Elsevier Editorial System(tm) for FEBS Letters
Manuscript Draft

Manuscript Number: FEBSLETTERS-D-14-01428R1

Title: Enzyme processivity changes with the extent of recalcitrant polysaccharide degradation

Article Type: Research Letter

Keywords: Processivity; recalcitrant polysaccharides

Corresponding Author: Prof. Morten Sorlie,

Corresponding Author's Institution:

First Author: Anne Grethe Hamre

Order of Authors: Anne Grethe Hamre; Silje Benedicte Lorentzen; Priit Väljamäe; Morten Sorlie

Manuscript Region of Origin: NORWAY

Abstract: Polysaccharide depolymerization in nature is primarily accomplished by processive glycoside hydrolases which abstract single carbohydrate chains from polymer crystals and cleave glycosidic bonds without dissociating from the substrate after each catalytic event. Processivity is thought to conserve energy during enzymatic polysaccharide degradation. Herein, we compare two processive chitinases, ChiA and ChiB, one mutant, ChiB-W97A, and the endochitinase ChiC of the well-characterized chitinolytic machinery of Serratia marcescens by monitoring the extent of degradation on three different chitin substrates, and using the [(GlcNAc)2]/[GlcNAc] product ratio as a measure of processivity. The results show that the apparent processivity (Papp) greatly diminishes with the extent of degradation and confirm the hypothesis that Papp is limited by the length of obstacle free path on the substrate.

Suggested Reviewers: Kiyohiko Igarashi
The University of Tokyo
aquarius@mail.ecc.u-tokyo.ac.jp
Expert in the field

Claus Felby
University of Copenhagen
cf@life.ku.dk
Expert in the field

Birte Svensson
Technical University of Denmark
bis@bio.dtu.dk
Expert in the field

Opposed Reviewers:

Dear Editor,

Please find enclosed our manuscript "Enzyme processivity changes with the extent of recalcitrant polysaccharide degradation", which we would like to be considered for publication in *FEBS Letters*.

Polysaccharide depolymerization in nature is primarily accomplished by processive glycosyl hydrolases (GHs) as this is postulated to be more efficient than by non-processive GHs. Our manuscript details how apparent processivity changes with the extent of polysaccharide degradation and the nature of the substrate for a single GH. Moreover, efficiency and rate of degradation with respect to the degree of processivity are shown to correlate. 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

Detailed Response to Reviewers

Dear Editor,

We are grateful for the thorough and positive review of our manuscript “Enzyme processivity changes with the extent of recalcitrant polysaccharide 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

Reviewer #1: The manuscript by Hamre et al., seeks out parse out the determinants for processivity for glycoside hydrolyses on crystalline, recalcitrant substrates. The role of processivity in polysaccharide depolymerization is only now being seriously explored, given their importance as catalysts for the generation of biofuel precursors. While none of the findings of the authors are surprising or unexpected, works such as this are necessary for formulating an understanding what accounts for processivity of these enzymes. The only issues that I have with this manuscript is that it is written for an audience that is intimately familiar with carbohydrate chemistry, and I would suggest that the authors modify the document suitable for non-specialists. For example, it would be useful to have chemical structures of the sugars. Similarly, for the Figure, the symbols used for the different enzymes are drawn in such a way that it is not easy to follow what is happening during the initial time points due to overlap.

Response: We have added a Figure that shows the structures of chitin and cellulose, as suggested. This Figure is now Fig. 1. Moreover, we have made another Figure (now Fig. 2.) that explains why a high ratio of [$(GlcNAc)_2$]/[GlcNAc] indicates a high degree of apparent processivity.

We also agree with the reviewer that it is not easy to follow what is happening during the initial time points, especially in what was Fig. 1. (now Fig. 3.) and what was Fig. 5. (now Fig. 7.). For Fig. 3., we have added another Figure (Fig. 3B.) showing initial degradation. In Fig. 7. (synergy when several chitinases are added, there are no differences initially and we now state this explicitly in the text.

Reviewer #2: The manuscript of Hamre et al. deals with the apparent processecivity of 3 glycoside hydrolases from the chitinolityc machinery. The work is nicely reported and the manuscript is of high interest.
Experiments sound perfectly suitable for supporting the hypothesis. Conclusions are of utmost importance for experts of the field and even to a larger audience.
The current manuscript is to mind highly suitable for publication in FEBS Letters.

Response: We appreciate the positive evaluation.

Reviewer #5: The investigation aims at quantifying and comparing the degree of processivity, (i.e. number of catalytic events per enzyme-to-substrate attachment) for three chitinase enzymes (and one mutant) which appear to represent distinct classes of enzymes in terms of structure, behavior and role in chitin degradation. The results show distinct and different patterns for the enzymes, which is highly interesting and well worth publication, and FEBS Letters is a suitable forum for this work. Experiments appear to be well performed and described and the results seem reliable and are clearly presented. Overall the paper is very well written. However, the manuscript can (and needs to) be further improved, as motivated below.

The major weakness of the work lies in the use of the ratio of disaccharide over monosaccharide product as a measure of processivity. It relies on a number of assumptions that have not been appropriately addressed in the paper. The authors must either provide convincing motivations for these assumptions, or clearly refer to previous papers were experimental evidence or convicing motivation can be found, or at least declare all possible factors other than processivity that may influence the disaccharide/monosaccharide ratio, and point out that results should be treated with caution.

In the Introduction (page 4) it is written:

"It has been proposed that the first cleavage from a polymer chain end will result in the release of an odd numbered oligosaccharide (e.g mono- or trisaccharide) whereas all subsequent processive cleavages result in the release of disaccharides. Assuming that the first product is a trisaccharide that is subsequently hydrolysed to a mono- and a disaccharide the Papp is given by Papp = [disaccharide] / [monosaccharide]."

- Convincing motivation is needed for this assumption, either in the paper or by reference.

Intuitively, I would expect an equal probability for even and odd number of residues in the first product from a chain end, rather than trisaccharide alone. An equal even/odd probability in turn relies on a number of assumptions:
1) That the association of chain ends to the surface of the insoluble substrate, and thereby accesibility for enzyme attachment, is independent of the orientation of the chain end relative to the surface. If that is the case, or how much it may differ for different polymers and aggregation forms, and to which extent it may affect the probability for even/odd first product, is poorly known.
2) That there is an equal probability for both orientations of the chain end to associate with the enzyme. If that is the case, and the possible extent of inequality, is also poorly known. It could very well be so that each enzyme displays a certain preference for one or the other orientation, and that this may differ with substrate and aggreagation form.
3) That the probability for cleavage, and thereby continued processive action, is equal for both chain orientations, once associated to the enzyme. Again this is poorly known, and possible inequality might be a unique property for each enzyme/substrate combination.

The above statement (Intro, page 4) is directly followed by:
"Although this approach has several pitfalls [17] a recent high speed atomic force microscopy (HS AFM) study has revealed a good consistency between Papp values of cellobiohydrolases measured using product profiles and HS AFM results [18]. To assess the degree of processivity we have therefore quantified the soluble hydrolytic products GlcNAc and (GlcNAc)2."

- It is good that a reference is provided where possible pitfalls are discussed, but the reference to the HS-AFM study is not sufficient to rule out all these pitfalls. First of all, there is not yet a sufficient body of experimental results to extrapolate the results for individual enzyme/substrate combinations to general conclusions for all or groups of processive enzymes. What applies to the three CBHs on the two cellulose substrates investigated in the HS-AFM study, does not necessarily hold for the chitinases investigated here. Secondly, Nakamura et al have not calculated and compared Papp values from product profiles and do not claim that there is a good consistency with AFM results, beyond stating that the values place the three analysed enzymes in the same order (TrCel7A > PcCel7D > PcCel7C). When calculating Papp values from AFM data (34.0; 29.3; 28.7), the values are indeed similar to the values for product ratios (34.8; 33.3; 29.9), but a closer look also reveals that the values are rather similar for all three enzymes and actually differ less than the estimated errors.

- It is also important to consider here, how well processivity values from HS-AFM results are expected to correlate with Papp values from product ratios. In HS-AFM, single enzyme molecules are tracked when moving along the surface of the insoluble substrate. It is highly likely that enzyme molecules that bind transiently and only make a single or few processive cuts before dissociation, will be under-represented among observed enzyme molecules. For example, enzymes which are known or predicted to act processively prefentially in one direction may very well be able to associate with the "wrong" chain ends, and even act processivley in the "wrong" direction, but probably make only a single or few cuts before dissociation. Such events are less likely to be observed. Thus the method is expected to overestimate the average processivity (i.e. average for all enzyme molecules in the population).

- On the other hand, the even/odd product ratio may be affected by further factors other than processivity:
1) Endo-initiation, will generate even products and result in over-estimation of processivity. Väljamäe and coworkers have already shown for some cellobiohydrolases that endo initiation may be as common or even more common than exo-initiation, also for enzymes considered to be highly processive, and also that the probability of endo initiation may vary with both enzyme and substrate.
2) Disaccharide cleavage, will reduce the even/odd ratio, and thus underestimate processivity. Even a low activity against the disaccharide may have considerable effect in experiments with long incubation time.
Both these factors are likely to be enzyme-specific and may also depend on the conditions. For each of the investigated enzymes (ChiA, ChiB, ChiB-W97A, ChiC) it needs to be declared if it is known that endo-initiation and disaccharide hydrolysis activity is sufficiently low to have a neglible effect on product ratios in these experiments. In the case where it is not known, that should be declared.

Response: We agree with the reviewer. Caution must be taken when interpreting the results.

To address this, we have changed:

“Although this approach has several pitfalls [17] a recent high speed atomic force microscopy (HS AFM) study has revealed a good consistency between P_{app} values of cellobiohydrolases measured using product profiles and HS AFM results [18]. To assess the degree of processivity we have therefore quantified the soluble hydrolytic products GlcNAc and $(GlcNAc)_2$.”

with

“This approach has several pitfalls, like the assumption of the exclusive formation of odd numbered oligosaccharide 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 Despite these possible pitfalls, a recent high speed atomic force microscopy (HS AFM) study has revealed a good consistency between P_{app} values of cellobiohydrolases measured using product profiles and HS AFM results [18]. However, one may speculate that the fraction of enzymes performing very short runs on polymer is underestimated in single molecule tracking studies leading to the overestimate of processivity. Here we quantified the soluble hydrolytic products $(GlcNAc)_2$ and GlcNAc in hydrolysis of chitin by *Serratia marcescens* chitinases and used their ratio as a measure of P_{app}. Although the absolute values of P_{app} should be treated with caution our results demonstrated the drastic reduction of P_{app} with the degree of chitin conversion.”

We also agree with the Reviewer that it is important to address if any of the enzymes are able to cleave disaccharides into monomers at the given conditions. None of the enzymes is able to do this and this has been stated in the manuscript.

The following sentence has been added:

“It is important to note that none of the enzymes are capable of cleaving $(GlcNAc)_2$ into two GlcNAc-units”

Detailed comments, in order of appearance:

* Highlights, suggested changes:
Apparent enzyme processivity changes with extend of polysaccharide degradation
=> Apparent enzyme processivity decreases with the extent of polysaccharide degradation
Correlation between enzyme processivity and degradation efficiency
=> Enzyme processivity correlates with degradation efficiency

Response: These are very good suggestions so the Highlights have been changed.

* Abstract
- The current abstract does not fully summarise the content of the article. What was done and which enzymes were used can be better specified and would make it more clear to the reader. Could be something like this:
"Polysaccharide depolymerization in nature is primarily accomplished by processive glycoside hydrolases which abstract single carbohydrate chains from polymer crystals and cleave glycosidic bonds without dissociating from the substrate after each catalytic event. Processivity is thought to conserve energy during enzymatic polysaccharide degradation. Herein, we compare two processive chitinases, ChiA and ChiB, one mutant, ChiB-W97A, and the endochitinase ChiC of the well-characterized chitinolytic machinery of Serratia marcescens, by monitoring the extent of degradation on three different chitin substrate, and using the [GlcNAc2]/[GlcNAc] product ratio as measure of processivity. The results show that the apparent processivity

(Papp) greatly diminishes with the extent of degradation and confirms the hypothesis that Papp is limited by the length of obstacle free path on the substrate."

Response: We have changed the Abstract as suggested.

- Change "exo-processive chitinases" to "processive chitinases". As far as I understand, neither of the two enzymes are intrinsically restricted to only attack chain ends. Endo-type attachment and endolytic cleavage has been observed on certain substrates. It may very well be so that action on insoluble chitin is predominantly initiated from chain ends, but that is then dictated by the properties of the substrate, rather than an inherent property of the enzyme. Thus the term "exo-processive" should be avoided (throughout the whole article), unless an absolute restriction to exo-initiation has been experimentally proven for that particular enzyme. The probability of endo vs exo-initiation, on different substrates and different conditions, is in itself an important parameter for description of the property of an enzyme that may have large influence on the enzyme's efficieny and role in polymer degradation.

Response: We agree with the Reviewer and have changed this throughout the manuscript.

- Change "a nonprocessive endochitinase" to "an endochitinase" (or "a less processive endochitinase"). The results presented in this study indicate that also ChiC can act processively (albeit to a lower extent than ChiA and ChiB). It is thus not very wise to state the opposite already in the Abstract.

Response: We have done this as suggested by the Reviewer.

* Introduction
- Change "two exo-processive chitinases (ChiA and ChiB) and a nonprocessive endochitinase (ChiC)" => "two processive chitinases (ChiA and ChiB) and an endochitinase (ChiC)" as discussed above.

Response: We have done as suggested.

* Materials and methods
- Remove "All other chemicals were bought from standard manufacturers.", or rephrase to something meaningful, e.g. "All other chemicals were of analytical grade."

Response: We have changed as suggested.

- Declare the dimensions for the Fast fruit H+ column (length, inner diameter).

Response: We have added the dimensions.

* Results and discussion
- (page 7) Change "… their profiles are shown in Fig. 2." to e.g. "… progress curves of formation are shown in Fig. 2." to better define what is shown in Figure 2.

Response: We have changed as suggested.

- (page 7) "Thus the HS AFM single molecule tracking resulted in processivity values of 30.3 and 18.8 for ChiA and ChiB, respectively, which are in good agreement with the values found in this study by using the [(GlcNAc)2]/[GlcNAc] ratio." - Explain in which respect the results are in good agreement. Product ratios are from inital degradation of chitin. Were AFM results obtained under comparable conditions? Which values are in good agreement? For ChiA the values are indeed very similar (ratio 30.1 vs AFM 30.3), but for ChiB the ratio value is +29% compared to the AFM value. But should we expect that the two methods will generate similar values, if possible over-estimation of processivity by HS-AFM, and other effects are taken into account? I would limit the conclusions to stating that the results are in agreement insofar as also the even/odd ratio indicate a higher processivity for ChiA than ChiB.

Response: We appreciate this comment and agree with the Reviewer and have changed the text as suggested.

- (page 9) I guess that "a shared alpha-chitin" should be changed to "a sheared alpha-chitin"

Response: We appreciate the detection of this error.

* Figures

Figure 1
- Specify that beta-chitin was the substrate here.

- Change "The extent of degradation of chitin with respect to time for two processive and two non-processive chitinases of Serratia marcescens."
=> "The extent of degradation of beta-chitin with respect to time for Serratia marcescens chitinases.
- Note experimental conditions, i.e. substrate and enzyme concentrations, pH and temperature.
- Explain how extent of degradation is defined.
- Change "are more effective degrades" => "are more efficient degraders"
- Change "the two non-processive" to e.g. "the two less processive"

Figure 2
- Hydrolysis rates are not plotted in the diagram, and the legend needs to be changed.
- Change to e.g. "Progress curves of formation of GlcNAc2 (squares) and GlcNAc (circles) products from hydrolysis of beta-chitin by the chitinases. GlcNAc2 and GlcNAc were the only products detected.

Figure 3
- Remove "with dissimilar inherent degree of processivity."

Response: We have performed the corrections regarding the Figures 1-3 as suggested by the Reviewer.

Additional Reviewer (likely # 3 or 4): In the present manuscript, the authors demonstrated detailed analysis of product formation during chitin degradation by Serratia marcescens chitinases and discussed time course changes of processivity with the degree of the degradation. Processivity is very important factor to degrade polymers, and the estimation of processivity is one of the target for the researchers of such degrading enzymes. Since the results obtained in this study includes very important information for the degradation of recalcitrant polysaccharides such as cellulose and chitin, this manuscript will be becoming mile stone for biomass utilization. I have very positive impression for the manuscript while I would like to suggest the authors to revise the manuscript as follows:

Minor points
1. The authors have not draw the line for time course in Fig 1, 2 and 5.
Although there is argument what kind of function we should use for the curve fitting, I think "progress curve" should be lines but not plots.
Please consider to use double exponential or other function to track the plots.

Response: Some of the plots are a bit “crowded” in that it is not easy to follow i.e. what is happening during the initial time. Using lines makes them even more crowded. In our opinion, the way the plots are presented is the best even though they are not perfect.

2. I think readers will not understand why even sugar/odd sugar is an indicator of processivity. Please add some schematic representation for better understanding by the readers.

Response: We agree with the Reviewer and have added such a Figure (now Fig. 2).

3. In the discussion of Fig 4, the authors have not discuss the changes of chitin substrate, specially about hydration of chitin. If the content of water molecule is decreased inside of the substrate, same tendency will appear. Please discussed this point, too.

Response: We greatly appreciate this comment. The Reviewer makes a very interesting point. We have addressed this at the same time as we have expanded the discussion on our α-chitin results.

"Finally, the effect of the origin of the substrate on P_{app} was assessed by allowing ChiA to incubate with a shared α-chitin. Here, at the initial stage of hydrolysis, the $[(GlcNAc)_2]/[GlcNAc]$ ratio was observed to be 14.2 ± 0.5, significantly smaller than what was observed for the β-chitin (30.1 ± 1.5) used in this study. Interestingly, the addition of ChiB only slightly altered the initial P_{app} (15.5 ± 1.0).

have been exchanged with:

"Finally, the effect of the origin of the substrate on P_{app} was assessed by allowing ChiA to incubate with a sheared α-chitin. Here, at the initial stage of hydrolysis, the $[(GlcNAc)_2]/[GlcNAc]$ ratio was observed to be 14.2 ± 0.5, significantly smaller than what was observed for the β-chitin (30.1 ± 1.5) used in this study. The anti-parallel orientation of the polymer chains of α-chitin allows a high number of hydrogen bonds to be formed resulting in a tight packing of the polymeric strands and high stability of the crystalline structure [26, 27]. Moreover, this tight packing exclude the presence of water. The parallel orientation of the polymer strands in β-chitin allows for up to two water molecules pr. *N*-acetylglucosamin residue making this form less recalcitrant [28, 29], and hence likely easier to degrade in a processive manner. With this in mind, the decreased $[(GlcNAc)_2]/[GlcNAc]$ ratio with respect to extent of degradation as shown in Fig. 6 may also in part be connected with the hydration of the β-chitin in that the content of water molecules is decreased inside of the substrate making this part of the crystal more recalcitrant. The addition of ChiB to ChiA in α-chitin degradation only slightly altered the initial P_{app} (15.5 ± 1.0).

4. How much triose is produced during the incubation? Is the processivity becoming different if they add triose formation? The authors have not shown the raw data of HPLC, so they may add typical chromatogram whether their analysis is reliable enough.

Response: Triose is not detected at any time during degradation . If really important, we could add a typical chromatogram, but would prefer not to since we now already have 7 Figures.

Quite minor points
Page 3 lines 11-12, the unit of kcal/chitobiose kcal/cellobiose are inappropriate. Please change to kcal/mol of chitobiose or so.

Response: We are grateful for the detection of this error. It has been corrected.

Enzyme processivity changes with the extent of recalcitrant polysaccharide degradation

Anne Grethe Hamre,[1] Silje Benedicte Lorentzen,[1] Priit Väljamäe,[2] and Morten Sørlie[1,#]

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

[2]*Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia* .

[#] To whom correspondence should be addressed. E-mail: morten.sorlie@nmbu.no. Telephone: +47 64965902. Fax: +47 64965901.

ABSTRACT

Polysaccharide depolymerization in nature is primarily accomplished by processive glycoside hydrolases which abstract single carbohydrate chains from polymer crystals and cleave glycosidic bonds without dissociating from the substrate after each catalytic event. Processivity is thought to conserve energy during enzymatic polysaccharide degradation. Herein, we compare two processive chitinases, ChiA and ChiB, one mutant, ChiB-W97A, and the endochitinase ChiC of the well-characterized chitinolytic machinery of *Serratia marcescens* by monitoring the extent of degradation on three different chitin substrates, and using the [$(GlcNAc)_2$]/[GlcNAc] product ratio as a measure of processivity. The results show that the apparent processivity (P_{app}) greatly diminishes with the extent of degradation and confirm the hypothesis that P_{app} is limited by the length of obstacle free path on the substrate.

*Highlights

Highlights:

- Apparent enzyme processivity decreases with extend of polysaccharide degradation
- Apparent enzyme processivity changes with the nature of the substrate
- Enzyme processivity correlates with degradation efficiency

*Manuscript text
Click here to view linked References

1. Introduction

To understand the mechanisms behind enzymatic hydrolysis of recalcitrant polysaccharides such as cellulose (a 1,4-β-linked polymer of D-glucose) and chitin (a 1,4-β-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc) (Fig 1.) is of great biological and economic importance. Enzymes acting on cellulose or chitin face the challenges of associating with the insoluble substrate, disrupting the crystal packing, and guiding a single polymer chain into the catalytic center. Many polymer active enzymes act in a processive manner 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 [1-5]. The general idea is that catalytic efficiency is improved by keeping the enzyme closely associated to the substrate in between subsequent hydrolytic reactions. In the case of crystalline substrates, calculations show that the enzymes face a free energy penalty of 5.6 kcal/mol pr. chitobiose unit and 5.4 kcal/mol pr. cellobiose unit in decrystallization energies signifying the importance for processive enzymes being capable of keeping once-detached single chains from re-associating with the insoluble material [3, 5-8].

Intrinsic processivity of polymer active enzymes is governed by the rate constants k_{off} and k_{cat} meaning that it is the enzyme dissociation from the polymer chain and the catalytic constant that is important. This is summarized in the formula $P^{Intr} = (k_{cat} + k_{off}) / k_{off}$ [9, 10]. P^{Intr} values estimated for cellobiohydrolases are in the range of 1000 whereas the measured values of processivity, also referred to as apparent processivity (P_{app}), are more than an order of magnitude lower [9, 11]. These findings have led to the hypothesis that P_{app} (number of cleavages per one productive binding event) is limited by the length of obstacle free path on the substrate [9, 11-13]. Because processive enzymes have intrinsically low k_{off} values the encounter of an obstacle will cause an enzyme to halt. This limits the rate of enzyme

recruitment [12] and causes so called traffic jams [14, 15] on the polymer surface thus slowing the overall rate of polymer degradation. To test the possible correlation between the rate of polysaccharide degradation and P_{app}, we studied the changes of P_{app} upon a large range of the degree of chitin conversion. The well-characterized chitinolytic machinery of *Serratia marcescens* that contains two processive chitinases (ChiA and ChiB) and an endochitinase (ChiC) [16] was used. Chitin is an insoluble and heterogenous substrate, and therefore it is challenging to determine the processivity quantitatively [17]. The simplest way to measure P_{app} is to follow the profile of soluble products from hydrolysis. It has been proposed that the first cleavage from a polymer chain end will result in the release of an odd numbered oligosaccharide (e.g mono- or trisaccharide) whereas all subsequent processive cleavages result in the release of disaccharides (Fig. 2.). Assuming that the first product is a trisaccharide that is subsequently hydrolysed to a mono- and a disaccharide the P_{app} is given by P_{app} = [disaccharide] / [monosaccharide]. 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. Despite these possible pitfalls, a recent high speed atomic force microscopy (HS AFM) study has revealed a good consistency between P_{app} values of cellobiohydrolases measured using product profiles and HS AFM results [18]. However, one may speculate that the fraction of enzymes performing very short runs on the polymer is underestimated in single molecule tracking studies leading to an overestimate of the degree of processivity. Here we quantified the soluble hydrolytic products $(GlcNAc)_2$ and GlcNAc in hydrolysis of chitin by *Serratia marcescens* chitinases and used their ratio as a measure of P_{app}. Although the absolute values of P_{app} should be treated with caution our results demonstrated the drastic reduction of P_{app} with the degree of chitin conversion.

2. **Materials and methods**

2.1 Chemicals.

Chitooligosaccharides were obtained from Megazyme (Wicklow, Ireland). Squid pen β-chitin was purchased from France Chitin (180μm microparticulate, Marseille, France) and α-chitin was purchased from Yaizu Suisankagaku Industry (Tokyo, Japan) and was sheared using a converge mill to a crystallinity of 74 % as described by Nakagawa *et al.* [19]. All other chemicals were of analytical grade.

2.2 Protein expression and purification.

The ChiA and ChiB [20], the ChiC [21], and ChiB-W97A [8] genes were expressed in *Escherichia coli* as described previously. The 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 Degradation of chitin

Hydrolysis of chitin (2.0 mg/ml) was carried out in 50 mM sodium acetate buffer at pH 6.1. The chitin samples were sonicated for 20 minutes in a sonication bath (Transsonic, Elma) to increase the surface of the substrate and thereby increase the availability of chitin ends for the enzymes [22]. The reaction tubes were then incubated at 37 °C in an Eppendorf thermo mixer at 800 rpm to avoid settling of the chitin particles. The enzyme concentrations

were in total 2.5 μM in all experiments. Aliquots of 75 μl were withdrawn at regular time intervals, and the enzymes were inactivated by adding 75μl 20 mM H_2SO_4. Prior to further 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 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.

2.4 High performance liquid chromatography (HPLC).

Concentrations of chitooligosaccharides 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 mono/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. Peak areas were compared with peak areas obtained with standard samples with known concentrations of mono- and disaccharides.

3. Results and discussion

Progress curves of degradation of a β-chitin (180 μm) by the processive *S. marcescens* chitinases ChiA and ChiB along with the endochitinase ChiC and a ChiB variant (ChiB-W97A) with reduced processivity are shown in Fig. 3. As is characteristic to the enzymatic degradation of recalcitrant polysaccharides we can see drastic decreases in hydrolysis rates already at moderate degrees of conversion. $(GlcNAc)_2$ and GlcNAc were the only soluble products detected and progress curves of formation are shown in Fig. 4. It is important to note that none of the enzymes are capable of cleaving $(GlcNAc)_2$ into two GlcNAc-units. At the initial stage of hydrolysis, the $[(GlcNAc)_2]/[GlcNAc]$ ratio was constant with average values of 30.1 ± 1.5, 24.3 ± 2.0, 14.3 ± 1.4 and 11.0 ± 1.8 for ChiA, ChiB, ChiC and ChiB-W97A respectively (Fig. 5). In a recent HS AFM study, the velocity of movement and the duration of processive runs of ChiA and ChiB on β-chitin were measured [23]. The authors estimated the half-life of processivity to be 21 and 13 cleavages for ChiA and ChiB respectively. These values can be converted to processivity values by dividing with ln2 [18]. Thus the HS AFM single molecule tracking resulted in processivity values of 30.3 and 18.8 for ChiA and ChiB, respectively, which are in good agreement with ChiA being more processive than ChiB as seen in our study. This also indicates that the $[(GlcNAc)_2]/[GlcNAc]$ ratio can be used as a simple measure of the processivity of these enzymes.

Comparison of the extent of chitin degradation and the initial processivity values suggests that enzymes with a higher initial degree of processivity are more efficient degraders of β-chitin. Interestingly at higher degree of chitin degradation, analysis of $[(GlcNAc)_2]/[GlcNAc]$ ratios reveals differences between the processive enzymes ChiA and ChiB and the less processive endo acting ChiC and ChiB-W97A (Fig. 4). In the case of ChiA and ChiB the $[(GlcNAc)_2]/[GlcNAc]$ ratio continuously decreased with the degree of chitin

conversion whereas the effect of the extent of degradation to the processivity of ChiC and ChiB-W97A was less prominent. This suggests that endo enzymes rely less on processivity and that their rate is rather controlled by the presence of easily accessible regions on the chitin.

Since the $[(GlcNAc)_2]/[GlcNAc]$ ratio was time dependent, the processivity was also calculated based on the rates of $(GlcNAc)_2$ and GlcNAc formation ($P_{app} = \nu[(GlcNAc)_2]/\nu[GlcNAc]$). Rate based P_{app} values showed similar trends as the concentration based P_{app} values, but the drop of P_{app} values with conversion was more pronounced (data not shown). The difference was most evident for ChiA where the rates of $(GlcNAc)_2$ and GlcNAc formation were nearly equal at conversions above 50 %. Previously, studies of changes in product profiles (cellobiose/glucose ratio) in cellulose hydrolysis have revealed little or no changes in processivity with respect to conversion [18, 24, 25]. However, the degree of conversion remained below 10 % in these studies. At these low degrees of conversion, we did not see significant changes in P_{app} either (Fig. 3). In a study by Fox *et al*. it was found that P_{app} of an individual cellobiohydrolase (*Tl*Cel7A) increased with substrate degradation up to 60 % conversion [13]. The reasons behind the different behavior of *Tl*Cel7A and the different *S. marcescens* chitinases to this respect remain to be studied. Moreover, the P_{app} of *Tl*Cel7A decreased with increasing concentration in the presence of an endoglucanase (*Tem*GH5). The effect on P_{app} by addition of ChiB and ChiC, by themselves and together, with ChiA was also investigated in our study. At the initial stage of hydrolysis, the $[(GlcNAc)_2]/[GlcNAc]$ ratio was observed to be 27.1 ± 1.3 for ChiA and ChiB, 26.3 ± 2.0 for ChiA and ChiC, and 22.1 ± 2.0 for ChiA, ChiB, and ChiC combined. For ChiA in the presence of either ChiB or ChiC, these values are intermediate between those observed for ChiA and ChiB alone (30.1 ± 1.5 and 24.3 ± 2.0, respectively). When all three chitinases are present, the observed P_{app} value is close to that observed for ChiB alone. Even though initial P_{app} was smaller for ChiA in

combination with other chitinases, both the rate of hydrolysis (after the first initial 60 minutes) as well as the extent of degradation was larger (Fig. 7).

Finally, the effect of the origin of the substrate on P_{app} was assessed by allowing ChiA to incubate with a sheared α-chitin. Here, at the initial stage of hydrolysis, the [$(GlcNAc)_2$]/[GlcNAc] ratio was observed to be 14.2 ± 0.5, significantly smaller than what was observed for the β-chitin (30.1 ± 1.5) used in this study. The anti-parallel orientation of the polymer chains of α-chitin allows a high number of hydrogen bonds to be formed resulting in a tight packing of the polymeric strands and high stability of the crystalline structure [26, 27]. Moreover, this tight packing exclude the presence of water. The parallel orientation of the polymer strands in β-chitin allows for up to two water molecules pr. *N*-acetylglucosamin residue making this form less recalcitrant [28, 29], and hence likely easier to be degraded in a processive manner. With this in mind, the decreased [$(GlcNAc)_2$]/[GlcNAc] ratio with respect to extent of degradation as shown in Fig. 6 may also in part be connected with the hydration of the β-chitin in that the content of water molecules is decreased inside of the substrate making this part of the crystal more recalcitrant. The addition of ChiB to ChiA in α-chitin degradation only slightly altered the initial P_{app} (15.5 ± 1.0).

Together our data suggest that the substrate becomes more recalcitrant with conversion shown with a decrease in processivity of processive enzymes. Also, we see a difference between substrates. It is therefore essential to report both the nature of the substrate that is used as well as having control of the extent of substrate degradation when reporting the degree of processivity. As previously proposed for cellulose hydrolysis the degradation of chitin by processive chitinases is limited by the length of obstacle free paths on the substrate [9, 11-13]. Initially ChiA has a higher degree of processivity than ChiB while at the end points the [$(GlcNAc)_2$]/[GlcNAc] ratio is higher for ChiB than ChiA. Also, the end points for

ChiA and ChiB differes with approximately 30 %. These are several factors making us strongly suggesting that the initial degradation is the best measure of processivity and that having control of the extent of substrate degradation is important. Although the role of other factors, like product inhibition, cannot be excluded our data suggest that the length of obstacle free path during hydrolysis is an important contributor in controlling the rate of chitin degradation.

Acknowledgements

The research leading to these results has received funding from the Norwegian Financial Mechanism (Grant EMP171) and Grant 209335/F20 from the Norwegian Research Council

Reference List

1. Zakariassen, H., Eijsink, V. G. H. & Sørlie, M. (2010) Signatures of activation parameters reveal substrate-dependent rate determining steps in polysaccharide turnover by a family 18 chitinase, *CarbohydPolym.* **81**, 14-20.
2. Davies, G. & Henrissat, B. (1995) Structures and mechanisms of glycosyl hydrolases, *Structure.* **3**, 853-9.
3. Teeri, T. T. (1997) Crystalline cellulose degradation: new insight into the function of cellobiohydrolases, *Trends Biotechnol.* **15**, 160-167.
4. Varrot, A., Frandsen, T. P., von Ossowski, I., Boyer, V., Cottaz, S., Driguez, H., Schulein, M. & Davies, G. J. (2003) Structural Basis for Ligand Binding and Processivity in Cellobiohydrolase Cel6A from Humicola insolens, *Structure.* **11**, 855-864.
5. von Ossowski, I., Stahlberg, J., Koivula, A., Piens, K., Becker, D., Boer, H., Harle, R., Harris, M., Divne, C., Mahdi, S., Zhao, Y., Driguez, H., Claeyssens, M., Sinnott, M. L. & Teeri, T. T. (2003) Engineering the Exo-loop of Trichoderma reesei Cellobiohydrolase, Cel7A. A comparison with Phanerochaete chrysosporium Cel7D, *JMolBiol.* **333**, 817-829.
6. Beckham, G. T. & Crowley, M. F. (2011) Examination of the α-Chitin Structure and Decrystallization Thermodynamics at the Nanoscale, *J Phys Chem B.* **115**, 4516-4522.
7. Beckham, G. T., Matthews, J. F., Peters, B., Bomble, Y. J., Himmel, M. E. & Crowley, M. F. (2011) Molecular-Level Origins of Biomass Recalcitrance: Decrystallization Free Energies for Four Common Cellulose Polymorphs, *J Phys Chem B.* **115**, 4118-4127.
8. Horn, S. J., Sikorski, P., Cederkvist, J. B., Vaaje-Kolstad, G., Sørlie, M., Synstad, B., Vriend, G., Vårum, K. M. & Eijsink, V. G. H. (2006) Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides, *ProcNatlAcadSci USA.* **103**, 18089-18094.
9. Kurašin, M. & Väljamäe, P. (2011) Processivity of cellobiohydrolases is limited by the substrate, *J Biol Chem.* **286**, 169-177.
10. Payne, C. M., Jiang, W., Shirts, M. R., Himmel, M. E., Crowley, M. F. & Beckham, G. T. (2013) Glycoside hydrolase processivity is directly related to oligosaccharide binding free energy, *Journal of the American Chemical Society.* **135**, 18831-9.
11. Cruys-Bagger, N., Tatsumi, H., Ren, G. R., Borch, K. & Westh, P. (2013) Transient kinetics and rate-limiting steps for the processive cellobiohydrolase Cel7A: effects of substrate structure and carbohydrate binding domain, *Biochemistry.* **52**, 8938-48.
12. Jalak, J. & Valjamae, P. (2010) Mechanism of initial rapid rate retardation in cellobiohydrolase catalyzed cellulose hydrolysis, *Biotechnol Bioeng.* **106**, 871-883.
13. Fox, J. M., Levine, S. E., Clark, D. S. & Blanch, H. W. (2012) Initial- and Processive-Cut Products Reveal Cellobiohydrolase Rate Limitations and the Role of Companion Enzymes, *Biochemistry.* **51**, 442-452.
14. Igarashi, K., Uchihashi, T., Koivula, A., Wada, M., Kimura, S., Okamoto, T., Penttilä, M., Ando, T. & Samejima, M. (2011) Traffic Jams Reduce Hydrolytic Efficiency of Cellulase on Cellulose Surface, *Science.* **333**, 1279-1282.
15. Shang, B. Z., Chang, R. & Chu, J.-W. (2013) Systems-level Modeling with Molecular Resolution Elucidates the Rate-limiting Mechanisms of Cellulose Decomposition by Cellobiohydrolases, *J Biol Chem.* **288**, 29081-29089.
16. Vaaje-Kolstad, G., Horn, S. J., Sørlie, M. & 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.
17. Horn, S. J., Sørlie, M., Vårum, K. M., Väljamäe, P. & Eijsink, V. G. H. (2012) Measuring Processivity in *Meth Enzymol* pp. 69-95, Academic Press.

18. Nakamura, A., Watanabe, H., Ishida, T., Uchihashi, T., Wada, M., Ando, T., Igarashi, K. & Samejima, M. (2014) Trade-off between processivity and hydrolytic velocity of cellobiohydrolases at the surface of crystalline cellulose, *Journal of the American Chemical Society.* **136**, 4584-92.
19. Nakagawa, Y. S., Eijsink, V. G. H., Totani, K. & Vaaje-Kolstad, G. (2013) Conversion of α-Chitin Substrates with Varying Particle Size and Crystallinity Reveals Substrate Preferences of the Chitinases and Lytic Polysaccharide Monooxygenase of Serratia marcescens, *J Agric Food Chem.* **61**, 11061-11066.
20. Brurberg, M. B., Nes, I. F. & Eijsink, V. G. H. (1996) Comparative studies of chitinases A and B from Serratia marcescens, *Microbiology.* **142**, 1581-1589.
21. Synstad, B., Vaaje-Kolstad, G., Cederkvist, F. H., Saua, S. F., Horn, S. J., Eijsink, V. G. H. & Sørlie, M. (2008) Expression and Characterization of Endochitinase C from Serratia marcescens BJL200 and Its Purification by a One-Step General Chitinase Purification Method, *BiosciBiotechnolBiochem.* **72**, 715-723.
22. Fan, Y. M., Saito, T. & Isogai, A. (2008) Preparation of chitin nanofibers from squid pen beta-chitin by simple mechanical treatment under acid conditions, *Biomacromolecules.* **9**, 1919-1923.
23. Igarashi, K., Uchihashi, T., Uchiyama, T., Sugimoto, H., Wada, M., Suzuki, K., Sakuda, S., Ando, T., Watanabe, T. & Samejima, M. (2014) Two-way traffic of glycoside hydrolase family 18 processive chitinases on crystalline chitin, *Nature communications.* **5**, 3975.
24. Eriksson, T., Karlsson, J. & Tjerneld, F. (2002) A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (cel7A) and endoglucanase I (cel7B) of Trichoderma reesei, *Applied biochemistry and biotechnology.* **101**, 41-60.
25. Medve, J., Karlsson, J., Lee, D. & Tjerneld, F. (1998) Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from Trichoderma reesei: Adsorption, sugar production pattern, and synergism of the enzymes, *Biotechnol Bioeng.* **59**, 621-634.
26. Minke, R. & Blackwell, J. (1978) The structure of alpha-chitin, *J Mol Biol.* **120**, 167-181.
27. Sikorski, P., Hori, R. & Wada, M. (2009) Revisit of α-Chitin Crystal Structure Using High Resolution X-ray Diffraction Data, *Biomacromolecules.* **10**, 1100-1105.
28. Kobayashi, K., Kimura, S., Togawa, E. & Wada, M. (2010) Crystal transition between hydrate and anhydrous β-chitin monitored by synchrotron X-ray fiber diffraction, *Carbohydr Polym.* **79**, 882-889.
29. Sawada, D., Nishiyama, Y., Langan, P., Forsyth, V. T., Kimura, S. & Wada, M. (2012) Water in Crystalline Fibers of Dihydrate beta-Chitin Results in Unexpected Absence of Intramolecular Hydrogen Bonding, *PLoS One.* **7**, 8.
30. Petrov, M., Lymperakis, L., Friák, M. & Neugebauer, J. (2013) Ab Initio Based conformational study of the crystalline α-chitin, *Biopolymers.* **99**, 22-34.

Figure Captions

Fig. 1. Left: Chemical structure of chitin and how it stacks in an α-chitin polymer crystal structure. Right: Chemical structure of cellulose and how it stacks in a cellulose II polymer crystal structure [30]. Both chitin and cellulose have the sugar units rotated 180° relative to their neighboring residues, so that the smallest structural unit is a disaccharide.

Fig. 2. Crystal structure of ChiB (top) and a schematic picture of ChiB in complex with a single chitin chain. Highlighted in blue are surface exposed aromatic amino acids that stacks with sugar moieties (being individual subsites). The glycosidic bond between the sugar residues in subsite −1 and +1 is enzymatically cleaved. A correctly positioned N-acetyl group (shown as sticks) in the −1 subsite is essential for the substrate-assisted catalysis. Due to that the smallest structural unit of chitin is a disaccharide, the product of repeated processive enzymatic actions will be dimers, $(GlcNAc)_2$. Monomers, GlcNAc, originate from initial productive binding when the sugar in the non-reducing end occupies a subsite with an odd number. For these reasons, a high ratio of $[(GlcNAc)_2]/[GlcNAc]$ indicates a high degree of apparent processivity.

Fig 3. A: The extent of degradation of β-chitin (180 µm) with respect to time for two processive chitinases, an endochitinase, and a mutant with reduced degree of processivity from *Serratia marcescens*. The two processive chitinases (ChiA and ChiB) are more efficient degraders of chitin than the endochitinase and the mutant (ChiC and ChiB-W97A). B: The degradation at initial time points. Hydrolysis was undertaken with 2.5 µM enzyme in 50 mM, pH 6.1 sodium acetate buffer at t = 37 °C with 2.0 mg/ml chitin.

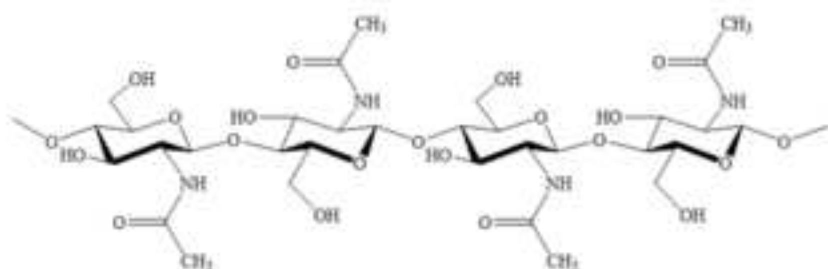
Fig. 4. Progress curves for the formation of $(GlcNAc)_2$ (squares) and GlcNAc (circles) after hydrolysis of β-chitin by ChiA (black), ChiB (red), ChiC (green) and ChiB W97A (blue). $(GlcNAc)_2$ and GlcNAc were the only products detected.

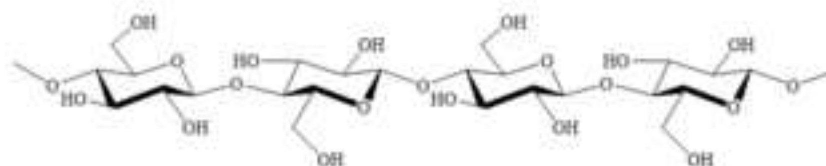
Fig 5. Comparison of initial $[(GlcNAc)_2]/[GlcNAc]$ ratios for the four different chitinases.

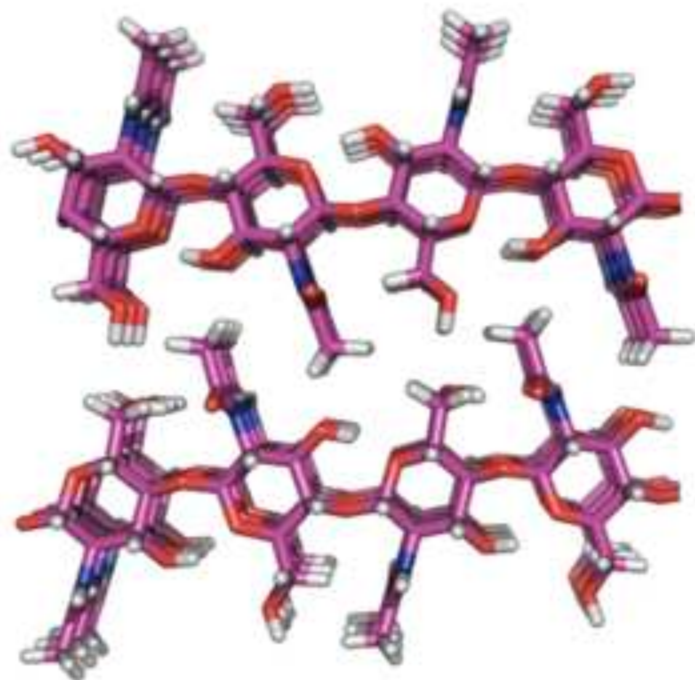
Fig 6. Comparison of the $[(GlcNAc)_2]/[GlcNAc]$ ratio against extent of degradation for the four different chitinases investigated.

Fig. 7. The extent of degradation of chitin with respect to time for ChiA alone, together with ChiB, together with ChiC, and together with both ChiB and ChiC, respectively.

Figure 1
Click here to download high resolution image







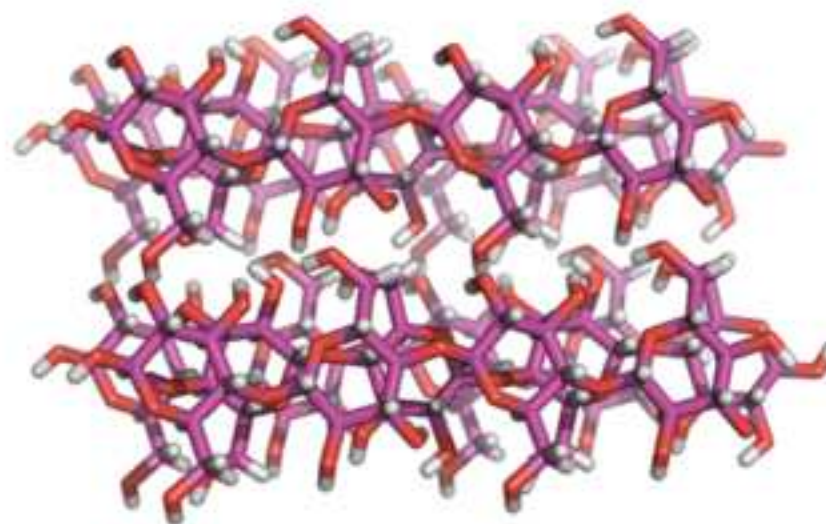


Figure 2
Click here to download high resolution image

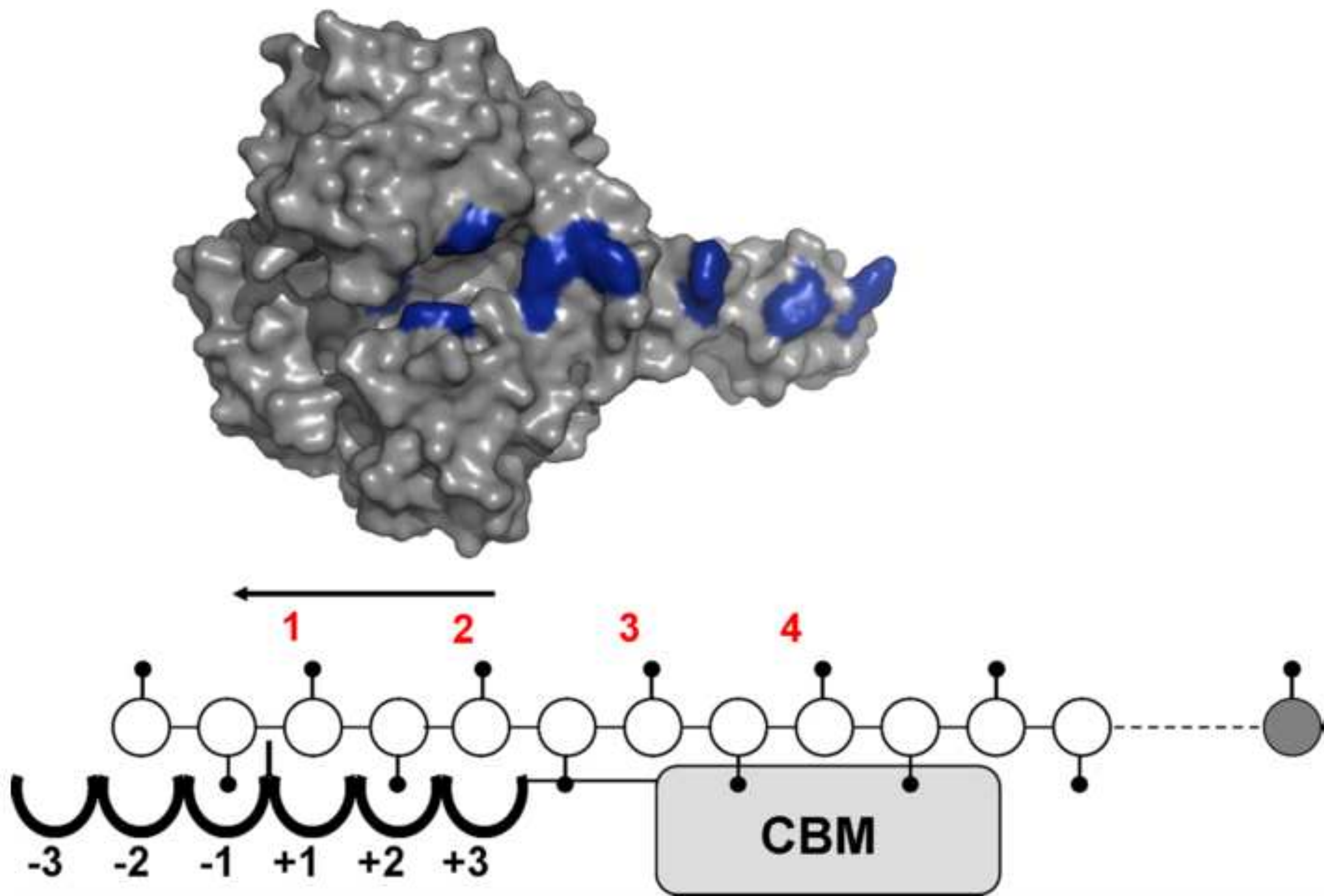


Figure 3A
Click here to download high resolution image

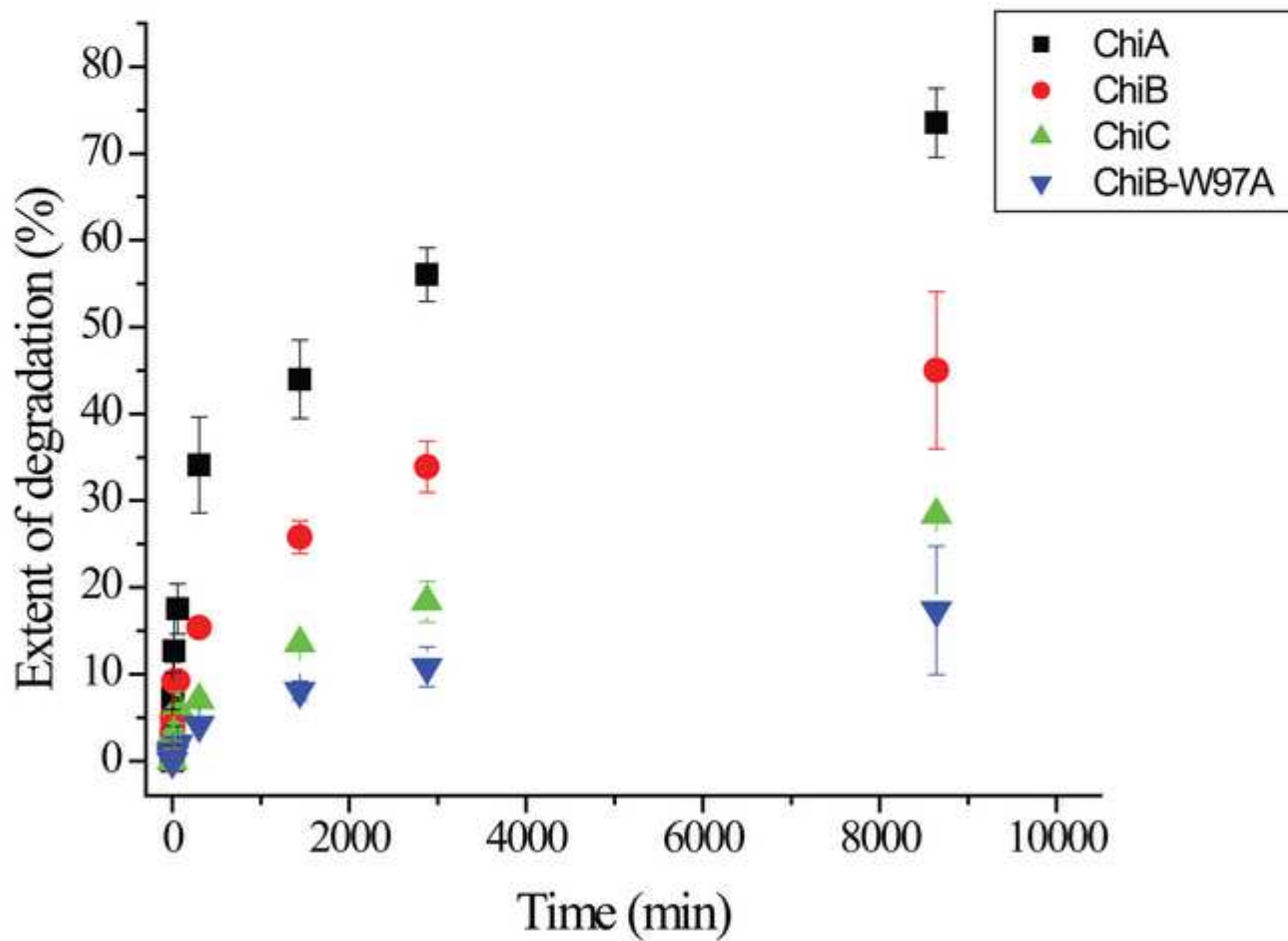


Figure 3B

Click here to download high resolution image

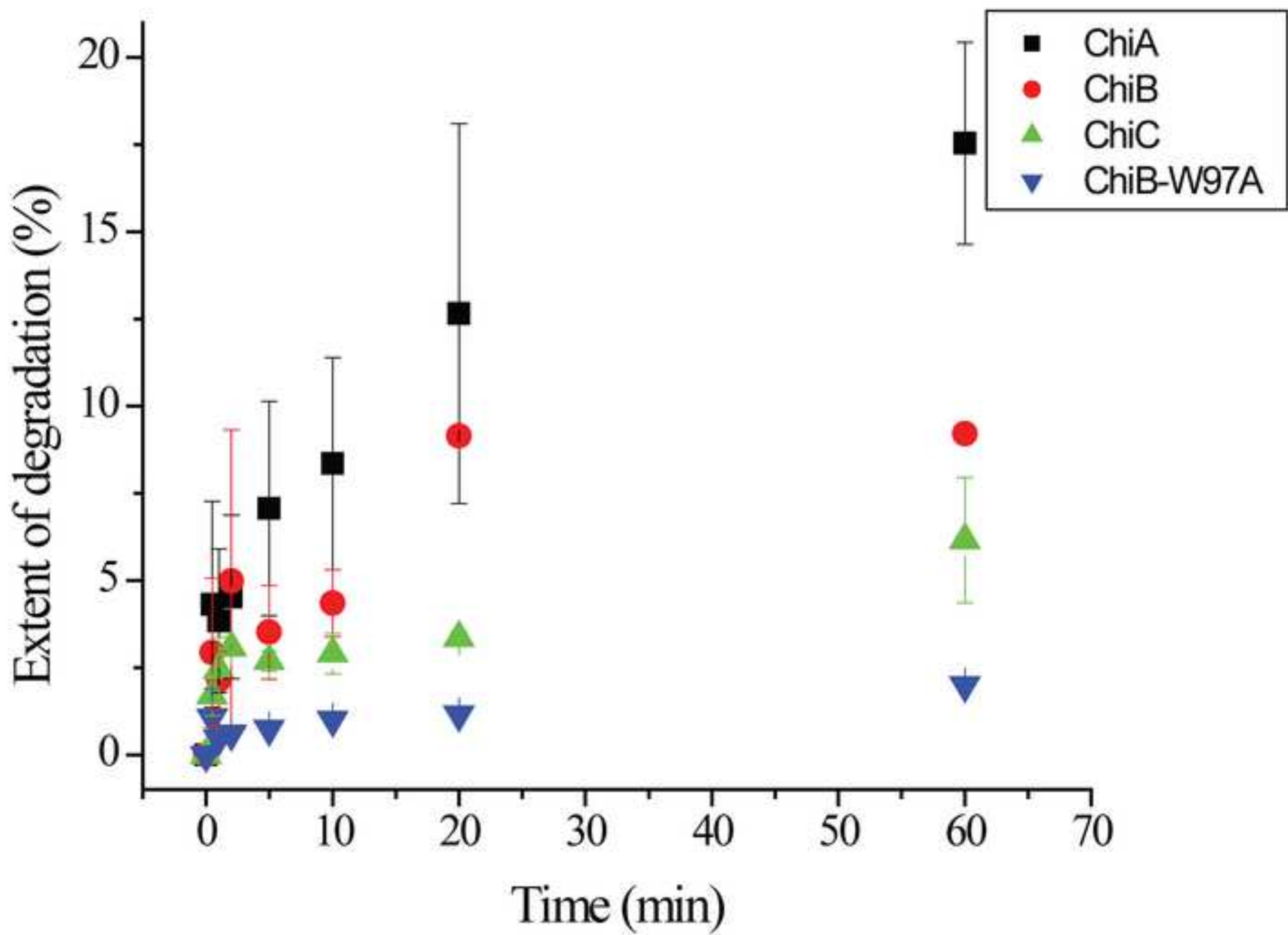


Figure 4
Click here to download high resolution image

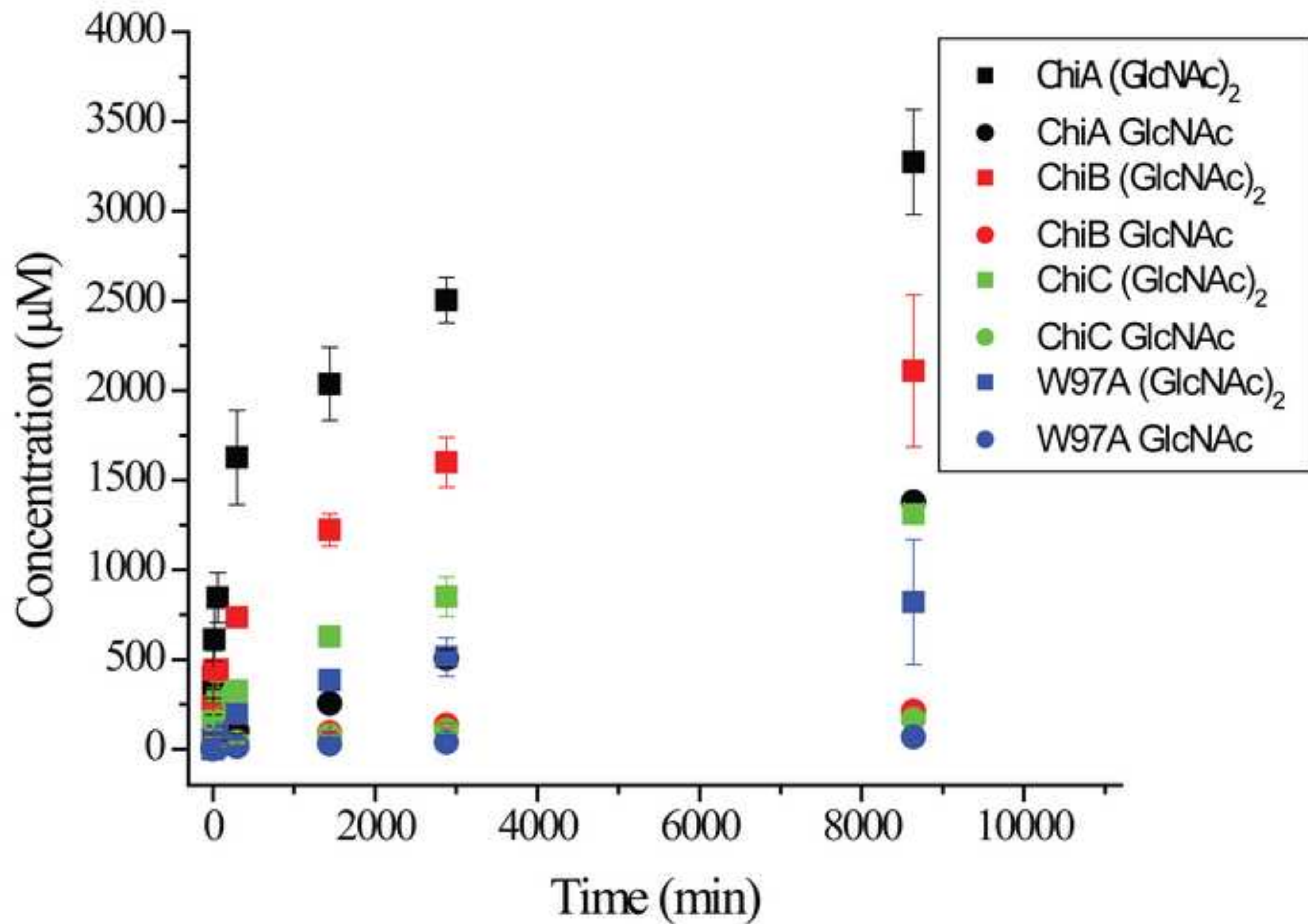


Figure 5

Click here to download high resolution image

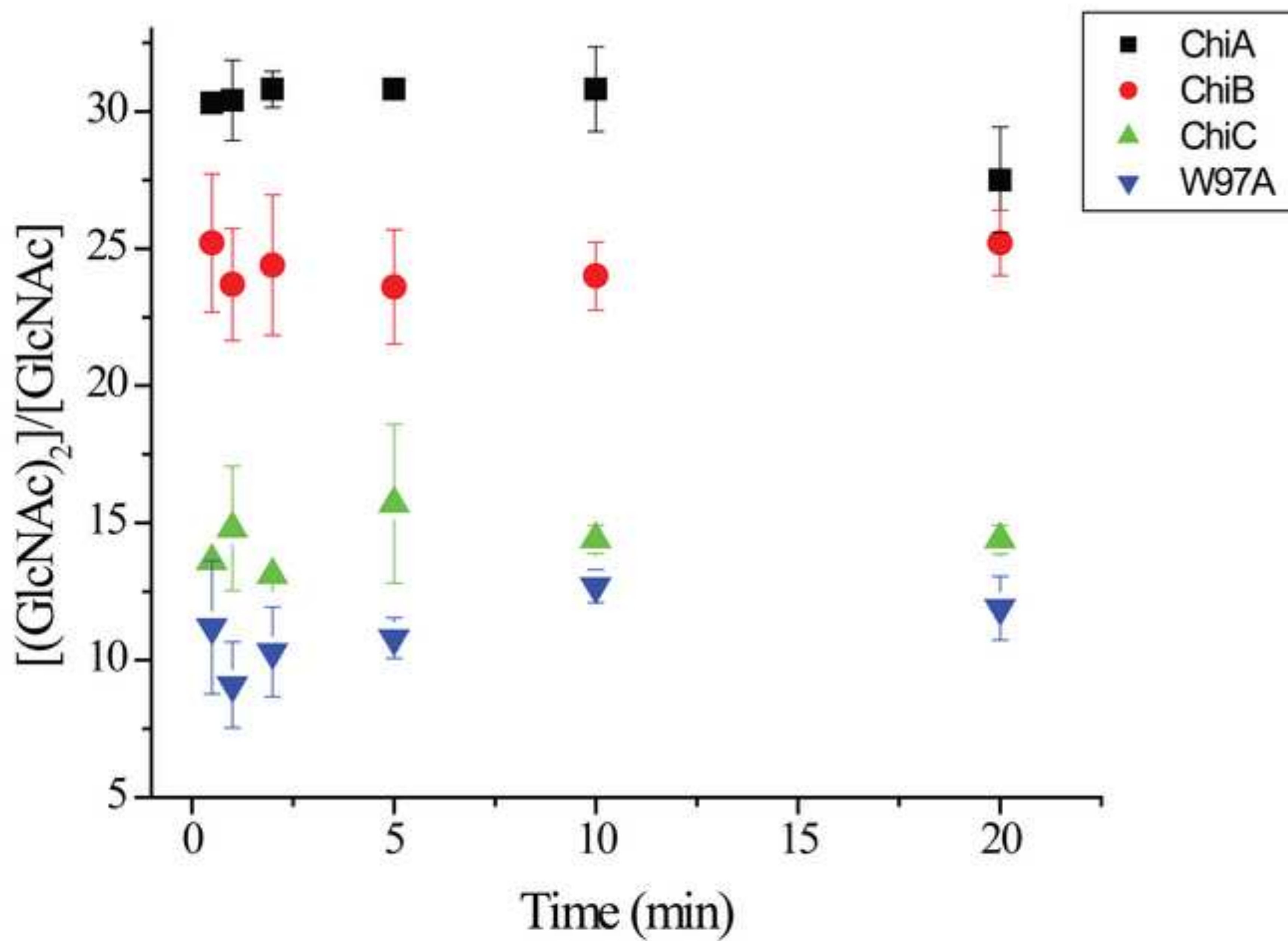


Figure 6
Click here to download high resolution image

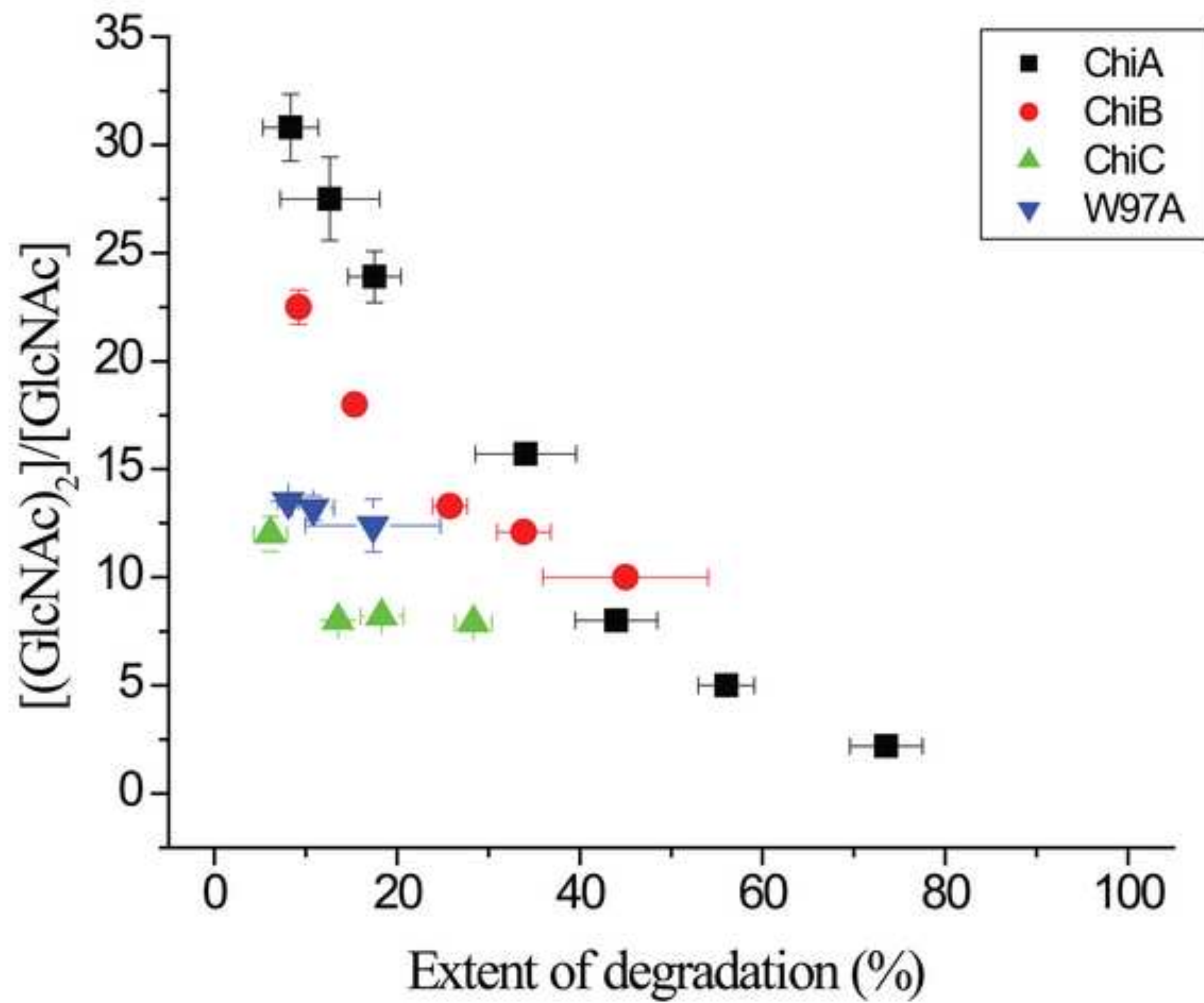


Figure 7
Click here to download high resolution image