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Effect of enzyme processivity on the efficacy of a competitive chitinase inhibitor

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ABSTRACT

Many glycoside hydrolases, such as chitinases and cellulases, degrade polysaccharides in a processive manner. Inhibition of chitinases is of great interest, because chitin-metabolizing pathogenic organisms such as certain fungi, insects and nematodes need chitinase activity for survival. Here we show how the processivity and the directionality of two chitinases, chitinase A (ChiA) and B (ChiB) from *Serratia marcescens*, affects the practical inhibition efficacy (IC_{50}) of allosamidin, a general competitive inhibitor of family 18 chitinases. The results show that there is a clear negative correlation between processivity and the efficiency of competitive inhibition, and that this effect of processivity (i.e. reducing inhibitor efficacy) is largest when allosamidin binds to those enzyme subsites that interact with the polymeric part of the substrate. Besides providing further insight into the processivity and directionality of the two *Serratia* enzymes, these results reveal important aspects of ligand binding that should be taken into account when designing inhibitors of processive enzymes.

1. Introduction

Enzymatic degradation of recalcitrant polysaccharides in biomass, such as cellulose (β-1,4-linked glucoses) and the cellulose derivative chitin (β-1,4-linked *N*-acetylglucosamines), is of great biological and economical importance. A common feature of many of the enzymes that degrade such polysaccharides is the ability to remain attached to the substrate in between subsequent hydrolytic reactions. In other words, such enzymes perform several hydrolytic cuts per each enzyme-substrate association (Davies & Henrissat, 1995; Rouvinen, Bergfors, Teeri, Knowles, & Jones, 1990). Such a processive mechanism is thought to be beneficial for the degradation of crystalline substrates because it prevents once-detached single polysaccharide chains from reassociating with the insoluble material (Teeri, 1997; von Ossowski et al., 2003). The substrate-binding clefts in processive chitinases and cellulases tend to be lined with aromatic residues, in particular tryptophan residues, which are thought to facilitate processivity by functioning as a flexible and hydrophobic sheath along which the polymer chain can slide during the processive mode of action (Divne, Ståhlberg, Teeri, & Jones, 1998; Katouno et al., 2004; Uchiyama et al., 2001; van Aalten et al., 2000; Varrot et al., 2003).

Chitin metabolism is essential in several major pathogenic organisms such as certain fungi, insects and nematodes, and chitin turnover has also been associated with the ability of humans to respond to such organisms (van Eijk et al., 2005). Humans possess at least two family 18 chitinases known as chitotriosidase and acidic mammalian chitinase (Bussink, Speijer, Aerts, & Boot, 2007). These chitinases show the typical features of processive enzymes, including deep substrate-binding clefts lined with aromatic residues (Fusetti et al., 2002; Olland et al., 2009). Inhibition of chitinases is of great interest, because this may lead to new ways to combat pathogenic organisms and because it has been suggested that inhibitors of the human chitinases may emerge as novel medicines for allergic and inflammatory disorders (Donnelly & Barnes, 2004; Zhu et al., 2004).

Chitinase A (ChiA; Brurberg, Eijsink, & Nes, 1994) and chitinase B (ChiB; Brurberg, Eijsink, Haandrikman, Venema, & Nes, 1995) from *Serratia marcescens* are two processive family 18 chitinases that degrade chitin chains in opposite directions (ChiA moves towards the non-reducing end and ChiB moves towards the reducing end, see Fig. 1) (Horn, Sørbotten, et al., 2006; Hult, Katouno, Uchiyama, & Sugiyama, 2005; Sikorski, Sørbotten, Horn, Eijsink, & Vårum, 2006; van Aalten et al., 2000). Recently, we have shown that aromatic residues close to the catalytic center are important for processivity in both ChiA and ChiB (Horn, Sikorski, et al., 2006; Zakariassen et al., 2009). In particular, it was shown that Trp^{167} in ChiA and Trp^{97}

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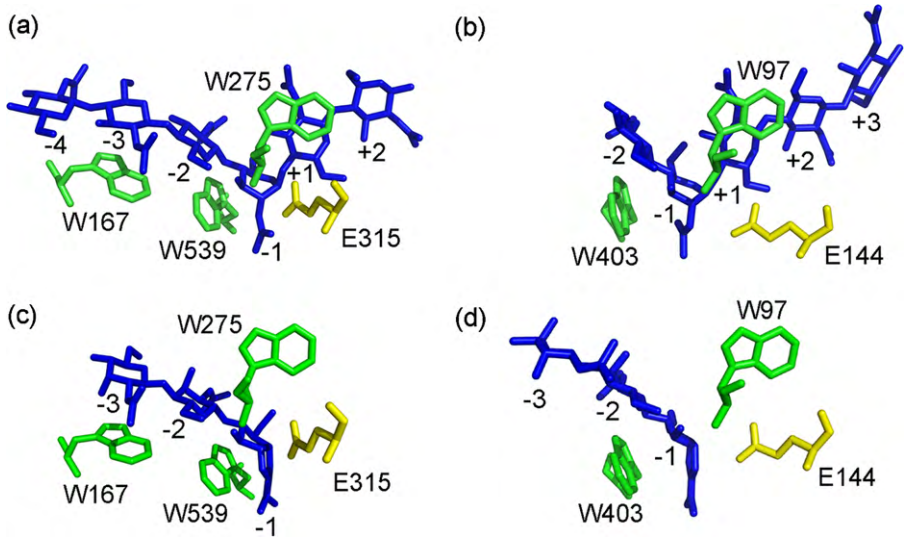

Fig. 1. Binding of substrate (a, b) and allosamidin (c, d) in ChiA and ChiB. The panels show the side chains of selected of aromatic amino acids (colored green) in the crystal structures of ChiA-E315QL in complex with $(GlcNAc)_6$ (a: PDB entry 1NH6) (Aronson et al., 2003), ChiB-E144Q in complex with $(GlcNAc)_5$ (b: PDB entry 1E6N) (van Aalten et al., 2001), ChiA in complex with allosamidin (c: PDB entry 1FFQ) (Papanikolau et al., 2003), and ChiB in complex with allosamidin (d: PDB entry 1E6R) (van Aalten et al., 2001). The GlcNAc oligomers and allosamidin are colored blue. The side chain of the catalytic acid (Glu315 in ChiA and Glu144 in ChiB) is shown in yellow. The positions of the catalytic acids in panels a and b were derived from superposing the structures of the corresponding wild-type apo enzymes onto the structures of the enzyme-substrate complexes. The numbers indicate the subsites to which the sugar monomers are bound. ChiA is thought to move towards the non-reducing end of the substrate (i.e. to the left, while releasing dimeric products from its +1 and +2 aglycon subsites), while ChiB is thought to move towards the reducing end (i.e. to the right, while releasing dimeric products from its −1 and −2 glycon subsites) (Hult et al., 2005; van Aalten et al., 2000). Thus, during processive action the polymeric part of the substrate would bind to glycon (−) subsites in ChiA and to the aglycon (+) subsites in ChiB. Trp539 (ChiA) and Trp403 (ChiB) are structurally equivalent and fully conserved in family 18 chitinases. It has so far not been possible to produce variants of ChiA and ChiB lacking this aromatic side chain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in ChiB are crucial for processivity. These residues are located in the −3 glycon and +1 aglycon subsites, respectively, meaning that they are thought to interact with the longer (polymeric) part of the substrate during processive action (see legend to Fig. 1 for a detailed explanation). Mutation of Trp^{275}, which is located in the +1 aglycon subsite of ChiA and structurally analogous to Trp^{97} in ChiB, led to a lesser reduction in processivity, in accordance with the notion that, in ChiA, this residue interacts with the dimeric product of processive hydrolysis (Fig. 1).

Considering the ideas and observations described above, we hypothesized that the practical efficacy of chitinase inhibitors depends on the degree of processivity and the directionality of the targeted enzyme. We have addressed this issue by analyzing inhibition of wild-type ChiA and ChiB and of two mutants with almost abolished processivity, ChiA-W167A and ChiB-W97A (Zakariassen et al., 2009; Horn, Sikorski, et al., 2006, respectively; for the sake of simplicity these two mutants are referred to as "non-processive mutants" in this paper). In addition, we have studied the ChiA-W275A mutant, which shows only a minor reduction in processivity (Zakariassen et al., 2009). As inhibitor, we used the natural product allosamidin, which is isolated from *Streptomyces* sp. and a well known and highly effective competitive inhibitor of family 18 chitinases (Banat, Kameyama, Yoshioka, & Koga, 1999; Horn, Sørlie, et al., 2006; Sakuda, Isogai, Matsumoto, Suzuki, & Koseki, 1986; Sakuda, Isogai, Matsumoto, & Suzuki, 1987). Allosamidin is a pseudotrisaccharide that binds to the −3 to −1 glycon subsites of family 18 chitinases with affinities in the low and sub-micromolar range and there is abundant structural data for chitinase–allosamidin complexes (Banat et al., 1999; Bortone, Monzingo, Ernst, & Robertus, 2002; Cederkvist et al., 2007; Papanikolau, Tavlas, Vorgias, & Petratos, 2003; Rao et al., 2003; Terwisscha van Scheltinga et al., 1995; van Aalten et al., 2001; Vaaje-Kolstad et al., 2004; PDB entry 1X6N (doi:10.2210/pdb1x6n/pdb)). Importantly, in ChiA allosamidin competes with the polymeric part of the substrate during processive hydrolysis, whereas in ChiB, the inhibitor competes with the dimeric part that is to be released as the product (see Fig. 1). In addition to measuring mutational effects on IC_{50} values for the different enzymes acting on polymeric substrates, we also determined binding affinities directly, to be able to correct for mutational effects on allosamidin and substrate affinity. We have also analyzed if processivity itself is influenced by the competitive inhibitor.

2. Experimental

2.1. Materials

Squid pen β-chitin (less than 75 μm particle size) was purchased from France Chitin (Marseille, France). Characterization of β-chitin from several squid species has shown that the number-average degree of polymerization (DP_n) is very high (in the order of thousands), and that the crystallinity index (C.I.) is approximately 80% (Chandumpai, Singhpibulporn, Faroongsarng, & Sornprasit, 2004; Jaworska, Sakurai, Gaudon, & Guibal, 2003; Susana Cortizo, Berghoff, & Alessandrini, 2008). The degree of acetylation was 92% ($F_A = 0.92$) (Karlsen, Heggset, & Sørlie, 2010). Chitosan, with a degree of acetylation of 63% ($F_A = 0.63$), an intrinsic viscosity [η] of 730 ml/g corresponding to a DP_n of 800, was prepared by homogeneous deacetylation of milled (1.0 mm sieve) shrimp shell chitin (Sannan, Kurita, Ogura, & Iwakura, 1978) and was converted to the chitosan hydrochloride salt (Draget, Vårum, Moen, Gynnild, & Smidsrød, 1992). This procedure results in a chitosan with a random distribution of *N*-acetylated and deacetylated units (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991). Allosamidin was isolated from *Streptomyces* sp., and the purity was controlled by ^1H NMR as described elsewhere (Sakuda et al., 1987). Previously, the structure of allosamidin had been verified by both NMR and crystallography (Sakuda et al., 1986). GlcNAc, $(GlcNAc)_2$ and all other chemicals were purchased from Sigma (St Louis, USA).

2.2. Enzymes

The wild-type chitinase genes *chia* (Brurberg et al., 1994) and *chib* (Brurberg et al., 1995) from *S. marcescens* strain BJL200 and mutant variants of these were expressed in *Escherichia coli* TOP10 (Invitrogen, CA, USA) by growing cells for 16–18 h at 37 °C in Luria-Bertani medium containing 50 μg/mL ampicillin. Periplasmatic extracts were produced as described by Brurberg, Nes, and Eijsink (1996) and the enzymes were purified using chitin affinity column chromatography, as described by Zakariassen et al. (2009). Enzyme purity was verified by SDS-PAGE and estimated to be >95% in all cases. Protein concentrations were determined by using the Quant-It™ protein assay kit and Qubit™ fluorometer from Invitrogen (CA, USA).

2.3. Isothermal titration calorimetry (ITC)

ITC was used to determine the affinity of the interaction between the different chitinases and allosamidin. The experiments were performed with a VP-ITC system from Microcal Inc. (Northampton, MA) (Wiseman, Williston, Brandts, & Lin, 1989). Solutions were thoroughly degassed prior to experiments to avoid air bubbles in the calorimeter. In a typical titration, 16 μM of chitinase in 50 mM sodium acetate buffer (pH 6.1) was placed in the reaction cell with a volume of 1.42 mL, and a 250 μM solution of allosamidin in the same buffer was placed in the ITC syringe. Aliquots of 8 μL were injected into the reaction cell at 140 s intervals with a stirring speed of 260 rpm. The titrations were normally complete after 20–25 injections. All experiments were performed at 30 °C. ITC data were collected automatically using the Microcal Origin v.7.0 software accompanying the VP-ITC system. Prior to further data analysis, all data were corrected for heat of dilution by subtracting the heat remaining after saturation of binding sites on the enzyme. Data were fitted using a non-linear least-squares algorithm using a single-site binding model employed by the Origin software that accompanies the VP-ITC system (Wiseman et al., 1989). All data from the binding reactions fitted well to a single-site binding model, yielding the stoichiometry (n), equilibrium binding association constant (K_a), and the enthalpy change (ΔH_r) of the reaction. Errors in the determined binding parameters represent standard deviations derived from at least three independent experiments.

2.4. Determination of IC_{50} values for allosamidin

IC_{50} values for the inhibition of chitinase-catalyzed hydrolysis of the polymeric substrates β-chitin and chitosan by allosamidin were determined. The experiments with β-chitin were performed by incubating 10 mg/mL β-chitin in 50 mM sodium acetate buffer (0.1 mg/mL BSA, pH 6.1) containing 100 nM chitinase and at least five different concentrations of allosamidin (ranging from 0 to 2.0 μM, depending on the chitinase studied), in a water bath at 37.0 °C. Reaction samples of 40 μL were withdrawn at regular time intervals, and the enzyme was inactivated by adding 40 μL acetonitrile. The reaction mixtures were mixed after each sample withdrawal. All reactions were performed at least in duplicate and samples were stored at −20 °C until further analysis. Chitin hydrolysis was approximated by analyzing the amount of liberated $(GlcNAc)_2$ as described previously (Horn, Sørbotten, et al., 2006). $(GlcNAc)_2$ is the absolutely dominant product for both the wild-type and the mutant enzymes (results not shown). Samples with known concentrations of $(GlcNAc)_2$ were used to prepare a standard curve. The initial rates were calculated from the slope of the initial linear phase of the reactions and were plotted as a Dixon plot ($[I]$ vs. $1/v$) to give the IC_{50} value as the opposite value of the x-intercept (Burlingham & Widlanski, 2003).

The experiments with chitosan, were performed by incubating 0.25 mg/mL chitosan in 50 mM sodium acetate buffer (0.1 mg/mL BSA, pH 6.1) containing 31 nM chitinase and at least five different concentration of allosamidin (ranging from 0 to 200 μM, depending on the chitinase studied), in a water bath at 37.0 °C. Reaction samples of 200 μL were withdrawn at regular time intervals, and the enzyme was inactivated by adding 200 μL 0.5 M NaOH. The reaction mixtures were mixed prior to each sample withdrawal. The concentrations of the newly formed reducing ends were determined using the 3-methyl-2-benzothiazolinone (MBTH) method, as described previously (Horn & Eijsink, 2004). All reactions were performed at least in duplicate. Samples with known concentrations of $(GlcNAc)_2$ were used to prepare a standard curve. The IC_{50} values were estimated as described above.

2.5. β-Chitin binding assay

First, for each purified chitinase variant, a solution with known protein concentration (Quant-It™ protein assay; see above) was diluted to various concentrations (0–11.1 μM) in 20 mM sodium acetate, pH 6.1. The A_{280} of these prepared solutions was then measured, thus creating an individual standard curve for each chitinase variant. β-Chitin (from a 20 mg/mL stock suspension in 20 mM sodium acetate, pH 6.1) was then added to each solution, bringing the reaction volume to 0.5 mL, the β-chitin concentration to 2.0 mg/mL, and the chitinase concentrations to 0–10 μM. The mixtures were incubated for 3 h, with mixing every 10 min (initial experiments, done for each enzyme variant, had shown that the binding reactions required up to 3 h to reach equilibrium; results not shown). To minimize hydrolysis of the chitin substrate, the reaction mixtures were kept at 4 °C. Subsequently, the sample tubes were spun for 5 min at 13,000 rpm in a microcentrifuge to pellet the chitin, and the A_{280} values of the supernatants were measured. Apparent extinction coefficients calculated from the respective A_{280} standard curves were then used to convert A_{280} values to chitinase concentrations. All assays were performed in triplicate, and values were corrected for release of absorbing compounds from the chitin (buffer + 1 mg/mL β-chitin) and for unspecific binding of the chitinases to the tubes (buffer + enzyme). The latter of these control measurements was done for each single chitinase variant used (wild-types and mutants). The equilibrium dissociation constants, K_d (μM) and binding capacities, B_{max} (μmol/g), were determined by fitting the binding isotherms to the one-site binding equation where P stands for protein: $[P_{bound}] = B_{max}[P_{free}]/K_d + [P_{free}]$, by non-linear regression using the Origin v7.0 software (OriginLab Corporation, Northampton, MA).

2.6. Degradation of chitosan—analysis of products formed during the initial phase of reaction in the presence and absence of allosamidin

Product patterns formed during the initial phase of chitosan degradation are diagnostic for processivity (Sørbotten, Horn, Eijsink, & Vårum, 2005; Horn, Sørbotten, et al., 2006). To analyze this, 0.25 mg/mL chitosan in 20 mM sodium acetate, pH 6.1 was incubated at 37.0 °C with 35 nM ChiA-WT or ChiB-WT in absence or presence of allosamidin, at concentrations of 160 and 90 μM, respectively. 1 μL samples were withdrawn from the reaction mixture after 45 s, 2 min and 4 min of reaction time, mixed with 2 μL of a matrix solution (15 mg/mL 2,5-dihydroxybenzoic acid, in 30% acetonitrile) and spotted directly on a target plate for MALDI-TOF mass spectrometry analysis. The spotted samples (in duplicate) were immediately dried using a heat gun. MS spectra of the products were acquired using an Ultraflex™ TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with gridless ion optics under control of Flexcontrol 4.1. The experiments were conducted

using an accelerating potential of 24 kV in the reflector mode. The intensities of the dominating DP4 products (D_3A_1, D_2A_2, D_1A_3, and A_4; D, glucosamine; A, *N*-acetylglucosamine) and DP5 products (D_2A_3 and D_1A_4) were summarized for all the samples (duplicate samples at three reaction times, meaning that $n = 6$) and used to obtain an estimate of the DP4/DP5 ratio.

3. Results and discussion

3.1. Binding of allosamidin to the chitinase variants

The binding of allosamidin to the various chitinases was studied by ITC (Table 1). The binding affinities (K_d) of ChiA-WT and ChiB-WT were found to be 0.17 ± 0.08 and 0.18 ± 0.05 μM, respectively (Table 1). The ChiA-W275A and ChiB-W97A mutants, which lack the tryptophan residue in the +1 subsite (Fig. 1), showed almost the same binding affinities for allosamidin as the wild-type enzymes, 0.21 ± 0.02 and 0.42 ± 0.02 μM, respectively (Table 1). No large effects were expected since the interactions between allosamidin and the enzymes are strongly dominated by interactions in the −3 to −1 subsites. The main chain NH group of Trp^{97}/Trp^{275} is close to O_3 of the allosamizoline group of allosamidin, and in the case of ChiB the structure indicates formation of a hydrogen bond (Papanikolau et al., 2003; van Aalten et al., 2001; this hydrogen bond is not observed in the ChiA structure). It is possible that the Trp → Ala mutation at position 97/275 leads to minor adjustments of the main chain, which again could lead to the minor effects on allosamidin affinity that were observed.

The K_d of binding allosamidin to ChiA-W167A was 2.40 ± 0.20 μM, representing a 14-fold reduction in affinity compared to the wild-type enzyme (Table 1). The main reason for this dramatic loss in binding affinity is the loss of a favorable hydrophobic stacking interaction between the sugar moiety in the −3 subsite and Trp^{167} (Fig. 1). The crystal structure of ChiA-W167A in complex with allosamidin (PDB entry: 1X6N; doi:10.2210/pdb1x6n/pdb) shows that the only noticeable structural consequence of the W167A mutation is the removal of this interaction. Otherwise, the structures of the wild-type and mutant ChiA–allosamidin complexes look the same.

3.2. Effects of processivity on efficacy of competitive inhibition

To examine if processivity affects the efficiency of a competitive inhibitor, we determined the IC_{50} values for inhibition of the hydrolysis of β-chitin and chitosan, by ChiA-WT, ChiB-WT, and the mutants with reduced processivity, ChiA-W275A, ChiA-W167A, and ChiB-W97A (Table 1). The Dixon plots used for the determination of the IC_{50} values are shown in Fig. 2.

Before comparison and interpretation of the IC_{50} values, it is important to know the effects of the mutation on the binding affinity towards polymeric substrates. As described above, the mutants lack a tryptophan residue in the −3 glycon subsite (Trp^{167}) or in the +1 aglycon subsite (Trp^{275} and Trp^{97}) (see Fig. 1). We have now determined the binding affinities of these mutants for β-chitin and the results show that the mutations lead to only a small decrease in chitin binding affinity (Fig. 3 and Table 2). For example, the K_d for the ChiA-W167A mutant was 8.2 ± 0.6 μM as compared to 5.0 ± 0.4 μM for the ChiA wild-type. These observations are consistent with previous results showing that aromatic residues close to the catalytic site of multi-domain chitinases and cellulases are important for the hydrolyzing activity towards crystalline substrates, but make only a minor contribution to the binding affinity for the substrate (Koivula et al., 1998; Pantoom, Songsiriritthigul, & Suginta, 2008; Uchiyama et al., 2001). Because of the comparable binding affinities towards β-chitin it is possible to do a meaningful comparison of the IC_{50} values without the need for correction for differences in substrate affinities (Table 1).

The IC_{50} values observed in the reactions with chitin were similar to the determined K_d values for allosamidin. This agrees well with what one would expect on the basis of the reaction kinetics. Firstly, it is highly likely that productive binding between enzyme and substrate, which includes the extraction of a single polymer chain to be guided into the active site cleft, is the rate-limiting step in the degradation of crystalline polysaccharides such as chitin and cellulose (Horn, Sikorski, et al., 2006; Koivula et al., 1998; von Ossowski et al., 2003; Zhang & Wilson, 1997; Zakariassen, Eijsink, & Sørlie, 2010). This means that the hydrolytic step in the enzyme mechanism, either it is processive or non-processive, is faster than the rate-limiting step and cannot be observed in the kinetic measurements. Secondly, a binding equilibrium between allosamidin and the enzymes is likely to be reached much faster than the binding equilibrium between the enzymes and β-chitin, due to the inaccessible nature of the crystalline chitin substrate and the low effective substrate concentrations. Due to these conditions, the binding equilibrium between allosamidin and the enzyme is a dominating factor underlying the determined IC_{50} values, which, thus, should be similar to the K_d values. This also implies that the effects of the Trp → Ala mutations on IC_{50} values would be similar to the effects on K_d values, as is indeed observed for all three mutants (Table 1).

A totally different situation is observed for the IC_{50} values obtained with the water soluble polymeric chitin-derivative chitosan. Generally, the IC_{50} values are much larger than the K_d values (Table 1), indicating higher substrate accessibility and a higher effective substrate concentration for the chitosan substrate, even though the actual concentration of β-chitin was 40-fold higher than the chitosan concentration (10 and 0.25 mg/mL, respectively). In the case of hydrolysis of chitosan, product release and displacement are the rate-limiting steps (Zakariassen et al., 2010). Thus, the processes of product release and substrate reorientation (during processive action) or reassociation (during non-processive action) are reflected in the overall reaction rates. Consequently, there would be a true competition between the substrate and allosamidin which can be assessed by looking at reaction rates and IC_{50} values.

The observed IC_{50} value for the processive ChiB-WT was about 4-fold higher than the IC_{50} value found for non-processive ChiB-W97A (91 ± 18 and 23 ± 3 μM, respectively). This difference cannot be explained by mutational effects on enzyme affinity for the

Table 1
Binding affinity and inhibitory power of allosamidin for chitinase variants.

Chitinase	K_d (μM)[a]	IC_{50} (μM)[b]	
		β-Chitin	Chitosan
ChiA-WT	0.17 ± 0.08	0.06 ± 0.01	164 ± 13
ChiA-W167A	2.40 ± 0.20	3.70 ± 0.23	133 ± 17
ChiA-W275A	0.21 ± 0.02	0.17 ± 0.04	148 ± 15
ChiB-WT	0.18 ± 0.05[c]	0.24 ± 0.01	91 ± 18
ChiB-W97A	0.42 ± 0.02	0.37 ± 0.03	23 ± 3

[a] From ITC measurements.
[b] From Dixon plots; see Fig. 2.
[c] In an independent study conducted previously in our laboratory at pH 6.0, $T = 30$ °C, Cederkvist et al. (2007) determined this value to be 0.16 ± 0.04 μM.

Table 2
Binding of wild-type and mutant chitinases to β-chitin.

Chitinase	K_d (μM)	B_{max} (μmol of chitinase/g)
ChiA-WT	5.0 ± 0.4	0.6 ± 0.1
ChiA-W167A	8.2 ± 0.6	0.5 ± 0.1
ChiA-W275A	9.6 ± 3.1	0.8 ± 0.1
ChiB-WT	8.7 ± 2.4	0.9 ± 0.1
ChiB-W97A	17.4 ± 4.7	0.8 ± 0.1

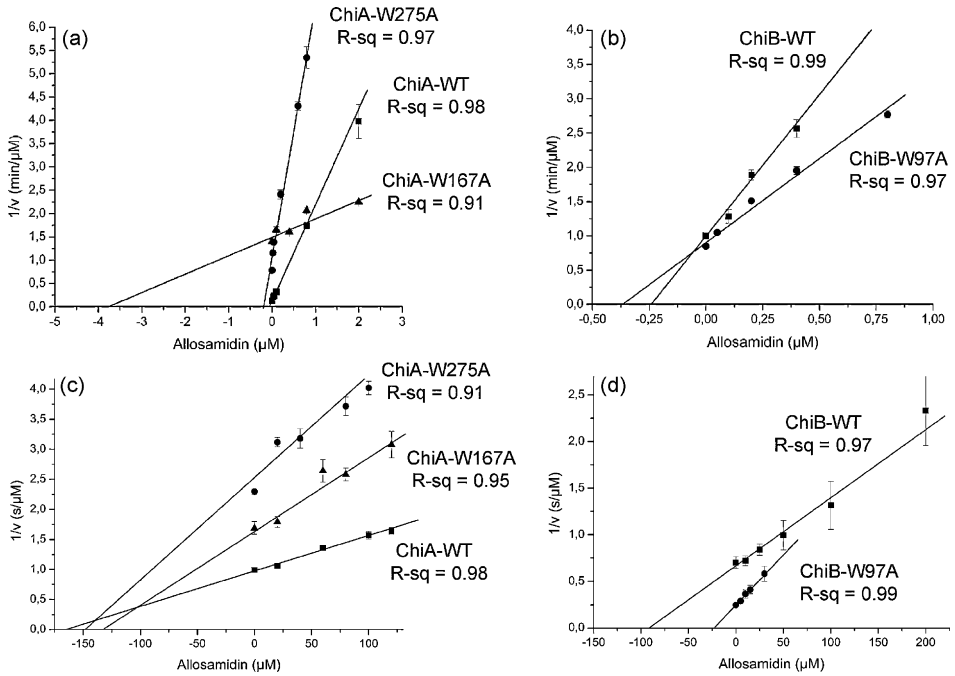

Fig. 2. Dixon plots for allosamidin inhibition of chitinases. Panels a and c show inhibition of ChiA-WT, ChiA-W167A, and ChiA-W275A when acting on β-chitin and chitosan, respectively. Panels b and d show inhibition of ChiB-WT and ChiB-W97A when acting on β-chitin and chitosan, respectively. The β-chitin assays contained 10 mg/mL β-chitin, a chitinase concentration of 100 nM, and allosamidin concentrations varying from 0 to 2.0 μM. The chitosan assays contained 0.25 mg/mL chitosan, a chitinase concentration of 31 nM, and allosamidin concentrations varying from 0 to 200 μM. Each point represents the mean ± SD of at least two independent experiments. *Note:* Because of overlapping data points in the Dixon plots, some data points are difficult to observe. It is important to emphasize that all the Dixon plots contained at least five data points. Correlation coefficients (*R*-sq) are given in the figures.

inhibitor and/or the substrate: the W97A mutation reduces the affinity for both the inhibitor (Table 1) and the substrate (Table 2) by a factor of about two, suggesting that there would be no major change in the IC_{50}. Thus, as discussed below, the observed change in IC_{50} is likely to be due to the non-processive character of the W97A mutant.

For ChiA, the chitosan experiments showed more dramatic effects. Despite the fact that the non-processive ChiA-W167A mutant showed a 14-fold reduced affinity for allosamidin (Table 1), the IC_{50} value for this mutant was similar to the IC_{50} value for the processive ChiA wild-type (133 ± 17 and 164 ± 13 μM, respectively). This indicates that the loss in inhibitor affinity is compensated for by the loss of processivity in the mutant enzyme.

Taken together, the results with chitosan show that loss of processivity increases the chitinases' sensitivity for competitive inhibition by allosamidin. This can be rationalized by looking at the principle behind the processive mode of action. After each catalytic cleavage, the polymeric product will remain associated with the enzyme and relocate to a new productive binding position, via intermediate binding modes that are facilitated by solvent mediated interactions and flexible hydrophobic residues (Davies & Henrissat, 1995; Teeri, 1997; Varrot et al., 2003). The polymer is thus never fully dissociated from the enzyme, and the active site remains unavailable for binding of a competitive inhibitor. Consequently, the more processive the enzyme is, the more difficult it is for the competitive inhibitor to compete with the substrate for binding to the active site. One might expect that the effect of processivity on inhibitor efficacy would be largest if the inhibitor competes with the polymeric part of the substrate, as is the case in ChiA but not in ChiB (Fig. 1). Indeed, when corrected for direct mutational effects on allosamidin binding affinity (K_d in Table 1), the W167A mutation in ChiA increased inhibitor efficacy to a larger extent than the W97A mutation in ChiB.

As a control, we also studied the effects of the W275A mutation in ChiA. The IC_{50} value for the ChiA-W275A mutant was not significantly different from the IC_{50} value for ChiA-WT (148 ± 15 and

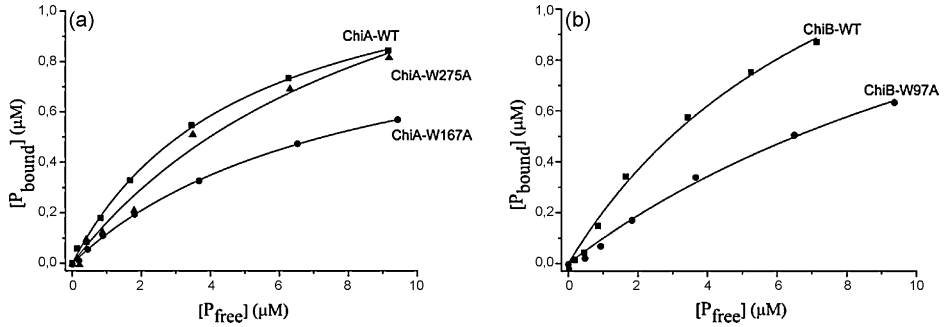

Fig. 3. Equilibrium isotherms for the adsorption of ChiA-WT, ChiA-W167A and ChiA-W275A (a), and of ChiB-WT and ChiB-W97A (b) to β-chitin. The reaction (0.5 mL) contained 1.0 mg β-chitin and the enzyme concentration varied from 0 to 10 μM. After 3 h of incubation at 4 °C, the reaction mixtures were centrifuged and concentrations of specifically bound protein (P_{bound}) and non-bound protein (P_{free}) were determined and plotted against each other. All data sets were fitted to the equation for one-site binding by non-linear regression (see Section 2 for details). Each data point represents the average of values obtained in three independent binding experiments.

Table 3
Effect of allosamidin on processivity.

Chitinase	β-Chitin $(GlcNAc)_2/(GlcNAc)_3$[a]		Chitosan (DP4/DP5)[b]	
	−Allosamidin	+Allosamidin[c]	−Allosamidin	+Allosamidin[c]
ChiA-WT	14.0 (0.9)	18.7 (2.7)	1.6 (0.7)	1.4 (0.5)
ChiB-WT	8.3 (1.3)	9.0 (3.0)	1.8 (0.4)	1.7 (0.2)

[a] Final $(GlcNAc)_2/(GlcNAc)_3$ ratios after degradation of β-chitin. Reaction conditions were such that the substrate was maximally degraded. Both enzymes convert $(GlcNAc)_3$ to $(GlcNAc)_2$ and GlcNAc, and only these latter two were observed in the end product mixtures. GlcNAc can only emerge from a previously formed $(GlcNAc)_3$ molecule. The $(GlcNAc)_2/(GlcNAc)_3$ ratio, indicative for the degree of processivity during degradation of the polymeric substrate can be calculated using the formulae $[(GlcNAc)_2 - GlcNAc]/[(GlcNAc)_3 + GlcNAc]$, which, in this case (with no remaining trimers present) equals $[(GlcNAc)_2 - GlcNAc]/[GlcNAc]$ (all amounts are molar amounts). The pros and cons of this method have been discussed elsewhere (Eijsink et al., 2008; see text).

[b] Ratios of DP4/DP5 products formed during the initial phase of chitosan degradation (less than ~20% of the chitosan substrate degraded). The values represent the ratios between the summed intensities of the dominating DP4 products versus the summed intensities of the dominating DP5 products, as found in MALDI-TOF-MS analysis (see Section 2 for details).

[c] The allosamidin concentrations were 0.5 μM in the reaction with β-chitin and 160 μM (ChiA) or 90 μM (ChiB) in the reaction with chitosan.

164 ± 13 μM, respectively). This is in agreement with the observation that this mutation has only minor effects on processivity (Zakariassen et al., 2009) combined with the fact that this mutation affects an aglycon subsite.

3.3. Effects of competitive inhibition on processivity

As shown above, the inhibitory effect of a competitive inhibitor depends on the processivity of the chitinases, and it is therefore interesting to see if competitive inhibition affects the processivity itself. Processivity towards crystalline substrates is notoriously difficult to assess even with "established" methods (reviewed by Eijsink, Vaaje-Kolstad, Vårum, & Horn, 2008). Here we approached the issue using two methods. In the first method, processivity towards chitin was assessed by analyzing the final $(GlcNAc)_2/(GlcNAc)_3$ ratios after complete degradation of β-chitin. This method has previously been used to evaluate the processivity of both cellulases and chitinases (Horn, Sørbotten, et al., 2006; Medve, Karlsson, Lee, & Tjerneld, 1998; Teeri et al., 1998). Table 3 shows that, within experimental error, this ratio was not changed by the presence of allosamidin, indicating that the degree of processivity of ChiA and ChiB is not affected by the presence of the inhibitor.

In the second method we exploited the fact that processive hydrolysis of chitosan gives a diagnostic dominance of even-numbered oligomeric products during the initial phase of the reaction, as shown and explained in detail in previous publications (Horn, Sørbotten, et al., 2006; Sørbotten et al., 2005). To obtain a rough estimate of this dominance we conducted MALDI-TOF-MS analysis of product mixtures obtained during the initial phases of degradation reactions with ChiA or ChiB and summarized the intensities of the DP4 and DP5 species that were detected (see Section 2). Table 3 shows that the DP4/DP5 ratios were not affected by the presence of allosamidin, indicating, again, that the degree of processivity is not affected by the presence of the inhibitor.

These two methods are by no means quantitative, but the results nevertheless strongly indicate that allosamidin is not capable of interfering significantly with processive hydrolysis. In other words, once the substrate is bound it will remain associated to the processive enzyme, regardless of the presence of an inhibitor. This is in accordance with the results and considerations concerning the effect of processivity on allosamidin binding that are described above, the key issue being that during processive action, the polymeric substrate remains associated with the enzyme in between hydrolytic steps.

4. Conclusion

The present results show that there is a correlation between the degree of processivity of a chitinase and the efficiency of competitive inhibition of this enzyme by allosamidin: the more processive the enzyme, the less effective the inhibitor. Furthermore, when it comes to designing inhibitors for processive enzymes, our results suggest that it may be wise to focus on creating binding interactions in those subsites that are most likely to become accessible during the processive hydrolysis, i.e. the subsites that harbor the oligomeric product (usually a dimer). This is shown by the fact that the effect of abolishing processivity was larger in ChiA, where allosamidin competes with the polymeric part of the substrate. Finally, the remarkable differences between the IC_{50} values obtained using chitin and those obtained using chitosan (Table 1) underpin the importance of using relevant substrates when testing inhibitor efficacies, e.g. when screening compound libraries.

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