_3, leading to an overall increase in k_{cat} (Table 1). Since the mutational effects in terms of lost binding energy should be similar in both enzymes, the only logic explanation for these observations is that k_3 is a rate-limiting factor in ChiB only. This explanation is in accordance with the observation that K_m effects are larger in ChiB than in ChiA, since Eq. 4 shows that the effect of changes in k_3 on K_m becomes larger as the relative magnitude of k_3 increases.

New text:
Removal of aromatic side chains in the +1 and +2 subsites is likely to reduce the substrate association rate, k_1, increase the substrate dissociation rate, k_{-1}, and increase the rate of product release, k_3. All these possible effects lead to an increase in K_m, as is indeed observed. The key difference between the two enzymes lies in the effect of the mutations on k_{cat}, which, considering Eq. 5 and the assumption that changes in k_2 are similar for both enzymes, must imply a difference

in the effect on k_3 and/or in the overall importance of k_3. Notably, the only alternative explanation would imply that the mutations have a positive effect on k_2 in ChiB, which is highly unlikely, for reasons explained above. In ChiA, the mutations reduce k_{cat}, which must be due to a negative effect on k_2 that, apparently, is not compensated by an increase in k_3. In ChiB, however, the presumed similar effect on k_2 is apparently more than compensated for by a positive effect of k_3, leading to an overall increase in k_{cat} (Table 1). Since the mutational effects in terms of lost binding energy should be similar in both enzymes, the only logic explanation for these observations is that k_3 is a rate-limiting factor in ChiB only. This explanation is in accordance with the observation that K_m effects are larger in ChiB than in ChiA, since Eq. 4 shows that the effect of changes in k_3 on K_m becomes larger as the relative magnitude of k_3 increases.

4. Page 10, paragraph 1: " The + subsites in chiA are likely to be optimized for the rapid release of dimeric products during processive action, hence mutational effects on k3 are not noticeable in the overall catalytic performance on (GlcNAc)4. On the other hand, the + subsites in ChiB are optimized to stay attached to the polymeric substrate in between catalytic steps..." I don't think that the authors can say that ChiA is generally insensitive to mutational effects; they have made on set of mutations. Their arguments would be better supported if they could mutate ChiA in a way where it held on to polymeric products longer (F396W?). Alternatively, could they make - site muations to ChiB that would cause them to release products faster? Such additional studies may aid in a more rigorous justification of the conclusions (or of the argument made in the paragraph referenced in point 3 above).

Response: We agree that we cannot state that ChiA is "generally insensitive to mutational effects" and we did not intend to do so. We also agree that more work could have been done, but we would like to point out that the beauty of the simple approach used so far is that we can compare the effects of essentially identical mutations (i.e mutation of the Trp in subsite +1) in different structural backgrounds (Fig. 3; discussed on page 10) that relate to different functionalities. We note that there must be some confusion since the reviewer connects position 396 in subsite +2 to the polymeric product, whereas this subsite +2 in fact is interacting with the dimeric product. For ChiB it is the other way around. In response to this comment we have adapted the text as follows:

Old text:

The + subsites in ChiA are likely to be optimized for rapid release of dimeric products during processive action, hence mutational effects on k_3 are not noticeable in the overall catalytic performance on $(GlcNAc)_4$. On the other hand the + subsites in ChiB are optimized to stay attached to the (polymeric) substrate in between catalytic steps, hence k_3 is rate-limiting and mutational effects on k_3 are noticeable in the overall catalytic performance on $(GlcNAc)_4$.

New text:

The + subsites in ChiA are likely to be optimized for rapid release of dimeric products during processive action, which may explain why expected beneficial effects of the W275A and F396A mutations on k_3 are not noticeable in the catalytic performance (k_{cat}) on $(GlcNAc)_4$. On the other hand the + subsites in ChiB are optimized to stay attached to the (normally polymeric) substrate

in between catalytic steps, hence k_3 is rate-limiting and mutational effects on k_3 are noticeable in the overall catalytic performance on $(GlcNAc)_4$.

Minor points:
1. Page 5, "adding 75 uL of 20 mM H2SO4": sulfuric acid will almost certainly hydrolyze some of the substrate (has a check been performed?). This is why sodium hydroxide is usually used to stop these reactions. Acid-catalyzed hydrolysis (by H2SO4) is not a deal breaker, but one or two sentences addressing the issue are required.

Response: There are no hydrolysis of $(GlcNAc)_4$ at such mildly acidic conditions and short time intervals. To address this, the following has been changed:

"Seven samples of 75 µl were withdrawn at regular time intervals up to 20 minutes, and the enzyme was inactivated by adding 75 µl 20 mM H_2SO_4. Prior to HPLC analysis, all samples were filtrated though a 0.45 µm Duapore membrane (Millipore) to remove denaturated protein. All samples were stored at –20 °C until HPLC analysis."

to

"Seven samples of 75 µl were withdrawn at regular time intervals up to 20 minutes, and the enzyme was inactivated by adding 75 µl 20 mM H_2SO_4. At such mildly acidic conditions and short time intervals before analysis, there are no significant acid catalyzed hydrolysis in line with the work of Einbu and Vårum where such rate constant has been found to be 1.5×10^{-4} s^{-1} in concentrated acid (12 M) [33]. Prior to HPLC analysis, all samples were filtrated though a 0.45 µm Duapore membrane (Millipore) to remove denaturated protein. All samples were stored at –20 °C until HPLC analysis."

2. Page 7: "less binding energy is available to overcome the free energy penalty of the conformational changes that accompany substrate binding." This statement is entirely speculative, yet it is stated as fact.

Response: We agree with the Reviewer. To justify the statement, we have added the following sentence:

"Weakening enzyme-substrate interactions in the +1 and +2 subsites is likely to reduce this binding energy (carbohydrate-aromate stacking interactions in GHs typically yield ~2 kcal/mol in favorable free energy change [35,44]."

The papers that are used as references are Baban *et al.*2010, J. Phys. Chem. B and Zolotnitsky *et al.* 2004, Proc. Natl. Acad. Sci. U.S.A.

3. The paper needs to be gone over 1-2 more times for typos (e.g. "economically importance" in the abstract, "from the reducing and"
on Page 3)

Response: We thank the Reviewer for finding typos. We will go through the paper carefully.

4. Page 10: "we show tha tthe +1 and +2 subsites in ChiB are tailored to remain attached to the product after catalysis, as one might expect for a processive enzyme moving towards the polymer's reducing end." I think that this should read "attached to the substrate" as the authors stated earlier that these are substrate (polymeric chain) binding

sites in ChiB. Additionally, even with this correction, I am not sure how this suggests anything about why the enzyme would move toward the polymer's reducing end.

Response: The intention of using “remain attached” is to emphasize that when a hydrolysis is completed, subsites +1 and +2 are still bound to what would be chitin in nature. Moreover, we do not suggest from our results that “a processive enzyme (i.e. ChiB) moving towards the polymers reducing end”. We simply state that ChiB does this (well-referenced in the paper) and that our findings goes along well with this.

***Title Page and Abstract**

The directionality of processive enzymes acting on recalcitrant polysaccharides is reflected in the kinetic signatures of oligomer degradation

Anne Grethe Hamre, Daniel Schaupp, Vincent G.H. Eijsink, and Morten Sørlie*

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

* To whom correspondence should be addressed, Morten Sørlie (morten.sorlie@nmbu.no)

ABSTRACT

The enzymatic degradation of the closely related insoluble polysaccharides cellulose (β(1–4)-linked glucose) by cellulases and chitin (β(1–4)-linked *N*-acetylglucosamine) by chitinases is of large biological and economical importance. Processive enzymes with different inherent directionalities, i.e. attacking the polysaccharide chains from opposite ends, are crucial for the efficiency of this degradation process. While processive cellulases with complementary functions differ in structure and catalytic mechanism, processive chitinases belong to one single protein family with similar active site architectures. Using the unique model system of *Serratia marcescens* with two processive chitinases attacking opposite ends of the substrate, we here show that different directionalities of processivity are correlated to distinct differences in the kinetic signatures for hydrolysis of oligomeric tetra-*N*-acetyl chitotetraose.

Keywords: Processivity; kinetics; glycoside hydrolases; recalcitrant polysaccharides.

Highlights:

- Comparison of two processive exochitinases with opposite directionality
- Directionalities of processivity are correlated to differences in the kinetic signatures
- Aromatic residues important for processivity greatly impact hydrolysis kinetics

*Manuscript text
Click here to view linked References

1. Introduction

Chitin, a β-1,4-linked linear polymer of N-acetyl glucosamine (GlcNAc), and cellulose, comprised of β-1,4-linked glucose, are the two most abundant biopolymers in Nature with an annual production amounting to 100 billion and one trillion tons respectively [1,2]. Thus, these polymers are an almost unlimited source of raw material for environmentally friendly and biocompatible products. The enzymatic degradation of these recalcitrant polysaccharides is therefore of great biological and economical importance.

Enzymes catalyzing the hydrolysis of *O*-glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety are called glycoside hydrolases (GHs) (www.cazy.org; [3]). The enzymatic hydrolysis of glycosidic bonds requires a proton donor and a nucleophile/base and leads to either retention or inversion of the stereochemistry on the anomeric oxygen at C1 [4-6]. Moreover, enzymes acting on polysaccharides can have different modes of action. Endo-acting enzymes randomly cleave the polymer chains, whereas exo-acting enzymes have a preference for acting from either the reducing or the non-reducing chain end [4]. Both endo and exo mechanisms can be combined with processive action meaning that the enzyme hydrolyzes a series of glycosidic linkages along the same polymer chain producing dimeric products before dissociation. In order to bind to and guide the insoluble substrate through the active site cleft, many GHs have a path of solvent exposed aromatic residues leading from a carbohydrate binding domain to the active site cleft [7-12]. It has been suggested that these residues function as a flexible and hydrophobic sheath along which the polymer chain can slide during the processive mode of action [13,14].

There are 21 different GH families that contain one or more cellulose degrading enzymes. Most of these cellulases are classified into GH family 5, 6, 7, 8, 9, 12, 44, 45 and 48 [3,15]. Processive exo-acting cellulases are found in families 6, 7, and 48 [16]. Families 7 and

48 contain exocellulases moving from the reducing end all use the retaining mechanism. Exocellulases moving in the opposite direction are found in family 6 and use the inverting mechanism [3,17]. Some processive endo-cellulases belonging to families 5 and 9 have recently been discovered [18,19].

Chitinases occur in GH families 18 and 19, and family 18 chitinases are thought to be Nature's primary instrument for degradation of recalcitrant chitinous biomass. Interestingly, while all GH18 enzymes use the same retaining substrate-assisted catalytic mechanism [20-22], members of the GH18 family differ in terms of endo versus exo activity, processive versus non-processive action, and the directionality of processivity [7,23-26]. A specific example is the chitinolytic machinery of *Serratia marcescens* that includes three well-characterized GH18 chitinases [24]. Chitinase A (ChiA) is processive and moves towards the non-reducing end, while chitinase B (ChiB) also is processive but moves towards the reducing end (Fig. 1) [26]. Chitinase C (ChiC) is a less processive endo-acting enzyme [27,28]. The two processive chitinases have aromatic residues in their +1 and +2 subsites. In ChiB, these subsites interact with the substrate during processive hydrolysis while in ChiA the product of a processive hydrolysis, chitobiose, is displaced from these subsites (Fig. 1). In this study, we show, by kinetic analyses of site-directed mutants in subsite +1 and +2 in ChiA and ChiB, that different directionalities of processivity are correlated to distinct differences in the kinetic signatures for hydrolysis of oligomeric tetra-*N*-acetyl chitotetraose.

2. Materials and Methods

2.1 Chemicals.

Chitooligosaccharides were obtained from Megazyme (Wicklow, Ireland). All other chemicals were of analytical grade.

2.2 Protein expression and purification.

The chitinases used were from *S. marcescens* strain BJL200 [29,30], ChiA-F396A and ChiA-W275A genes were expressed in *Escherichia coli* as described previously [25]. For protein purification, periplasmic extracts were loaded on a column packed with chitin beads (New England Biolabs) equilibrated in 50 mM Tris-HCl pH 8.0. After washing the column with the same buffer, the enzymes were eluted with 20 mM acetic acid. The buffer was then changed to 100 mM Tris-HCl pH 8.0 using Amicon Ultra- Centrifugal filters (Millipore). Enzyme purity was verified by SDS-PAGE and estimated to be > 95 %. Protein concentrations were determined by using the Bradford Protein Assay from Bio-Rad.

2.3 Kinetic analysis

The kinetic constants k_{cat} and K_m of the ChiA mutants were determined essentially as described previously [31,32]. In each experiment, 8-10 different $(GlcNAc)_4$ concentrations varying from 2 – 200 µM in 20 mM sodium acetate buffer, pH 6.1 and 0.1 mg/ml BSA were pre-incubated in 10 minutes at 37 °C in an Eppendorf thermo mixer at 800 rpm before the reactions were started by adding purified enzyme to the reactions. Final enzyme concentrations were 1 nM for ChiA-W275A and 0.5 nM for ChiA-F396A. Seven samples of 75 µl were withdrawn at regular time intervals up to 20 minutes, and the enzyme was inactivated by adding 75 µl 20 mM H_2SO_4. At such mildly acidic conditions and short time intervals before analysis, there are no significant acid catalyzed hydrolysis in line with the work of Einbu and Vårum where such rate constant has been found to be 1.5×10^{-4} s^{-1} in concentrated acid (12 M) [33]. Prior to HPLC analysis, all samples were filtrated through a 0.45 µm Duapore membrane (Millipore) to remove denaturated protein. All samples were stored at –20 °C until HPLC analysis.

2.4 High performance liquid chromatography (HPLC) of chito-oligosaccharides

Concentrations of chito-oligosaccharides were determined using HPLC with a Rezex Fast fruit H^+ column (100 mm length and 7.8 mm inner diameter) (Phenomonex). An 8 µl sample was injected on the column, and the 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. Peak areas were transferred to concentrations using standard samples with known concentrations of chitooligosaccharides.

2.5 Data analysis

Reaction conditions were such that the rate of hydrolysis of $(GlcNAc)_4$ was essentially constant over time, with the $(GlcNAc)_4$ concentration always staying above 80 % of the starting concentration. Data points were only discarded if more than 20 % of the initial $(GlcNAc)_4$ were hydrolyzed (to ensure initial rates only). If, for any reason, more than two data points, out of the seven, had to be removed, the whole set was discarded. The slopes of plots of 0.5 times the $(GlcNAc)_2$ concentration versus time were taken as the hydrolysis rate. The rates were plotted versus substrate concentration in a Michaelis-Menten plot, and the experimental data were fitted to either the Michaelis-Menten equation (Eq. 1) or the Michaelis-Menten equation with substrate inhibition (Eq. 2) [34] by nonlinear regression using Origin v7.0 (OriginLab Corp., Northampton, MA). Three independent measurements were performed for each mutant, and obtained parameters are presented as an average of these three measurements and their standard deviations.

$$v_0 = V_{\max} \frac{[\mathrm{S}]}{K_\mathrm{m} + [\mathrm{S}]} \quad (1)$$

$$v_0 = V_{\max} \frac{[\mathrm{S}]}{K_\mathrm{m} + \left(1 + \frac{[\mathrm{S}]}{K_\mathrm{i}}\right)[\mathrm{S}]} \quad (2)$$

3. Results and Discussion

Kinetic data have previously been obtained for ChiA-WT, ChiB-WT, ChiB-W97A (subsite +1), and ChiB-W220A (subsite +2) using $(\mathrm{GlcNAc})_4$ as the substrate (Table 1) [31]. In this work, we have obtained kinetic data for ChiA-W275A (subsite +1) and ChiA-F396A (subsite +2) allowing comparative analyses of effects on the same mutations in ChiB to address the connection between directionality and structural features of the +1 and +2 subsites (Fig. 1). Note that, due to the difference in directionality [23,26], the +1 and +2 subsites are *product* binding sites in ChiA, whereas they are *substrate* binding sites in ChiB. In other words, in ChiB, these subsites bind to the polymeric part of the chitin molecule that is being processively degraded.

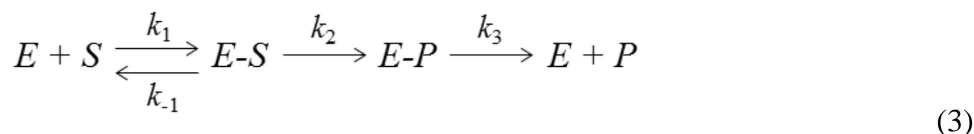
Hydrolysis of $(\mathrm{GlcNAc})_4$ always yielded two $(\mathrm{GlcNAc})_2$ molecules showing that productive binding exclusively takes place at −2 to +2 subsites. Moreover, in the substrate concentration range studied, all enzymes with the exception of ChiA-W275A showed straightforward Michaelis-Menten kinetics, and the experimental data were therefore fitted to the standard Michaelis-Menten equation (Eq. 1; Fig. 2). ChiA-W275A, however, displayed substrate inhibition necessitating the use of a version of the Michaelis-Menten equation (Eq. 2) that is adapted to this situation (Fig. 2) [34]. Upon removal of Trp^{275}, less binding energy is available to overcome the free energy penalty of the conformational changes that accompany substrate binding (see below), and it is conceivable that, consequently, other, non-productive

binding modes become more prominent. Notably, ChiA has an extended substrate-binding cleft and surface, displaying considerable substrate affinities in many subsites [35-37]. MacDonald *et al*. observed from a crystal structure the binding of two chitotriose thiazolines in ChiA, one molecule binding in the −3 to −1 subsites (as expected), and a second molecule in the "leaving-group subsites" +1 and +2 (with the third moiety of the ligand disordered in solvent) [36]. It may therefore be that for a fraction of available ChiA-W275A two molecules of $(GlcNAc)_4$ bind of either side of the catalytic acid in the same manner as the chitotriose thiazolines causing the substrate inhibition. Other substrate inhibition binding modes cannot of course be completely ruled out. It is unlikely that we observe product inhibition since our data are obtained at initial rates (always between 0 to 20 % of the substrate being consumed), and that product inhibition by and binding of $(GlcNAc)_2$ to ChiA has been found to be 0.45 mM (K_i) and 0.4 mM (K_d). respectively [38]. Fitting of the data to Eq. 2 yielded a substrate inhibition constant, K_i, of 25 μM.

The kinetic data (Table 1) show that while the wild type enzymes have similar k_{cat} and K_m values, the kinetic effects of mutations in their quite conserved +1 and +2 subsites are different. K_m values increase for both enzymes, but the increase is much more pronounced in ChiB (200-fold and 18-fold for W97A and W220A, respectively) than in ChiA (17-fold and 2.3-fold for W275A and F396A, respectively). Furthermore, in ChiB the mutations lead to an increase in $k_{cat,}$ (from 28 s^{-1} to 126 s^{-1} and 45 s^{-1} for W97A and W220A, respectively), whereas in ChiA the mutations resulted in decreased k_{cat} values (from 33 s^{-1} to 8 s^{-1} and 13 s^{-1} for W275A and F396A, respectively). For both enzymes, the effects of mutating the +1 subsite were more prominent than the effects of mutating the +2 subsite.

A previous study on the temperature-dependency of catalytic rate for ChiA led to the conclusion that the rate-determining step is substrate association when the substrate is insoluble while it is product release when the substrate is soluble [39]. Generally, product

release should be considered when analyzing the catalytic properties of polysaccharide degrading enzymes, as exemplified by product inhibition issues encountered in the industrial saccharification of cellulose [40]. Including product release as a potentially rate-limiting factor, the equations for Michaelis-Menten kinetics look as follows [41]:



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E\text{-}S \xrightarrow{k_2} E\text{-}P \xrightarrow{k_3} E + P \tag{3}$$

$$K_\mathrm{m} = \frac{k_3}{k_2 + k_3} \cdot \frac{k_2 + k_{-1}}{k_1} \tag{4}$$

$$k_\mathrm{cat} = \frac{k_2 k_3}{k_2 + k_3} \tag{5}$$

Notably, Eq. 5 shows that the rate constant for product displacement, k_3, is part of k_{cat} and may even dominate the term if it is much lower than the rate of the catalysis of the chemical reaction, k_2 [41].

It is likely that mutations in the +1 and +2 subsites affect the rate constant for product formation, k_2. Productive binding of $(GlcNAc)_4$ involves that the sugar moiety in the −1 subsite undergoes a conformational change with a considerable free energy penalty [42]. Classical work of lysozyme [43] has shown that in such cases, catalysis depends on this energy penalty being compensated by binding energy resulting from interactions between other sugar moieties and the enzyme. Weakening enzyme-substrate interactions in the +1 and +2 subsites is likely to reduce this binding energy (carbohydrate-aromate stacking interactions in GHs typically yield ~2 kcal/mol in favorable free energy change [35,44]). In addition to affecting binding energies, changes in the +1 and +2 subsites may affect the stereochemistry of the enzyme-substrate complex, with possible additional consequences on k_2. Importantly, ChiA and ChiB have very similar catalytic centers, including a fully conserved −1 subsite.

Structural data for their Michaelis complexes [22,45] show almost identical enzyme-substrate interactions in subsites near the catalytic center, including similar stacking interactions of the Trp (Trp97/Trp275) in subsite +1 and the aromate (Trp220/Phe396) in subsite 2 (Fig. 1). Thus, it seems reasonable to assume that the effects of the mutations on k_2 are similar for the two enzymes.

Removal of aromatic side chains in the +1 and +2 subsites is likely to reduce the substrate association rate, k_1, increase the substrate dissociation rate, k_{-1}, and increase the rate of product release, k_3. All these possible effects lead to an increase in K_m, as is indeed observed. The key difference between the two enzymes lies in the effect of the mutations on k_{cat}, which, considering Eq. 5 and the assumption that changes in k_2 are similar for both enzymes, must imply a difference in the effect on k_3 and/or in the overall importance of k_3. Notably, the only alternative explanation would imply that the mutations have a positive effect on k_2 in ChiB, which is highly unlikely, for reasons explained above. In ChiA, the mutations reduce k_{cat}, which must be due to a negative effect on k_2 that, apparently, is not compensated by an increase in k_3. In ChiB, however, the presumed similar effect on k_2 is more than compensated for by a positive effect of k_3, leading to an overall increase in k_{cat} (Table 1). Since the mutational effects in terms of lost binding energy should be similar in both enzymes, the only logic explanation for these observations is that k_3 is a rate-limiting factor in ChiB only. This explanation is in accordance with the observation that K_m effects are larger in ChiB than in ChiA, since Eq. 4 shows that the effect of changes in k_3 on K_m becomes larger as the relative magnitude of k_3 increases.

Previous studies have shown that surface exposed tryptophans close to the catalytic center are important for the degree of processivity in chitinases [7,25]. In ChiB, Trp^{97} is the most important residue for processivity [7] while Trp^{167}, in the – 3 subsite, has the same importance in ChiA [25]. Mutation of the Trp^{275} in ChiA, the equivalent of Trp^{97} in ChiB,

hardly affects processivity. These existing data show that tryptophans interacting with the polymeric part of the substrate, i.e. in the – subsites in ChiA and the + subsites in ChiB determine the degree of processivity. The present comparative analysis shows that the tailoring of the enzymes to opposite directionalities is reflected in the kinetics of $(GlcNAc)_4$ degradation. The + subsites in ChiA are likely to be optimized for rapid release of dimeric products during processive action, which may explain why expected beneficial effects of the W275A and F396A mutations on k_3 are not noticeable in the catalytic performance (k_{cat}) on $(GlcNAc)_4$. On the other hand the + subsites in ChiB are optimized to stay attached to the (normally polymeric) substrate in between catalytic steps, hence k_3 is rate-limiting and mutational effects on k_3 are noticeable in the overall catalytic performance on $(GlcNAc)_4$.

Inspection of the structures of ChiA and ChiB in complex with substrate shows that the +1 and +2 sites in ChiA are more open than in ChiB (Fig. 3) [22,45]. This is in line with our conclusion that product release from the +1 and +2 subsites is more restricted and rate-limiting in ChiB compared to ChiA.

In conclusion, the present study provides further insight into how chitinases of the same glycoside hydrolase family are fine-tuned to the directionality of processivity. We show that relatively simple kinetic studies with oligomeric substrates reveal differences between such enzymes that are in accordance with the directionality of their processivity. In particular, we show that the +1 and +2 subsites in ChiB are tailored to remain attached to the product after catalysis, as one might expect for a processive enzyme moving towards the polymers reducing end.

Acknowledgements

This work was supported by Grant 209335/F20 from the Norwegian Research Council.

Reference List

[1] Kim, J., Yun, S. and Ounaies, Z. (2006). Discovery of cellulose as a smart material. Macromolecules 39, 5583-5583.

[2] Tharanathan, R.N. and Kittur, F.S. (2003). Chitin - The undisputed biomolecule of great potential. Crit. Rev. Food Sci. Nutr. 43, 61-87.

[3] Lombard, V., Ramulu, H.G., Drula, E., Coutinho, P.M. and Henrissat, B. (2014). The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 42, D490-D495.

[4] Davies, G. and Henrissat, B. (1995). Structures and mechanisms of glycosyl hydrolases. Structure 3, 853-9.

[5] Koshland, D.E. (1953). Stereochemistry and the Mechanism of Enzymatic Reactions. Biological Reviews of the Cambridge Philosophical Society 28, 416-436.

[6] Sinnott, M.L. (1990). Catalytic Mechanisms of Enzymatic Glycosyl Transfer. Chemical Reviews 90, 1171-1202.

[7] Horn, S.J. et al. (2006). Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides. Proc. Natl. Acad. Sci U.S.A 103, 18089-18094.

[8] Katouno, F., Taguchi, M., Sakurai, K., Uchiyama, T., Nikaidou, N., Nonaka, T., Sugiyama, J. and Watanabe, T. (2004). Importance of exposed aromatic residues in chitinase B from Serratia marcescens 2170 for crystalline chitin hydrolysis. J Biochem. 136, 163-168.

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

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

[11] Watanabe, T. et al. (2003). Aromatic residues within the substrate-binding cleft of Bacillus circulans chitinase A1 are essential for hydrolysis of crystalline chitin. Biochem. J. 376, 237-244.

[12] Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J.K. and Jones, T.A. (1990). Three-dimensional structure of cellobiohydrolase II from Trichoderma reesei. Science 249, 380-6.

[13] Divne, C., Stahlberg, J., Teeri, T.T. and Jones, T.A. (1998). High-resolution crystal structures reveal how a cellulose chain is bound in the 50 angstrom long tunnel of cellobiohydrolase I from Trichoderma reesei. Journal of Molecular Biology 275, 309-325.

[14] Varrot, A., Frandsen, T.P., von Ossowski, I., Boyer, V., Cottaz, S., Driguez, H., Schulein, M. and Davies, G.J. (2003). Structural Basis for Ligand Binding and Processivity in Cellobiohydrolase Cel6A from Humicola insolens. Structure 11, 855-864.

[15] Lee, J.W. (2013) Advanced Biofuels and Bioproducts, Springer New York. New York, NY.

[16] Wilson, D.B. and Kostylev, M. (2012). Cellulase processivity. Methods Mol Biol 908, 93-9.

[17] Barr, B.K., Hsieh, Y.L., Ganem, B. and Wilson, D.B. (1996). Identification of two functionally different classes of exocellulases. Biochemistry 35, 586-592.
[18] Sakon, J., Irwin, D., Wilson, D.B. and Karplus, P.A. (1997). Structure and mechanism of endo/exocellulase E4 from *Thermomonospora fusca*. Nat. Struct. Mol. Biol. 4, 810-818.
[19] Watson, B.J., Zhang, H.T., Longmire, A.G., Moon, Y.H. and Hutcheson, S.W. (2009). Processive endoglucanases mediate degradation of cellulose by *Saccharophagus degradans*. J. Bacteriol. 191, 5697-5705.
[20] Terwisscha van Scheltinga, A.C., Armand, S., Kalk, K.H., Isogai, A., Henrissat, B. and Dijkstra, B.W. (1995). Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and X-ray structure of a complex with allosamidin: evidence for substrate assisted catalysis. Biochemistry 34, 15619-15623.
[21] Tews, I., Terwisscha van Scheltinga, A.C., Perrakis, A., Wilson, K.S. and Dijkstra, B.W. (1997). Substrate-assisted catalysis unifies two families of chitinolytic enzymes. J. Am. Chem. Soc. 119, 7954-7959.
[22] van Aalten, D.M.F., Komander, D., Synstad, B., Gåseidnes, S., Peter, M.G. and Eijsink, V.G.H. (2001). Structural insights into the catalytic mechanism of a family 18 exo-chitinase. Proc. Natl. Acad. Sci. U.S.A 98, 8979-8984.
[23] Hult, E.L., Katouno, F., Uchiyama, T., Watanabe, T. and Sugiyama, J. (2005). Molecular directionality in crystalline beta-chitin: hydrolysis by chitinases A and B from Serratia marcescens 2170. Biochem. J. 388, 851-856.
[24] Vaaje-Kolstad, G., Horn, S.J., Sørlie, M. and Eijsink, V.G.H. (2013). The chitinolytic machinery of Serratia marcescens – a model system for enzymatic degradation of recalcitrant polysaccharides. FEBS J. 280, 3028-3049.
[25] Zakariassen, H., Aam, B.B., Horn, S.J., Vårum, K.M., Sørlie, M. and Eijsink, V.G.H. (2009). Aromatic residues in the catalytic center of chitinase A from *Serratia marcescens* affect processivity, enzyme activity, and biomass converting efficiency. J. Biol. Chem. 284, 10610-10617.
[26] Igarashi, K. et al. (2014). Two-way traffic of glycoside hydrolase family 18 processive chitinases on crystalline chitin. Nat. Commun. 5
[27] Hamre, A.G., Lorentzen, S.B., Väljamäe, P. and Sørlie, M. (2014). Enzyme processivity changes with the extent of recalcitrant polysaccharide degradation. FEBS Lett. 588, 4620-4624.
[28] Horn, S.J., Sørbotten, A., Synstad, B., Sikorski, P., Sørlie, M., Vårum, K.M. and Eijsink, V.G.H. (2006). Endo/exo mechanism and processivity of family 18 chitinases produced by Serratia marcescens. FEBS J. 273, 491-503.
[29] Brurberg, M.B., Eijsink, V.G.H., Haandrikman, A.J., Venema, G. and Nes, I.F. (1995). Chitinase B from Serratia marcescens BJL200 is exported to the periplasm without processing. Microbiology 141, 123-131.
[30] Brurberg, M.B., Eijsink, V.G.H. and Nes, I.F. (1994). Characterization of a chitinase gene (chiA) from Serratia marcescens BJL200 and one-step purification of the gene product. FEMS Microbiol. Lett. 124, 399-404.
[31] Krokeide, I.M., Synstad, B., Gåseidnes, S., Horn, S.J., Eijsink, V.G.H. and Sørlie, M. (2007). Natural substrate assay for chitinases using high-performance liquid chromatography: A comparison with existing assays. Anal. Biochem. 363, 128-134.
[32] Zakariassen, H., Hansen, M.C., Jøranli, M., Eijsink, V.G.H. and Sørlie, M. (2011). Mutational effects on transglycosylating activity of family 18 chitinases and construction of a hypertransglycosylating mutant. Biochemistry 50, 5693-5703.
[33] Einbu, A. and Vårum, K.M. (2008). Characterization of Chitin and Its Hydrolysis to GlcNAc and GlcN. Biomacromol. 9, 1870-1875.

[34] Papežová, K., Němec, T., Chaloupková, R. and Glatz, Z. (2007). Study of substrate inhibition by electrophoretically mediated microanalysis in partially filled capillary. J. Chromatogr. A 1150, 327-331.
[35] Baban, J., Fjeld, S., Sakuda, S., Eijsink, V.G.H. and Sørlie, M. (2010). The Roles of Three Serratia marcescens Chitinases in Chitin Conversion Are Reflected in Different Thermodynamic Signatures of Allosamidin Binding. J. Phys. Chem. B 114, 6144-6149.
[36] Macdonald, J.M., Tarling, C.A., Taylor, E.J., Dennis, R.J., Myers, D.S., Knapp, S., Davies, G.J. and Withers, S.G. (2010). Chitinase inhibition by chitobiose and chitotriose thiazolines. Angew. Chem. Int. Ed. Engl. 49, 2599-2602.
[37] Norberg, A.L., Dybvik, A.I., Zakariassen, H., Mormann, M., Peter-Katalinic´, J., Eijsink, V.G.H. and Sørlie, M. (2011). Substrate positioning in chitinase A, a processive chito-biohydrolase from *Serratia marcescens*. FEBS Lett. 585, 2339-2344.
[38] Kuusk, S., Sørlie, M. and Väljamäe, P. (2015). The predominant molecular state of bound enzyme determines the strength and type of product inhibition in the hydrolysis of recalcitrant polysaccharides by processive enzymes. J. Biol. Chem.
[39] Zakariassen, H., Eijsink, V.G.H. and Sørlie, M. (2010). Signatures of activation parameters reveal substrate-dependent rate determining steps in polysaccharide turnover by a family 18 chitinase. Carbohyd.Polym. 81, 14-20.
[40] Teugjas, H. and Valjamae, P. (2013). Product inhibition of cellulases studied with C-14-labeled cellulose substrates. Biotechnol. Biofuels 6, 14.
[41] Fersht, A. (1999) Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding, Freeman. New York.
[42] Biarnes, X., Ardevol, A., Planas, A., Rovira, C., Laio, A. and Parrinello, M. (2007). The Conformational Free Energy Landscape of ƒ-D-Glucopyranose. Implications for Substrate Preactivation in ƒ-Glucoside Hydrolases. J. Am. Chem. Soc. 129, 10686-10693.
[43] Honda, Y. and Fukamizo, T. (1998). Substrate binding subsites of chitinase from barley seeds and lysozyme from goose egg white. Biochim.Biophys.Acta 1388, 53-65.
[44] Zolotnitsky, G., Cogan, U., Adir, N., Solomon, V., Shoham, G. and Shoham, Y. (2004). Mapping glycoside hydrolase substrate subsites by isothermal titration calorimetry. Proc. Natl. Acad. Sci. U.S.A 101, 11275-11280.
[45] Papanikolau, Y., Prag, G., Tavlas, G., Vorgias, C.E., Oppenheim, A.B. and Petratos, K. (2001). High resolution structural analyses of mutant chitinase A complexes with substrates provide new insight into the mechanism of catalysis. Biochemistry 40, 11338-11343.

Table 1. Kinetic parameters of wild-type and mutant ChiA and ChiB for the hydrolysis of $(GlcNAc)_4$ at pH 6.1 and 37 °C.

	k_{cat} (s^{-1})[a]	K_m (μM)[a]	k_{cat}/K_m (s^{-1} $μM^{-1}$)
ChiA-WT[b]	33 ±1	9 ± 1	4
ChiA-W275A[c]	8 ± 1	157 ± 8	0.1
ChiA-F396A	13 ± 2	21 ± 8	0.6
ChiB-WT[b]	28 ± 2	4 ± 2	7
ChiB-W97A[b]	126 ± 4	807 ± 40	0.2
ChiB-W220A[b]	45 ± 2	71± 3	0.6

[a] Average of three measurements. [b] Data from Krokeide *et al*. [31]. [c] The kinetic parameters were calculated with respect to the Michaelis-Menten equation for substrate inhibition; see text for details.

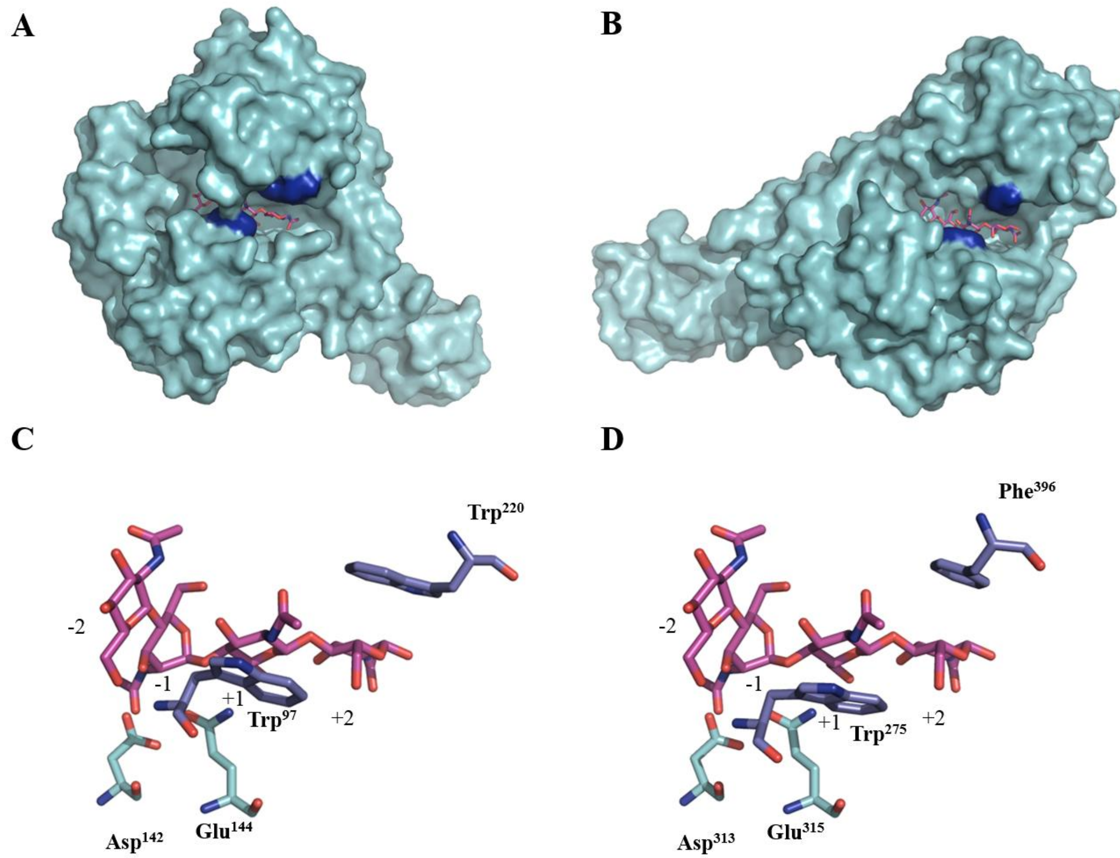


Fig. 1. Enzyme-substrate interactions for ChiA and ChiB. Panel A and C show the structure of exo-processive ChiB (PDB ID code 1e6n, [22]) that degrades chitin from the non-reducing end. Panels B and D show the structure of exo-processive ChiA (PDB ID code 1ehn, [45]) that degrades chitin from the reducing end. Panels A and C show surface representations of the complete protein; the surface-exposed aromatic amino acids in subsites +1 and +2 are highlighted in blue, whereas crystallographically observed substrate molecules are shown in magenta. Bothe chitinases contain a carbohydrate-binding module, a CBM5/12 pointing to the right in ChiB and and FnIII domain pointing to the left in ChiA (for more details, see Vaaje-Kolstad et al., 2013 [24]) (C) Close up of the active site of ChiB. Asp^{142} and Glu^{144} are part of the diagnostic DXDXE motif containing the catalytic acid/base (Glu144). (D) Close up of the active site of ChiA.

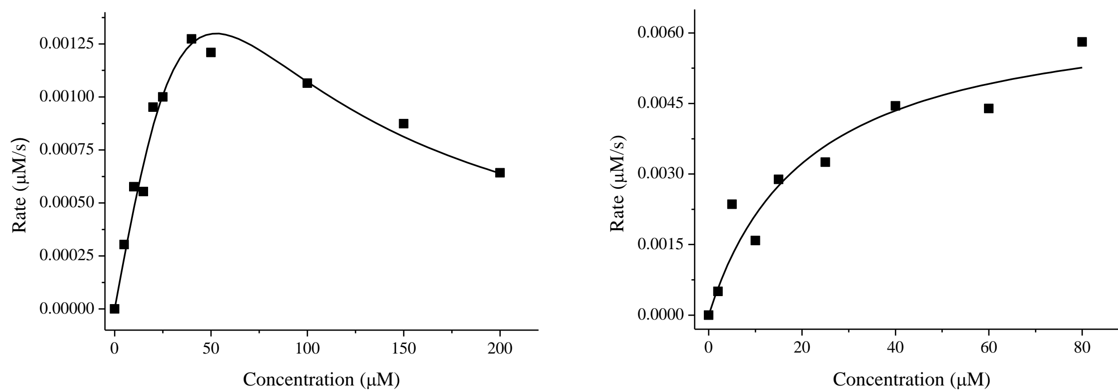


Fig. 2. Examples of Michaelis-Menten plots for degradation of $(GlcNAc)_4$ by ChiA-W275A (left) and ChiA-F396A (right). The left plot shows substrate inhibition; see text for details.

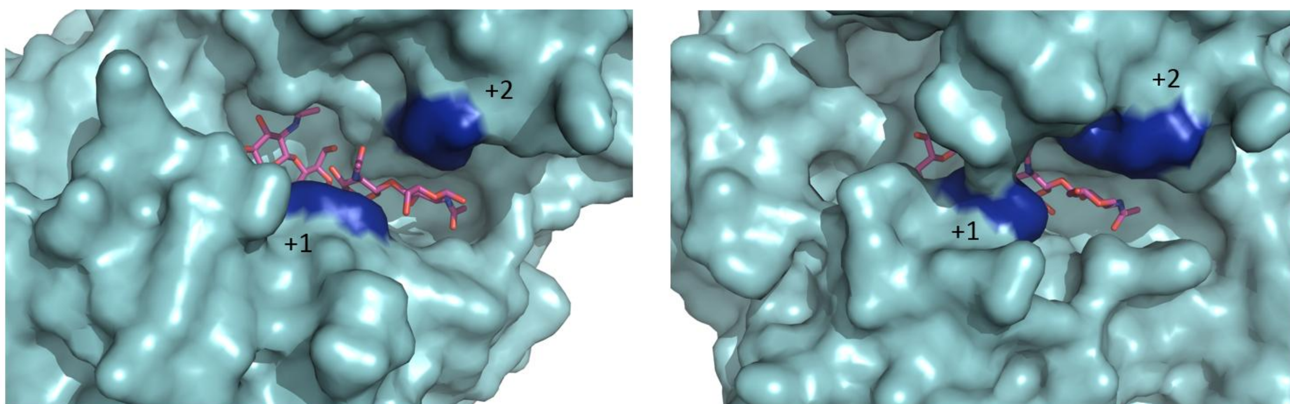


Fig. 3. A close-up of a surface presentation of the +1 and +2 subsites of ChiA (left; PDB ID code 1ehn, [45]) with Trp^{275} and Phe^{396} colored blue and ChiB (right; PDB ID code 1e6n, [22]) with Trp^{97} and Trp^{220} colored blue, respectively. The structures show that ChiA has a more open active site cleft compared to ChiB; in ChiB, part of the cleft has a tunnel-like shape.

***Authors contributions**

Author contributions: Sørlie and Eijsink planned project and experiments. Hamre and Schaupp executed experiments, Sørlie, Eijsink, Hamre, and Schaupp wrote the manuscripts.