Processivity and substrate-binding in family 18 chitinases

Morten Sørlie[#], Henrik Zakariassen, Anne Line Norberg, and Vincent G.H. Eijsink.

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

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

Abstract

Enzymatic depolymerization of polysaccharides is a key technology in the biorefining of biomass. The enzymatic conversion of the abundant insoluble polysaccharides cellulose and chitin is of particular interest and complexity, because of the bi-phasic nature of the process, the seemingly complicated tasks faced by the enzymes, and the importance of these conversions for the future biorefinery. Here we review recent work on family 18 chitinases that sheds light on important aspects of the catalytic action of these depolymerizing enzymes, including the structural basis of processivity and its direction, the energies involved in substrate-binding and displacement.

Introduction

Chitin, a β-1,4-linked polymer of *N*-acetylglucosamine (GlcNAc), is among the most abundant biopolymers in nature, and hence, of large biological and economical importance. The degradation of chitin to di- and monosaccharides is catalyzed by glycosyl hydrolases called chitinases. Chitin, and, particularly its partially deacetylated and water soluble analogue chitosan, are important in the food and feed industry, pharmacological industry, in water purification systems, and as antimicrobial additives (Synowiecki and Al-Khateeb 2003). Chitin metabolism is essential in several major plague organisms such as certain fungi, insects and nematodes, and chitin turnover has been associated with the ability of humans to respond to such organisms (van Eijk et al. 2005). Inhibition of chitinases belonging to glycosyl hydrolase family 18 (Henrissat and Davies 1997) is a target area in the development of medicines for allergic and inflammatory disorders (Donnelly and Barnes 2004; Zhu et al. 2004). Hydrolytic products of chitin and chitosan, chitooligosaccharides (CHOS), have interesting biological activities (Aam et al. 2010), for example as elicitors of plant defense against fungal infections (Roby et al. 1987). CHOS are known to affect several cellular processes and direct enzymatic inhibition by CHOS has been observed for a prolyl endopeptidase as well as a family 18 chitinase (Cederkvist et al. 2008; Je et al. 2007; Rahman et al. 2008).

Some of the most important characteristics that define chitinase functionality are: i) the degree of processivity, which is the ability of the enzyme to remain attached to the substrate in between subsequent hydrolytic reactions (Davies and Henrissat 1995; Teeri 1997; von Ossowski et al. 2003), (Davies and Henrissat 1995; Teeri 1997; von Ossowski et al. 2003), ii) the tendency to cleave the polymeric substrate at chain ends (exo-action) or at random positions (endo-action), iii) the directionality of the degradation, which is a relevant

parameter for chitinases that are exo-acting and/or processive, and iv) the kinetics and thermodynamics of substrate and inhibitor binding. Below, we discuss several of these characteristics, as well as their structural basis, links between them and their functional implications.

Processivity in Glycoside Hydrolysases

The ability to remain attached to the substrate in between subsequent hydrolytic cleavages is a common feature of glycosidases that degrade recalcitrant, crystalline polysaccharides such as cellulose and chitin (Davies and Henrissat 1995; Rouvinen et al. 1990). Such a 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 (Harjunpaa et al. 1996; Teeri 1997; von Ossowski et al. 2003), thus reducing the number of times the enzymes has to carry out the energetically unfavorable process of gaining access to a single polymer chain (Beckham and Crowley 2011). Because of the 180° rotation between consecutive sugar units in chitin and cellulose (Fig. 1), sliding of a single polymer chain through the enzyme`s active site will result in productive binding only for every second sugar, and the processive degradation of these polymers thus yield disaccharides (Davies and Henrissat 1995; Rouvinen et al. 1990). The processive mechanism of cellulases from *Trichoderma reesei* has been extensively studied (Divne et al. 1998; Harjunpaa et al. 1996; Igarashi et al. 2009; Jalak and Valjamae 2010; Kipper et al. 2005; Koivula et al. 1998; Kurašin and Väljamäe 2011; von Ossowski et al. 2003; Zou et al. 1999), and so has this mechanism in cellulases from *Humicola insolens* (Varrot et al. 2003; Varrot et al. 1999b), *Thermobifidia fusca* (Li et al. 2007; Vuong and Wilson 2009; Zhou et al. 2004) and *Clostridium cellulyticum* (Mandelman et al. 2003; Parsiegla et al. 1998; Parsiegla et al. 2000;

Parsiegla et al. 2008). Despite this considerable body of experimental work, the mechanism of processivity is not completely understood. Remaining questions concern the structural basis of processivity and its directionality, as well as uncertainty as to how favourable processivity really is. As to the latter, it may be noted that the same properties that make cellulases processive also may contribute to the enzymes getting "stuck" on their substrates (Kurašin and Väljamäe 2011). Since processive enzymes are dominant compounds of currently known and commercially interesting enzyme cocktails for biomass conversion (Merino and Cherry 2007), creating a deeper understanding of these enzymes is of considerable interest.

Processivity and active site topology

Processive enzymes tend to have long and deep, sometimes "tunnel-like", substrate-binding clefts that enclose their substrate substrates to different degrees, as illustrated by the first crystal structures of two processive cellulases (cellobiohydrolases) from *T. reesei* (Divne et al. 1998; Rouvinen et al. 1990). Crystallographic studies of two Cel6A enzymes showed that the loops that enclose the substrate-binding cleft are flexible and can move away from the active site, exposing it to solvent (Varrot et al. 1999b; Zou et al. 1999). This would allow initial endo-cleavage of the substrate, followed by a processive exo-action after the loops comprising the "roof" on the active site tunnel have been "closed". A similar mechanism has been proposed for the action of ChiB on water-soluble chitosan (Sikorski et al. 2006). The importance of these tunnel-forming loops for processivity has been addressed in several studies. For example, the fact that endo-glucanases E2 from *Thermomononspora fusca* (Spezio et al. 1993) and Cel7B from *H. insolens* (Mackenzie et al. 1998) have deep substrate-binding clefts, but lack tunnel-forming loops and seem to be non-processive has been taken to suggest that the loops are important for processivity. A study on processive *Tr*Cel7A showed that deletion of the tip of a loop that forms the roof of the active site tunnel led to a less

processive enzyme (von Ossowski et al. 2003). There are, however, examples of processive glycosidases that do not seem to use flexible loops tunnel-forming loops. Examples include the processive endo-cellulase E4 from *T. fusca*, which has a relatively open and shallow active site cleft (Li et al. 2007; Sakon et al. 1997), and the processive family 18 chitinase ChiA from *Serratia marcescens*, which has a deep substrate-binding cleft but lacks flexible loops that enclose the substrate (Papanikolau et al. 2001; Perrakis et al. 1994) (Fig 2). It should be noted that experimental assessment of processivity is rather complicated and prone to errors as discussed by Eijsink *et al*. and Horn *et al*. (Eijsink et al. 2008; Horn et al. 2006b). This should be kept in mind when assessing literature data.

Processivity and enzyme-substrate interactions

Although having some structural differences in their active site structures, processive glycosidases share the common feature of having their substrate-binding clefts and surfaces lined with aromatic residues, in particular tryptophan residues (Fig 2). Aromatic residues are well-known to be involved in carbohydrate-protein interactions and are together with hydrogen bonding the dominant interactions in protein-carbohydrate complexes (Quiocho 1989; Vyas 1991). Aromatic side chains interact with both sides of the sugar ring through hydrophobic stacking (Hu et al. 2002; Quiocho 1989; Williams and Davies 2001). If the side chain of the aromatic residue and the sugar ring are co-planar, the π electrons of the aromatic residue can form several CH-π interactions with the sugar ring (Nishio et al. 1998). Such hydrophobic stacking interactions offer a solution to the problem of how processive enzymes manage to remain attached to the substrates while at the same time retaining the ability to slide during the processive mode of action. Compared to e.g. hydrogen bonds, hydrophobic stacking interactions are non-specific and involve larger interaction surfaces. Therefore this type of interactions is thought to facilitate processivity by reducing the sliding energy of the

polymer chain and by functioning as a flexible and ”fluid-like” sheath along which the polymer chain can slide (Breyer and Mattthews 2001; Divne et al. 1998; Meyer and Schulz 1997; Parsiegla et al. 2000; Parsiegla et al. 2008; Varrot et al. 2003).

More insight into this concept may be derived from a study by Meyer and Schulz (1997), who used a combined structural and modeling approach to propose a model for how malto-oligosaccharides ”slide” through the pores of maltoporin (Meyer and Schulz 1997). The surface of the pore is lined with aromatic residues, and it was shown that the presence of these residues results in a binding-profile that is smoothened (i.e. contains less high-energy barriers) compared to if binding had only involved hydrogen-bonds (which in fact make up most of the carbohydrate-protein interactions in this system). The energy profiles contributed by hydrogen bonding interactions and hydrophobic stacking interactions are shown in Fig. 3 Together these profiles result in a smoothening of the total energy profile (Fig 3.), which promotes sliding.

The mechanism underlying processivity was also addressed in a study by Varrot *et al*. who solved five high-resolution structures of the *Hi*Cel6A in complex with non-hydrolyzable thio-oligosaccharides (Varrot et al. 2003). Varrot *et al*. pointed out that the enzyme must tolerate intermediate states of non-productive binding during processive action, meaning that the –1 subsite, which is highly optimized for productive binding, and the other subsites, must be able to accommodate both faces of the pyranose rings meaning that the –1 subsite, which is highly optimized for productive binding, and the other subsites, must be able to accommodate both faces of the pyranoside rings. The structures of the *Hi*Cel6A-ligand complexes, revealing binding modes in which the subsites see both sugar faces, as well as an intermediate binding mode in between the two extremes showed which interactions are involved and how sliding may occur. The productive binding-mode is characterized by having a large number of hydrogen-bonds between the ligand and the protein, whereas hydrogen bonding is much more indirect in the non-productive binding-modes that are characterized by complex networks of

solvent-mediated interactions. For example, productive binding to the –1 subsite involves three direct hydrogen-bonds, compared with only one in the non-productive binding-mode. The structures also showed that the tryptophan residues in the substrate-binding cleft are flexible (the aromatic planes tilt by up to around 5°), such that the aromatic planes align with the sugar rings in both binding modes. Varrot *et al*. concluded that these two effects (solvent-mediated interactions and aromatic flexibility), together with small rigid-body movements of secondary structural elements, and a flexible catalytic acid, allows the polymeric substrate to slide through the catalytic cleft of *Hi*Cel6A without dissociation (Varrot et al. 2003).

In another study, Parsiegla *et al*. determined the structure of the catalytic domain of the processive Cel48F from *C. cellulolyticum* in complex with non-hydrolyzable thio-oligosaccharides and of an inactive mutant (E55Q; Glu^{55} is the catalytic acid) in complex with oligosaccharide substrates (Parsiegla et al. 2000). The substrate-enzyme complexes showed well-defined substrate-binding subsites, and within these sites, the sugar moieties had hydrophobic stacking interactions with several aromatic residues and formed hydrogen bonds to water molecules and polar/charged residues. The inhibitor-enzyme complexes showed that the ligands occupied a second set of sites that were somewhat less well defined and shifted half the length of a sugar moiety, compared to the enzyme-substrate complexes. The latter complexes revealed different stacking interactions with the aromatic residues. On the basis of these results, the authors suggested that such a second set of binding sites may reduce the energy barrier for translocation of the substrate, which would promote sliding. In a follow-up study, Parsiegla *et al*. determined the structures of two inactive mutants of *Cc*Cel48F (E55Q and E44Q) in complex with longer thio-oligosaccharides (Parsiegla et al. 2008). The structures confirmed that ligands may occupy two slightly different positions and that aromatic residues play important roles in both these binding modes.

The experimental observations of different binding modes by Varrot *et al*. (2003) and Parsiegla *et al*. (2008) provide important insight into processivity. It is clear that aromatic residues play central roles.

Processivity in Family 18 Chitinases

Structural comparisons (Perrakis et al. 1994; van Aalten et al. 2000) as well as microscopy studies with labeled enzymes (Hult et al. 2005)suggest that ChiA and ChiB (Fig. 2) degrade chitin chains processively, moving in opposite directions. ChiB is thought to move toward the reducing end (releasing dimeric products from its –1 and –2 subsites), and ChiA is thought to move toward the non-reducing end (releasing products from its +1 and +2 subsites).

Enzyme processivity on substrates such as cellulose and chitin is generally difficult to measure and observe for reasons that have been discussed extensively elsewhere (Eijsink et al. 2008; Horn et al. 2006b). For example, exo-acting non-processive enzymes and endo-acting processive dimer-producing enzymes will give similar product profiles. Ratios between monomeric and dimeric products that are sometimes used to get an indication of processivity are strongly influenced by binding preferences for oligomeric intermediate products (e.g. a hexamer could become three dimers or to dimers and two monomers, depending on how the hexamer preferentially binds). The processivity of family 18 chitinases can be assessed by studying the degradation of chitosan, a partially deacetylated polymeric chitin derivative that is soluble (the studies referred to below employed chitosan which was 65 % acetylated). Because of the substrate-assisted catalytic mechanism of family 18 chitinases (Terwisscha van Scheltinga et al. 1995; Tews et al. 1997; van Aalten et al. 2001), requiring an acetyl group being present on the –1 sugar, chitosan binding modes that position a deacetylated sugar in the -1 subsite are non-productive. Because of this, processive enzymes acting on chitosan will yield diagnostic product patterns that are dominated by oligomers comprising am even

number of sugars (except for the first cleavage product, every other product resulting from the same initial enzyme-substrate association will be even-numbered; see (Sørbotten et al. 2005) & (Sikorski et al. 2006).

All three *Serratia* family 18 chitinases predominantly bind in an endo-mode to chitosan (Sikorski et al. 2006) and the two processive enzymes. ChiA (Fig 4.) and ChiB product patterns indicative of processivity, whereas the non-processive ChiC yields a product pattern with random length distribution (Horn et al. 2006b). Using this system, we have studied the contribution of aromatic residues to chitinase processivity.

In line with the idea that the polymeric part of the substrate binds to the aglycon (+) subsites in ChiB, it has been shown that the processivity of ChiB is mainly controlled by aromatic residues in the +1 (Trp^{97}) and +2 (Trp^{220}). Judged by chitosan digestion pattern mutation of Trp^{97} to Ala greatly reduces processivity (Horn et al. 2006a). ChiB does not have aromatic acids in the glycon (product releasing) subsites while ChiA interestingly does. To address the contributions of the aromatic residues close to the catalytic center in ChiA on processivity, Trp^{167} (–3), Trp^{275} (+1), and Phe^{396} (+2) were mutated and the hydrolytic activities were characterized against chitin and chitosan (Zakariassen et al. 2009). The main finding was that the W167A mutation, situated where the polymeric substrate is attached during hydrolysis, almost abolished processivity, the W275A and F396A mutations showed only modest reductions in processivity. Thus, in ChiA a “ChiA-typical” tryptophan in a glycon susbite is crucial for processivity, in contrast to ChiB where tryptophans in the aglycon subsites are vital for a processive mechanism. These crucial residues are located on the side where the polymeric part of the substrate is thought to bind; these mutational results thus provide insight into the structural basis of the directionality of processivity.

Another interesting finding for both ChiA and ChiB was that all Trp -> Ala mutations resulted in enzymes that were slower and less efficient in chitin degradation while activity for

the soluble chitosan increased. These results show that the processive mechanism is essential for an efficient conversion of crystalline substrates but comes at a large cost in terms of intrinsic enzyme speed. To illustrate this further the double mutant ChiA-W167A/W275A was shown to have no activity on crystalline chitin while displaying a 20-fold increase in activity towards chitosan compared to the wild type enzyme.

The results of the Trp -> Ala mutations on reaction kinetics indicate that the rate-limiting steps for hydrolysis of insoluble and soluble polymers are different. This was probed by determining activation parameters for the degradation of chitin and chitosan for several ChiA variants, including wild-type and W167A. The activation parameters were derived from Eyring analysis of the temperature dependency of the apparent catalytic rate constants (k_{cat}^{app}) of both chitin and chitosan degradation (Zakariassen et al. 2010a). For hydrolysis of β-chitin, both ChiA variants showed low activation enthalpy changes ($\Delta H^{\ddagger}$) and relatively large negative changes in activation entropy ($\Delta S^{\ddagger}$), characteristic for a bimolecular (associative) rate-limiting step (Table 1). Furthermore, ChiA-WT had a 1.5-fold higher k_{cat}^{app} compared to ChiA-W167A, due to a more favorable $\Delta H^{\ddagger}$ (4.7 ± 0.5 and 7.4 ± 0.6 kcal/mol, respectively), in accordance with having more aromatic residues available for association to the insoluble polymer. These findings strongly suggest that substrate binding is the rate-limiting step during hydrolysis of insoluble β-chitin as has been suggested before for such insoluble substrates (Koivula et al. 1998; von Ossowski et al. 2003; Zhang and Wilson 1997).

Changing the substrate to chitosan led to remarkable changes in the activation parameters for the wild-type enzyme. Firstly, $\Delta S^{\ddagger}$ was dramatically reduced ($-T\Delta S^{\ddagger}$ went from 13.9 ± 0.4 from chitin to 0.7 ± 0.5 kcal/mol for chitosan), suggesting that enzyme-substrate association was no longer the rate-limiting step. Secondly, $\Delta H^{\ddagger}$ was significantly increased (from 4.7 ± 0.5 to 15.2 ± 0.6 kcal/mol). These values are compatible with sliding of the (glycon) polymer and displacement of the (aglycon) dimeric product comprising the rate-

limiting step. The entropic term is small because of the substrate is already in the active-site and because water molecules involved in the enzyme-substrate interaction remain "trapped" in the interaction. Also, for sliding, all weak interactions between the polymeric substrate and the enzyme need to be temporarily disrupted (Varrot et al. 2003), explaining the high $\Delta H^{\ddagger}$ value.

Importantly, confirming the ideas about rate-limiting steps described above, the effect of the W167A mutation on activation parameters was totally different when comparing chitin and chitosan. Whereas this mutation led to a higher enthalpic barrier in the degradation of chitin ($\Delta H^{\ddagger}$ from 4.7 ± 0.5 to 8.7 ± 0.6 kcal/mol), the same mutation lowered the enthalpic barrier for degradation of chitosan ($\Delta H^{\ddagger}$ from 15.2 ± 0.6 kcal/mol to 7.9 ± 0.7 kcal/mol). The latter decrease is in accordance with the idea that substrate displacement or "sliding" is rate-limiting during chitosan hydrolysis. Removal of Trp^{167} leads to an enzyme that is less "sticky", less processive and, due to a lower enthalpic activation barrier, faster. The W167A mutant showed a clearly increased $\Delta S^{\ddagger}$ value ($-T\Delta S^{\ddagger}$ = 7.3 ± 0.7 vs 0.7 ± 0.5 kcal/mol for the wild-type enzyme), which is compatible with the idea that association with substrate to a larger extend contributes to the rate-determining step, which again is compatible with the observation that this mutant showed reduced processivity.

Processive enzymes that remain attached to the polymeric substrate in between hydrolytic steps could be less susceptible to competitive inhibition, especially if the inhibitor would compete with the polymeric part of the substrate. Indeed, we found that allosamidin a pseudotrisaccharide that acts as a competitive inhibitor of family 18 chitinases by binding to the –3 to –1 subsites (Terwisscha van Scheltinga et al. 1995), is more effective towards the non-processive variants of ChiA and ChiB, especially in the case of ChiA where allosamidin competes with the polymeric part of the substrate (Zakariassen et al. 2010b) (Table 2). The non-processive enzyme variants ChiA-W167A and ChiB-W97A showed approximately wild-

type affinities for the substrate. As expected, the K_d for allosamidin (determined by ITC) had hardly changed for ChiB-W97A (mutation in the +1 subsite), whereas it had increased approximately 14-fold (from 0.17 ± 0.2 μM to 2.4 ± 0.2 μM) for ChiA-W167A (mutation in –3 subsite).

The effect of processivity on allosamidin efficacy cannot be assessed using chitin as a substrate because in this case the hydrolytic step, either it is processive or non-processive, is faster than the rate-limiting (i.e. substrate-binding) step and cannot be observed in the kinetic measurements. For chitosan, with another rate-limiting step, one would expect a true competition between the substrate and allosamidin that can be assessed by looking at IC_{50} values. The inhibition experiments showed that the observed IC_{50} value for ChiB-WT was about 4-fold higher than for the almost non-processive ChiB-W97A mutant, despite the two enzymes having approximately similar K_d values. This indicates that the non-processive variant is slightly more prone to competitive inhibition. In the case of ChiA, the IC_{50} value for the non-processive W167A mutant was similar to that of ChiA-WT, despite the fact that the non-processive mutant showed a 14-fold reduced affinity for allosamidin. So, in this case, the loss of processivity increased the sensitivity for competitive inhibition by allosamidin quite substantially.

These observations make sense if one realizes what processivity implies: during processive action the polymeric substrate is never fully dissociated from the enzyme (Davies and Henrissat 1995; Teeri 1997; Varrot et al. 2003), and the active site thus remains unavailable for binding of a competitive inhibitor.

Due to the opposite directionalities in ChiA and ChiB, it was expected that the effect of processivity on inhibitor efficacy would be largest in ChiA. Indeed, when corrected for

direct mutational effects on allosamidin binding affinity (K_d), the W167A mutation in ChiA increased inhibitor efficacy to a much larger extent than the W97A mutation in ChiB.

Thermodynamics of binding

The catalytic centers of ChiA and ChiB (and of other family 18 chitinases such as the non-processive endochitinase ChiC) are essentially identical. In all these enzymes there are several subsites for sugar binding, and among those is the highly conserved –1 subsite where there are many interactions that ensuring binding of the –1 sugar in an energetically unfavorable distorted conformation (Biarnes et al. 2007; Synstad et al. 2004). It is interesting to note that, this –1 subsite is a "product" site in ChiB, where dimers are being released from the –2 and –1 subsites during processive action on chitin, whereas it is a "substrate" site in ChiA, where the dimeric product is released from the +1 and +2 subsites, whereas the polymeric part binds to (and "slides" along) the glycon (–) subsites. Clearly, the active sites of ChiA and ChiB must be adapted to these different directionalities, as shown by the work on aromatic residues discussed above. To gain more insight into the more subtle differences in active site architecture, we have conducted various studies on the thermodynamic of ligand binding in ChiA and ChiB, as well as in the non-processive ChiC with its much more open active site architecture (Fig. 2).

The most important parameter in the thermodynamic description of binding is the Gibbs free energy change ($\Delta G_r°$) that holds information on the binding affinity of a ligand to an enzyme. The free energy change is related to the equilibrium constant of the binding reaction and can be divided into an enthalpic ($\Delta H_r°$) and entropic ($\Delta S_r°$) part (Equation 1).

$$\Delta G_r° = -\,RT\,\ln K_a = \Delta H_r° - T\Delta S_r° \qquad (1)$$

The enthalpy change of a binding reaction reflects the changes in weak interactions the ligand and the enzyme have to each other compared to that of the solvent. The entropic change is normally divided into three separate terms; the loss of translational entropy from combining two entities to one (ΔS_{mix}), the change in solvation upon ligand binding (ΔS_{solv}), and conformational changes within both ligand and enzyme upon binding. This can be summarized as shown in Equation 2.

$$\Delta S_r^\circ = \Delta S_{solv} + \Delta S_{mix} + \Delta S_{conf} \qquad (2)$$

Individual enthalpic and entropic contributions upon binding can vary, but often in a compensatory manner giving small variations in the free energy that ultimately determines the thermodynamic stability of the complex. This phenomenon is known as the enthalpy-entropy compensation and is often observed in aqueous systems where noncovalent interactions dominate (Cooper et al. 2001; Dunitz 1995).

The solvatization entropy, ΔS_{solv}, is directly linked to the heat capacity (ΔC_p) of a system and can be derived from ΔC_p by recognizing that ΔS_{solv} is close to zero for proteins near 385 K (Baker and Murphy 1997; Baldwin 1986; Murphy 1994). ΔC_p may be determined from the temperature dependency of ΔH_r°. Equation 3 gives ΔS_{solv} at i.e. t = 20 °C.

$$\Delta S_{solv} = \Delta C_p \ln (T_{293K} / T_{385K}) \qquad (3)$$

The mixing entropy change, ΔS_{mix}, can be calculated as a statistical correction that reflects the mixing of solute and solvent molecules and accounts for the change in entropy due to changes in translational/rotational degrees of freedom (Murphy 1994). For a bimolecular binding reaction this can be given as:

$$\Delta S_{mix} = R \ln (1 / 55.5) \qquad (4)$$

where R is the gas constant.

Table 3 shows data for the thermodynamics of ligand binding to the three *S. marcescens* chitinases (Baban et al. 2010; Cederkvist et al. 2007; Norberg et al. 2010a; Norberg et al. 2010b). Binding of allosamidin is of particular interest because this ligand binds in a virtually identical manner to all three chitinases, occupying the –3 to –1 subsites and interacting strongly with the catalytic glutamate (e.g. Glu144 in ChiB) and the nearby conserved aspartate (Asp142 in ChiB) (Papanikolau et al. 2003; Terwisscha van Scheltinga et al. 1995; van Aalten et al. 2001; Vaaje-Kolstad et al. 2004). Despite this similarity, the thermodynamic signatures of allosamidin binding differed considerably between the three enzymes.

Allosamidin binds to ChiA and ChiB with almost equal affinities ($\Delta G_r° \approx -9.3$ kcal/mol), but the thermodynamic signatures of the binding are very different. The interaction with ChiA was driven equally by enthalpic ($\Delta H_r°$ −6.2 ± 0.2 kcal/mol) and solvation entropic changes ($-T\Delta S_{solv} = -4.9 \pm 0.7$ kcal/mol) (Baban et al. 2010). The beneficial enthalpic term is in agreement with the "stickiness" discussed above in relation to ChiA being processive with the polymeric part of the substrate being bound to the glycon (–) subsites. Mutation of Trp^{167} to Ala resulted in a 20-fold increase in the K_d for allosamidin binding that is translated into differences in free energy change of –1.8 kcal/mol and enthalpic change of –4.4 kcal/mol (Baban et al. 2010), confirming the importance of the residue for binding affinity. Much in contrast to the situation in ChiA, binding of allosamidin to ChiB was driven by changes in conformational entropy ($-T\Delta S_{conf} = -10.8 \pm 0.5$ kcal/mol), while being accompanied by an enthalpic penalty ($\Delta H_r° = 3.8 \pm 0.2$ kcal/mol). Thus, binding of allosamidin to what are "product-binding sites" in ChiB (i.e., where chitobiose is released from the enzyme during hydrolysis) has a fundamentally different thermodymanic signature than binding of

allosamidin to "substrate-binding sites" in ChiA (i.e., where the polymeric chitin chain would be binding during hydrolysis).

The contribution of conformational entropy to allosamidin binding in ChiBis atypical for binding to glycosyl hydrolases and lectins (Cederkvist et al. 2007). Two factors that may contribute to such favorable conformational changes may be that allosamidin interacts only with residues that have low *B*-factors in the structure of the ligand-free enzyme (van Aalten et al. 2001) and that allosamidin is "preformed" meaning it must not undergo a chair – boat conformational transformation upon binding and, hence, does not lose much conformational entropy.

For comparison, the energetics of allosamidin binding to Chitinase C (ChiC) of *S. marscescens* have been determined as well (Baban et al. 2010). ChiC is a true endo-chitinase with a more shallow and open substrate binding cleft than ChiA and ChiB (Fig. 2) and with a lower affinity for allosamidin (Table 3). The thermodynamics of allosamidin binding are completely dominated by a large and positive solvation entropy change ($-T\Delta S_{solv}$ of -9.4 ± 0.7 kcal/mol). Considering the open character of the substrate-binding cleft of ChiC it is conceivable that this cleft is more solvated than the more closed substrate-binding clefts of ChiA and ChiB, meaning that more water molecules are expulsed upon ligand binding.

Further studies on the thermodynamics of ligand binding have been conducted for $(GlcNAc)_6$ binding to an inactive ChiB mutant (ChiB-E144Q). The binding preferences for the hexameric ligand are such that it binds 80% to subsites –2 to +4 and 20% to subsites –3 to +3) (Horn et al. 2006c). Binding of the hexamer is primarily driven by solvation entropy ($-T\Delta S_{solv}$ of -12.5 ± 0.4 kcal/mol), with a small enthalpic penalty ($\Delta H_r° = 1.7 \pm 0.3$ kcal/mole). The positive effect of ΔS_{solv} is likely due to a combination of desolvation of a heavily solvated ligand as well as desolvation of interacting aromatic residues in the aglycon

subsites (Trp^{97}, Trp^{220}, and Phe^{190}). Moreover, it is in agreement with the binding of allosamidin that only occupies three subsites and is accompanied by a $-T\Delta S_{solv}$ of -5.0 ± 0.3 kcal/mol. This value is similar to the one obtained for allosamidin binding to ChiA ($-T\Delta S_{solv}$ of -4.9 ± 0.7 kcal/mol). A priori, one would ascribe the low enthalpic penalty compared to allosamidin binding to favorable aromatic stacking interactions in these aglycon subsites involving the same tryptophans that make ChiB processive (Trp^{97}, Trp^{220}) as well as a Phe^{190} in subsite +3 (van Aalten et al. 2001). However, a study on the binding of oligomers of different length indicated that interactions in the +2 and +3 subsites (Trp^{220}, Phe^{190}) in fact are enthalpically unfavorable (Norberg et al., 2010; see below).

While conformational entropy changes are beneficial are important for allosamidin binding to ChiB, they are neglectable for $(GlcNAc)_6$ binding ($-T\Delta S_{conf} = -0.1 \pm 0.6$ kcal/mol). This is probably due to the hexamer being much more flexible and less "preformed" than allosamidin and to the fact that parts of the aglycon subsites show higher *B*-factors than the glycon subsites. Rigidification of flexible regions upon ligand binding is typical for ligand binding to glycoside hydrolases (Davies et al. 1995; Varrot et al. 2000; Zou et al. 1999).

To further assess individual subsite binding energetics, thermodynamic parameters for binding of N-acetylglucosamine (GlcNAc) oligomers of varying lengths to ChiB-E144Q have been determined using isothermal titration calorimetry (Norberg et al. 2010b). While binding to subsites –2 to +1 yielded a free energy change of –4.7 kcal/mol with a dominating enthalpy change (–4.9 kcal/mol), the stacking interactions in glycon subsites +2 (Trp) and +3 (Phe) were surprisingly endothermic ($\Delta H_r°$ of 3.9 and 1.8 kcal/mol, respectively) and entirely driven by entropy changes ($-T\Delta S_r°$ of –6.7 and –2.6 kcal/mol, respectively). The Trp^{220} mediated affinity in the +2 subsite ($\Delta G_r° = -2.7$ kcal/mol) is comparable to the binding affinity by a similar Trp-GlcNAc stacking in the +2 subsite of Chit42 of *T.harzianum* ($\Delta G_r° \approx -2.5$

kcal/mol) (Lienemann et al. 2009). The endothermic enthalpic contribution is counterintuitive considering the favorable stacking interactions by the aromatic residues (Baban et al 2010). However, substrate binding to subsites +2 and "+3" induces considerable conformational changes and it is conceivable that enthalpically favorable interactions in the apo-enzyme are disrupted upon binding (van Aalten et al. 2001). As an example, Phe^{190} rotates –91° around χ_1 when stacking with a GlcNAc moiety. The fact that binding of the trimer to the –2 to +1 subsites yields a beneficial enthalpic effect whereas allosamidin binding to subsites –3 to –1 comes with an enthalpic penalty, despite the fact the substrate distortion only needs to happen for the trimer, is quite surprising. It should be noted though that the two systems compared are quite different in terms of the enzymes used (wild-type for allosamidin versus the E144Q mutant for oligomer binding) and ligand (charged and titratable allosamidin versus non charged oligomers). The observed difference in enthalpic effects may indicate that interactions in the –3 subsite are enthalpically unfavorable and/or that interactions in the +1 subsite are enthalpically highly favorable. The latter interaction involves Trp^{97}, the residue that was shown to be crucial for processivity in ChiB (see above).

Recently, the free energies of decrystalliing a polymer chain in α-chitin has been calculated to be 5.6 ± 0.22 kcal/mol per chitobiose-unit for an edge chain (exo-attack) and 8.0 ± 0.60 kcal/mol per chitobiose-unit for a middle chain (endo-attack) (Beckham and Crowley 2011). The experimentally determined values of ligand-binding free energy in the thermodynamic studies described in this review are experimental measures of the compensating binding free energy that enzymes must exhibit to decrystallize individual chitin chains. The calculated and experimentally determined values are in the same order of magnitude. Nevertheless, the experimental values seem small, because productive binding of the substrate requires decrystallization of more than one chitobiose unit. Perhaps, additional

compensating binding energy is derived from the binding interactions exerted by the substrate-binding domains that are attached to the catalytic modules (Fig. 2).

Concluding remarks

Taken together, the studies that are reviewed above provide insight into structural features of chitinases that underlie processivity and its directionality. Aromatic residues clearly play crucial roles. The conclusions drawn from the mutational studies are to some extent supported by the results of the thermodynamic analyses of ligand binding to ChiA and ChiB, which seem to make sense in the context of the ideas and conclusions derived from the studies of mutational effects on enzyme functionality. Nevertheless, the results from the binding analyses indicate that there are more differences between the two enzymes (i.e. in addition to the presence of specific aromatic residues) that may determine processivity and directionality, although these differences have not yet been identified. The simple fact that the tryptophan residue that is crucial for processivity in ChiB (Trp^{97}) is conserved but much less important for processivity in ChiA (Trp^{275}) indicates that the tryptophans do not tell the whole story. One clear difference between ChiA and ChiB is the presence of loops that form a "roof" over the active site. Both enzymes are relatively open (Fig. 2) and lack the long "tunnels" seen in e.g. cellobiohydrolase II from *T. reesei* (Rouvinen et al. 1990) cellobiohydrolase II from *H. insolens* (Varrot et al. 1999a). Still, ChiA has some loops that are absent in ChiB and that affect the glycon subsites, while ChiB has some loops, which actually form a very short "tunnel" in the enzyme-substrate complex, that affect the aglycon subsites. It has been claimed that such substrate-covering loops are important for processivity (Li et al. 2007; von Ossowski et al. 2003; Vuong and Wilson 2009).

It is important to note that the "stickyness" provided by aromatic residues in ChiA and ChiB slows down the enzymes when they act on easily accessible substrates where the rate-limiting step involves product release and substrate displacement. It would be of interest to study systems comprising a series of analogous enzymes with varying degrees of processivity and substrates with varying degrees of accessibility (from purely crystalline to soluble). Such studies could yield more insight into the practical pros and cons of processivity and would provide guidelines for the development of effective enzymes for biomass processing.

The studies discussed above reveal the importance of aromatic residues in processive chitinases [note that such residues are much rarer in non-processive chitinases such as ChiC or family 19 chitinases (Heggset et al. 2009; Hoell et al. 2006). Interestingly, these crucial resiues have several other (putative) roles. Firstly, several studies suggest or show that aromatic residues, especially those that are slightly more remote for the catalytic center, play important roles in initial, not immediately productive binding of the substrate (Koivula et al. 1998; Norberg et al. 2011; Uchiyama et al. 2001). The idea is that these residues contribute to guiding glycan chains towards the catalytic center, a dynamic type of binding and sliding action that in fact is inherent to processivity. Secondly, recent studies show that the transglycosilation activity of family 18 chitinases is affected quite considerably by aromatic residues that contribute to affinity in the in aglycon (sugar acceptor) subsites (Lü et al. 2008; Taira et al. 2010; Zakariassen et al. 2011). While transglycosilation activity may not be desirable in biomass conversion processes, it is of great interest for the enzymatic production of chito-oligosaccahrides that have a variety of interesting (potential) applications (Aam et al. 2010).

Another issue for future results concerns the possible presence and functional roles of additional subsites, i.e. subsites are not visible in the crystal structures of enzyme-substrate complexes, because they are not occupied. There are several indications that the substrate-

binding clefts/surfaces of chitinases may be even more extended than available structural information suggests. For example, there are several indications that ChiA has sugar-binding affinity in "subsites +3 and +4 (Aronson et al. 2003; Norberg et al. 2011) and even more remote subsites have been proposed in other polysaccharide depolymerizing enzymes (Bozonnet et al. 2007; Robert et al. 2005). Such remote subsites may be important for enzyme functionality on true polymeric substrates and their interaction with the substrate may involve conformational changes (see Norberg et al., 2011 for further discussion).

Finally, another subject not addressed in the review but potentially of major importance is the role of the chitin-binding domains in the chitinases. It is clear that these domains contribute to substrate-binding (Boraston et al. 2004; Hervé et al. 2010; Watanabe et al. 1994). However, the roles of these domains on enzyme functionality, be it on catalytic efficiency as such, on processivity, and/or on possible substrate-accessibility effects remain to a large extent unresolved.

References

Aronson, N. N., B. A. Halloran, M. F. Alexyev, L. Amable, J. D. Madura, L. Pasupulati, C. Worth and P. Van Roey. 2003. Family 18 chitinase-oligosaccharide substrate interaction: subsite preference and anomer selectivity of Serratia marcescens chitinase A. Biochem. J. 376:87-95.

Baban, J., S. Fjeld, S. Sakuda, V. G. H. Eijsink and M. Sørlie. 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(18):6144-6149.

Baker, B. M. and K. P. Murphy. 1997. Dissecting the energetics of a protein-protein interaction: the binding of ovomucoid third domain to elastase. J.Mol.Biol. 268(2):557-569.

Baldwin, R. L. 1986. Temperature dependence of the hydrophobic interaction in protein folding. Proc.Natl.Acad.Sci.USA 83(21):8069-8072.

Beckham, Gregg T. and Michael F. Crowley. 2011. Examination of the α-Chitin Structure and Decrystallization Thermodynamics at the Nanoscale. J. Phys. Chem. B 115:4516-4522.

Biarnes, X., A. Ardevol, A. Planas, C. Rovira, A. Laio and M. Parrinello. 2007. The Conformational Free Energy Landscape of *ƒ*-D-Glucopyranose. Implications for Substrate Preactivation in *ƒ*-Glucoside Hydrolases. J. Am. Chem. Soc. 129(35):10686-10693.

Boraston, A. B., D. N. Bolam, H. J. Gilbert and G. J. Davies. 2004. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem. J. 382:769-781.

Bozonnet, S., M. T. Jensen, M. M. Nielsen, N. Aghajari, M. H. Jensen, B. Kramhøft, M. Willemoës, S.l Tranier, R. Haser and B. Svensson. 2007. The 'pair of sugar tongs' site on the non-catalytic domain C of barley α-amylase participates in substrate binding and activity. FEBS J. 274(19):5055-5067.

Breyer, W.A. and B.W. Mattthews. 2001. A structural basis for processivity. Protein Sci. 10:1699-1711.

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

Cederkvist, F. H., S. F. Saua, V. Karlsen, S. Sakuda, V. G. H. Eijsink and M. Sørlie. 2007. Thermodynamic Analysis of Allosamidin Binding to a Family 18 Chitinase. Biochemistry 46(43):12347-12354.

Cooper, A., C. M. Johnson, J. H. Lakey and M. Nollmann. 2001. Heat does not come in different colours: entropy-enthalpy compensation, free energy windows, quantum

confinement, pressure perturbation calorimetry, solvation and the multiple causes of heat capacity effects in biomolecular interactions. Biophys. Chem. 93(2-3):215-230.
Davies, G. and B. Henrissat. 1995. Structures and mechanisms of glycosyl hydrolases. Structure 3(9):853-859.
Davies, G. J., S. P. Tolley, B. Henrissat, C. Hjort and M. Schulein. 1995. Structures of oligosaccharide-bound forms of the endoglucanase V from Humicola insolens at 1.9 A resolution. Biochemistry 34(49):16210-16220.
Divne, Christina, Jerry Ståhlberg, Tuula T. Teeri and T. Alwyn Jones. 1998. High-resolution crystal structures reveal how a cellulose chain is bound in the 50 Å long tunnel of cellobiohydrolase I from *Trichoderma reesei*. J. Mol. Biol. 275(2):309-325.
Donnelly, L. E. and P. J. Barnes. 2004. Acidic mammalian chitinase - a potential target for asthma therapy. Trends Pharmacol Sci. 25(10):509-511.
Dunitz, J. D. 1995. Win some, lose some - Enthalpy-entropy compensation in weak intermolecular interactions. Chem. Biol. 2(11):709-712.
Eijsink, V. G.H., G. Vaaje-Kolstad, K. M. Vårum and S. J. Horn. 2008. Towards new enzymes for biofuels: lessons from chitinase research. Trends Biotechnol. 26(5):228-235.
Harjunpaa, V., A. Teleman, A. Koivula, L. Ruohonen, T. T. Teeri, O. Teleman and T. Drakenberg. 1996. Cello-oligosaccharide hydrolysis by cellobiohydrolase II from Trichoderma reesei. Association and rate constants derived from an analysis of progress curves. Eur.J Biochem. 240(3):584-591.
Heggset, E. B., I. A. Hoell, M. Kristoffersen, V. G. Eijsink and K. M. Vårum. 2009. Degradation of chitosans with chitinase G from Streptomyces coelicolor A3(2): production of chito-oligosaccharides and insight into subsite specificities. Biomacromolecules 10(4):892-899.
Henrissat, B. and G. J. Davies. 1997. Structural and sequence-based classification of glycoside hydrolases. Curr.Opin.Struct.Biol. 7(5):637-644.
Hervé, C., A. Rogowski, A. W. Blake, S. E. Marcus, H. J. Gilbert and J. P. Knox. 2010. Carbohydrate-binding modules promote the enzymatic deconstruction of intact plant cell walls by targeting and proximity effects. Proc.Natl.Acad.Sci U.S.A 107(34):15293-15298.
Hoell, I. A., B. Dalhus, E. B. Heggset, S. I. Aspmo and V. G. H. Eijsink. 2006. Crystal structure and enzymatic properties of a bacterial family 19 chitinase reveal differences from plant enzymes. FEBS J 273(21):4889-4900.

Horn, S. J., P. Sikorski, J. B. Cederkvist, G. Vaaje-Kolstad, M. Sørlie, B. Synstad, G. Vriend, K. M. Vårum and V. G. H. Eijsink. 2006a. Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides. Proc.Natl.Acad.Sci U.S.A 103(48):18089-18094.

Horn, S. J., A. Sørbotten, B. Synstad, P. Sikorski, M. Sørlie, K. M. Vårum and V. G. H. Eijsink. 2006b. Endo/exo mechanism and processivity of family 18 chitinases produced by Serratia marcescens. FEBS J. 273(3):491-503.

Horn, S. J., M. Sørlie, G. Vaaje-Kolstad, A. L. Norberg, B. Synstad, K. M. Vårum and V. G. H. Eijsink. 2006c. Comparative studies of chitinases A, B and C from Serratia marcescens Biocatal.Biotransfor. 24(1-2):39-53.

Hu, G. H., A. Oguro, C. Z. Li, P. D. Gershon and F. A. Quiocho. 2002. The "cap-binding slot" of an mRNA cap-binding protein: Quantitative effects of aromatic side chain choice in the double-stacking sandwich with cap. Biochemistry 41(24):7677-7687.

Hult, E. L., F. Katouno, T. Uchiyama, T. Watanabe and J. Sugiyama. 2005. Molecular directionality in crystalline beta-chitin: hydrolysis by chitinases A and B from Serratia marcescens 2170. Biochem. J. 388(Pt 3):851-856.

Igarashi, K., A. Koivula, M. Wada, S. Kimura, M. Penttila and M. Samejima. 2009. High speed atomic force microscopy visualizes processive movement of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. J. Biol. Chem. 284(52):36186-36190.

Jalak, J. and P. Valjamae. 2010. Mechanism of initial rapid rate retardation in cellobiohydrolase catalyzed cellulose hydrolysis. Biotechnol. Bioeng. 106(6):871-883.

Je, J. Y., E. K. Kim, C. B. Ahn, S. H. Moon, B. T. Jeon, B. Kim, T. K. Park and P. J. Park. 2007. Sulfated chitooligosaccharides as prolyl endopeptidase inhibitor. Int.J.Biol.Macromol. 41(5):529-533.

Kipper, K., P. Väljamäe and G. Johansson. 2005. Processive action of cellobiohydralese Cel7A from *Trichoderma reesei* is revealed as "burst" kinetics on fluoroscent polymeric model substrates. Biochem J 385:527-535.

Koivula, Anu, Tiina Kinnari, Vesa Harjunpaa, Laura Ruohonen, Anita Teleman, Torbjorn Drakenberg, Juha Rouvinen, T. Alwyn Jones and Tuula T. Teeri. 1998. Tryptophan 272: an essential determinant of crystalline cellulose degradation by *Trichoderma reesei* cellobiohydrolase Cel6A. FEBS Lett. 429(3):341-346.

Kurašin, Mihhail and Priit Väljamäe. 2011. Processivity of cellobiohydrolases is limited by the substrate. J. Biol. Chem. 286(1):169-177.

Li, Y., D. C. Irwin and D. B. Wilson. 2007. Processivity, substrate binding, and mechanism of cellulose hydrolysis by Thermobifida fusca Cel9A. Appl.Environ.Microbiol. 73(10):3165-3172.

Lienemann, M., H. Boer, A. Paananen, S. Cottaz and A. Koivula. 2009. Toward understanding of carbohydrate binding and substrate specificity of a glycosyl hydrolase 18 family (GH-18) chitinase from *Trichoderma harzianum*. Glycobiology 19(10):1116-1126.

Lü, Y., H. Yang, H. Hu, Y. Wang, Z. Rao and C. Jin. 2008. Mutation of Trp137 to glutamate completely removes transglycosyl activity associated with *Aspergillus fumigatus* AfChiB1. Glycoconj. J. 26:525-534.

Mackenzie, L. F., G. Sulzenbacher, C. Divne, T. A. Jones, H. F. Woldike, M. Schulein, S. G. Withers and G. J. Davies. 1998. Crystal structure of the family 7 endoglucanase I (Cel7B) from *Humicola insolens* at 2.2 angstrom resolution and identification of the catalytic nucleophile by trapping of the covalent glycosyl-enzyme intermediate. Biochem. J. 335:409-416.

Mandelman, D., A. Belaich, J. P. Belaich, N. Aghajari, H. Driguez and R. Haser. 2003. X-ray crystal structure of the multidomain endoglucanase Cel9G from *Clostridium cellulolyticum* complexed with natural and synthetic cello-oligosaccharides. J. Bacteriol. 185(14):4127-4135.

Merino, Sandra and Joel Cherry. 2007. Progress and challenges in enzyme development for biomass utilization. Adv. Biochem. Eng. Biotechnol. 108:95-120.

Meyer, J. E. and G. E. Schulz. 1997. Energy profile of maltooligosaccharide permeation through maltoporin as derived from the structure and from a statistical analysis of saccharide-protein interactions. Protein Sci. 6(5):1084-1091.

Murphy, K. P. 1994. Hydration and convergence temperatures - on the use and interpretation of correlation plots. Biophys.Chem. 51(2-3):311-326.

Nishio, M., M. Hirota and Y. Umezwa. 1998. The CH/p Interaction: Evidence, Nature, and Consequences. Wiley, New York.

Norberg, A. L., A. I. Dybvik, H. Zakariassen, M. Mormann, J. Peter-Katalinic´, V.G.H. Eijsink and M. Sørlie. 2011. Substrate positioning in chitinase A, a processive chito-biohydrolase from *Serratia marcescens*. FEBS Letters DOI: 10.1016/j.febslet.2011.06.002.

Norberg, A. L., V. G. H. Eijsink and M. Sørlie. 2010a. Dissecting Factors that Contribute to Ligand-Binding Energetics for Family 18 Chitinases. Thermochim.Acta 511:189-193.

Norberg, Anne Line, Vigdis Karlsen, Ingunn Alne Hoell, Ingrid Bakke, Vincent G. H. Eijsink and Morten Sørlie. 2010b. Determination of substrate binding energies in individual subsites of a family 18 chitinase. FEBS Lett. 584(22):4581-4585.

Papanikolau, Y., G. Prag, G. Tavlas, C. E. Vorgias, A. B. Oppenheim and K. Petratos. 2001. High resolution structural analyses of mutant chitinase A complexes with substrates provide new insight into the mechanism of catalysis. Biochemistry 40(38):11338-11343.

Papanikolau, Y., G. Tavlas, C. E. Vorgias and K. Petratos. 2003. De novo purification scheme and crystallization conditions yield high-resolution structures of chitinase A and its complex with the inhibitor allosamidin. Acta Crystallographica Section D-Biological Crystallography 59:400-403.

Parsiegla, G., M. Juy, C. Reverbel-Leroy, C. Tardif, J. P. Belaich, H. Driguez and R. Haser. 1998. The crystal structure of the processive endocellulase CelF of *Clostridium cellulolyticum* in complex with a thiooligosaccharide inhibitor at 2.0 angstrom resolution. EMBO J. 17(19):5551-5562.

Parsiegla, G., C. Reverbel-Leroy, C. Tardif, J. P. Belaich, H. Driguez and R. Haser. 2000. Crystal structures of the cellulase Ce148F in complex with inhibitors and substrates give insights into its processive action. Biochemistry 39(37):11238-11246.

Parsiegla, G., C. Reverbel, C. Tardif, H. Driguez and R. Haser. 2008. Structures of mutants of cellulase Cel48F of *Clostridium cellulolyticum* in complex with long hemithiocellooligosaccharides give rise to a new view of the substrate pathway during processive action. J. Mol. Biol. 375(2):499-510.

Perrakis, A., I. Tews, Z. Dauter, A. B. Oppenheim, I. Chet, K. S. Wilson and C. E. Vorgias. 1994. Crystal structure of a bacterial chitinase at 2.3 A resolution. Structure. 2(12):1169-1180.

Quiocho, F. A. 1989. Protein-Carbohydrate Interactions - Basic Molecular-Features. Pure and Applied Chemistry 61(7):1293-1306.

Rahman, A., S. G. Kumar, S. W. Kim, H. J. Hwang, Y. M. Baek, S. H. Lee, H. S. Hwang, Y. H. Shon, K. S. Nam and J. W. Yun. 2008. Proteomic analysis for inhibitory effect of chitosan oligosaccharides on 3T3-L1 adipocyte differentiation. Proteomics 8(3):569-581.

Robert, X., R. Haser, H. Mori, B. Svensson and N. Aghajari. 2005. Oligosaccharide Binding to Barley α-Amylase 1. J. Biol. Chem. 280(38):32968-32978.

Roby, D., A. Gadelle and A. Toppan. 1987. Chitin oligosaccharides as elicitors of chitinase activity in melon plants. Biochem.Biophys.Res.Commun. 143(3):885-892.

Rouvinen, J., T. Bergfors, T. Teeri, J. K. Knowles and T. A. Jones. 1990. Three-dimensional structure of cellobiohydrolase II from Trichoderma reesei. Science 249(4967):380-386.
Sakon, Joshua, Diana Irwin, David B. Wilson and P. Andrew Karplus. 1997. Structure and mechanism of endo/exocellulase E4 from *Thermomonospora fusca*. Nat. Struct. Mol. Biol. 4(10):810-818.
Sikorski, P., A. Sørbotten, S. J. Horn, V. G. H. Eijsink and K. M. Vårum. 2006. Serratia marcescens chitinases with tunnel-shaped substrate-binding grooves show endo activity and different degrees of processivity during enzymatic hydrolysis of chitosan. Biochemistry 45(31):9566-9574.
Spezio, M., D. B. Wilson and P. A. Karplus. 1993. Crystal-structure of the catalytic domain of a thermophilic endocellulase Biochemistry 32(38):9906-9916.
Synowiecki, J. and N. A. Al-Khateeb. 2003. Production, properties, and some new applications of chitin and its derivatives. Crit.Rev.Food Sci. 43(2):145-171.
Synstad, B., S. Gåseidnes, D. M. F. van Aalten, G. Vriend, J. E. Nielsen and V. G. H. Eijsink. 2004. Mutational and computational analysis of the role of conserved residues in the active site of a family 18 chitinase. Eur.J.Biochem. 271(2):253-262.
Sørbotten, A., S. J. Horn, V. G. H. Eijsink and K. M. Vårum. 2005. Degradation of chitosans with chitinase B from Serratia marcescens. Production of chito-oligosaccharides and insight into enzyme processivity. FEBS J 272(2):538-549.
Taira, Toki, Maho Fujiwara, Nicole Dennhart, Hiroko Hayashi, Shoko Onaga, Takayuki Ohnuma, Thomas Letzel, Shohei Sakuda and Tamo Fukamizo. 2010. Transglycosylation reaction catalyzed by a class V chitinase from cycad, Cycas revoluta: A study involving site-directed mutagenesis, HPLC, and real-time ESI-MS. Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics 1804(4):668-675.
Teeri, T. T. 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol. 15(5):160-167.
Terwisscha van Scheltinga, A. C., S. Armand, K. H. Kalk, A. Isogai, B. Henrissat and B. W. Dijkstra. 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(48):15619-15623.
Tews, I., A. C. Terwisscha van Scheltinga, A. Perrakis, K. S. Wilson and B. W. Dijkstra. 1997. Substrate-assisted catalysis unifies two families of chitinolytic enzymes. J. Am. Chem. Soc. 119(34):7954-7959.

Uchiyama, T., F. Katouno, N. Nikaidou, T. Nonaka, J. Sugiyama and T. Watanabe. 2001. Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase a from Serratia marcescens 2170. J. Biol. Chem. 276(44):41343-41349.

van Eijk, M., C. P. A. A. van Roomen, G. H. Renkema, A. P. Bussink, L. Andrews, E. F. C. Blommaart, A. Sugar, A. J. Verhoeven, R. G. Boot and J. M. F. G. Aerts. 2005. Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. Int.Immun. 17(11):1505-1512.

van Aalten, D. M. F., D. Komander, B. Synstad, S. Gåseidnes, M. G. Peter and V. G. H. Eijsink. 2001. Structural insights into the catalytic mechanism of a family 18 exo-chitinase. Proc.Natl.Acad.Sci.U.S.A 98(16):8979-8984.

van Aalten, D. M. F., B. Synstad, M. B. Brurberg, E. Hough, B. W. Riise, V. G. H. Eijsink and R. K. Wierenga. 2000. Structure of a two-domain chitotriosidase from Serratia marcescens at 1.9-angstrom resolution. Proc.Natl.Acad.Sci.U.S.A 97(11):5842-5847.

Varrot, A, S Hastrup, M Schülein and G J Davies. 1999a. Crystal structure of the catalytic core domain of the family 6 cellobiohydrolase II, Cel6A, from Humicola insolens, at 1.92 A resolution. Biochem. J. 337(2):297-304.

Varrot, A., T. P. Frandsen, I. von Ossowski, V. Boyer, S. Cottaz, H. Driguez, M. Schulein and G. J. Davies. 2003. Structural Basis for Ligand Binding and Processivity in Cellobiohydrolase Cel6A from Humicola insolens. Structure 11(7):855-864.

Varrot, A., M. Schulein and G. J. Davies. 1999b. Structural changes of the active site tunnel of Humicola insolens cellobiohydrolase, Cel6A, upon oligosaccharide binding. Biochemistry 38(28):8884-8891.

Varrot, A., M. Schulein and G. J. Davies. 2000. Insights into ligand-induced conformational change in Cel5A from Bacillus agaradhaerens revealed by a catalytically active crystal form. J.Mol.Biol. 297(3):819-828.

von Ossowski, I., J. Stahlberg, A. Koivula, K. Piens, D. Becker, H. Boer, R. Harle, M. Harris, C. Divne, S. Mahdi, Y. Zhao, H. Driguez, M. Claeyssens, M. L. Sinnott and T. T. Teeri. 2003. Engineering the Exo-loop of Trichoderma reesei Cellobiohydrolase, Cel7A. A comparison with Phanerochaete chrysosporium Cel7D. J.Mol.Biol. 333(4):817-829.

Vuong, T. V. and D. B. Wilson. 2009. Processivity, synergism, and substrate specificity of *Thermobifida fusca* Cel6B. Appl. Environ. Microbiol. 75(21):6655-6661.

Vyas, Nand K. 1991. Atomic features of protein-carbohydrate interactions. Curr. Opin. Struct. Biol. 1(5):732-740.

Vaaje-Kolstad, G., D. R. Houston, F. V. Rao, M. G. Peter, B. Synstad, D. M. F. van Aalten and V. G. H. Eijsink. 2004. Structure of the D142N mutant of the family 18 chitinase ChiB from Serratia marcescens and its complex with allosamidin. Biochim.Biophys.Acta 1696(1):103-111.

Watanabe, T., Y. Ito, T. Yamada, M. Hashimoto, S. Sekine and H. Tanaka. 1994. The Roles of the C-Terminal Domain and Type-Iii Domains of Chitinase A1 from Bacillus-Circulans Wl-12 in Chitin Degradation. J.Bacteriol. 176(15):4465-4472.

Williams, S. J. and G. J. Davies. 2001. Protein-carbohydrate interactions: learning lessons from nature. Trends Biotechnol. 19(9):356-362.

Zakariassen, H., V. G. H. Eijsink and M. Sørlie. 2010a. Signatures of activation parameters reveal substrate-dependent rate determining steps in polysaccharide turnover by a family 18 chitinase. Carbohyd.Polym. 81(1):14-20.

Zakariassen, H., M. C. Hansen, M. Jøranli, V. G. H. Eijsink and M. Sørlie. 2011. Mutational Effects on Transglycosylating Activity of Family 18 Chitinases and Construction of a Hypertransglycosylating Mutant. Biochemistry 50:5693-5703.

Zakariassen, H., L. Klemetsen, S. Sakuda, G. Vaaje-Kolstad, K. M. Vårum, M. Sørlie and V. G. H. Eijsink. 2010b. Effect of enzyme processivity on the efficacy of a competitive chitinase inhibitor. Carbohyd.Polym. 82(3):779-785.

Zakariassen, H., B. B. Aam, S. J. Horn, K. M. Vårum, M. Sørlie and V. G.H. Eijsink. 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(16):10610-10617.

Zhang, Sheng and David B. Wilson. 1997. Surface residue mutations which change the substrate specificity of *Thermomonospora fusca* endoglucanase E2. J. Biotechnol. 57(1-3):101-113.

Zhou, W., D. C. Irwin, J. Escovar-Kousen and D. B. Wilson. 2004. Kinetic studies of *Thermobifida fusca* Cel9A active site mutant enzymes. Biochemistry 43(30):9655-9663.

Zhu, Z., T. Zheng, R. J. Homer, Y. K. Kim, N. Y. Chen, L. Cohn, Q. Hamid and J. A. Elias. 2004. Acidic Mammalian Chitinase in Asthmatic Th2 Inflammation and IL-13 Pathway Activation. Science 304(5677):1678-1682.

Zou, J., G. J. Kleywegt, J. Stahlberg, H. Driguez, W. Nerinckx, M. Claeyssens, A. Koivula, T. T. Teeri and T. A. Jones. 1999. Crystallographic evidence for substrate ring distortion and

protein conformational changes during catalysis in cellobiohydrolase Ce16A from trichoderma reesei. Structure 7(9):1035-1045.

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

Table 1

Activation parameters for substrate hydrolysis in 50 mM sodium acetate buffer, pH 6.1.[a]

Enzyme	k_{cat}^{app}[b]	$\Delta G^{\ddagger}$[c,d]	$\Delta H^{\ddagger}$[b]	$-T\Delta S^{\ddagger}$[b,c]
		β-Chitin		
ChiA-WT	0.54 ± 0.04	18.6 ± 0.1	4.7 ± 0.5	13.9 ± 0.4
ChiA-W167A	0.34 ± 0.02	18.9 ± 0.1	8.7 ± 0.6	10.2 ± 0.6
		Chitosan		
ChiA-WT	40 ± 2	15.9 ± 0.1	15.2 ± 0.6	0.7 ± 0.5
ChiA-W167A	130 ± 17	15.2 ± 0.1	7.9 ± 0.7	7.3 ± 0.7

[a]Adapted from (Zakariassen et al. 2010a), [b] s^{-1}, [c] kcal/mol, [d] T = 37 °C

Table 2

Binding affinity and inhibitory power of allosamdin for chitinase variants.[a]

Chitinase	K_d (μM)[b]	IC_{50} (μM)[c]	
		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	0.16 ± 0.043[d]	0.24 ± 0.01	91 ± 18
ChiB-W97A	0.42 ± 0.02	0.37 ± 0.03	23 ± 3

[a] Adapted from(Zakariassen et al. 2010b), [b] from ITC experiments, [c] from Dixon plots, [d] from (Cederkvist et al. 2007).

Table 3

Thermodynamic parameters for ligand binding to ChiA, ChiB,a nd ChiC as determined by isothermal titration calorimetry at pH 6.0.

K_d[a]	ΔG_r°[b]	ΔH_r°[b]	$-T\Delta S_r^\circ$[b]	$-T\Delta S_{solv}^\circ$[b,c]	$-T\Delta S_{conf}^\circ$[b,d]	ΔS_r°[e]	$\Delta C_{p,r}^\circ$[e,f]
ChiB-E144Q with $(GlcNAc)_6$[g]							
0.13 ± 0.09	–9.2 ± 0.3	1.7 ± 0.3	–10.9 ± 0.4	–12.5 ± 0.4	–0.1 ± 0.6	37 ± 1	–158 ± 5
ChiB with allosamidin[h]							
0.16 ± 0.04	–9.4 ± 0.1	3.8 ± 0.2	–13.2 ± 0.4	–5.0 ± 0.3	–10.8 ± 0.5	44 ± 1	–63 ± 4
ChiA with allosamidin[h]							
0.17 ± 0.06	–9.4 ± 0.2	–6.2 ± 0.2	–3.2 ± 0.4	–4.9 ± 0.7	–1.2 ± 0.8	11 ± 1	–61 ± 13
ChiC with allosamidin[h]							
2.0 ± 0.2	–7.9 ± 0.1	–0.6 ± 0.1	–7.3 ± 0.4	–9.4 ± 0.7	–0.6 ± 0.1	24 ± 1	–120 ± 15

[a] μM, [b] kcal/mol, [c] $\Delta S_{solv}^\circ = \Delta C_p \ln(T/T_{385\,K})$ (Baker and Murphy 1997; Baldwin 1986; Murphy 1994); [d] derived using $\Delta S_r^\circ = \Delta S_{solv}^\circ + \Delta S_{mix}^\circ + \Delta S_{conf}^\circ$ where $\Delta S_{mix}^\circ = R\ln(1/55.5) =$ –8 cal/K mol ("cratic" term) (Baker and Murphy 1997); [e] (cal/K mol); [f] these data are derived from the temperature dependence of ΔH_r°, [g] at t = 20 °C, [h] at t = 30 °C. The table is adapted from (Baban et al. 2010; Cederkvist et al. 2007; Norberg et al. 2010a; Norberg et al. 2010b)

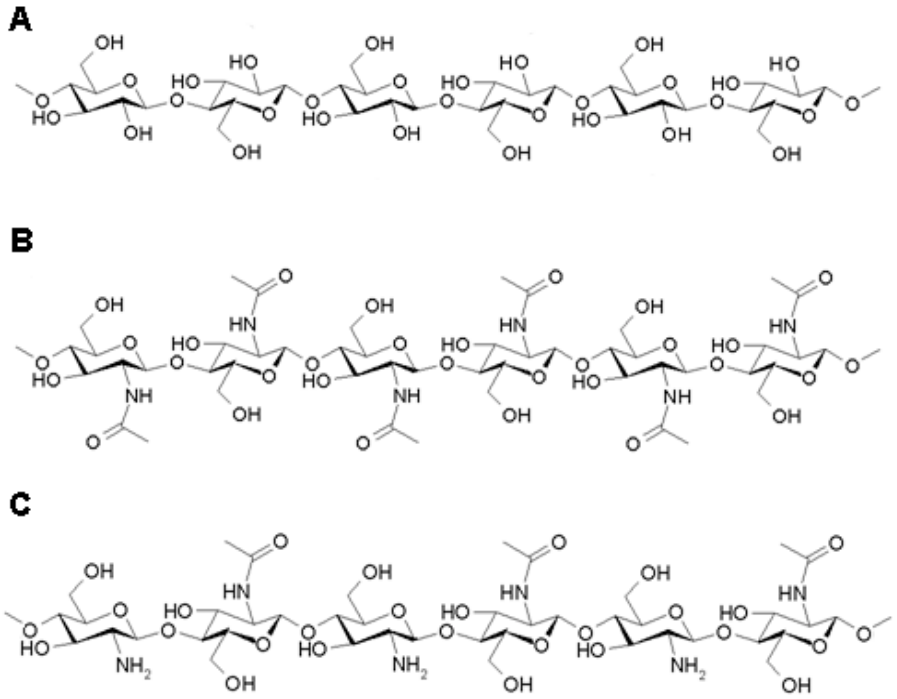

Figure 1. (A) Cellulose: β(1,4)-linked units of D-glucose (B) Chitin: β(1,4)-linked units of *N*-acetyl-D-glucosamine. (C) Chitosan: β(1,4)-linked units of *N*-acetyl-D-glucosamine and D-glucosamine residues. Note that the term chitosan is not precisely defined. Chitosan refers to soluble polymeric chitin derivatives with a degree of acetylation that may vary from 0 % to about 65 %.

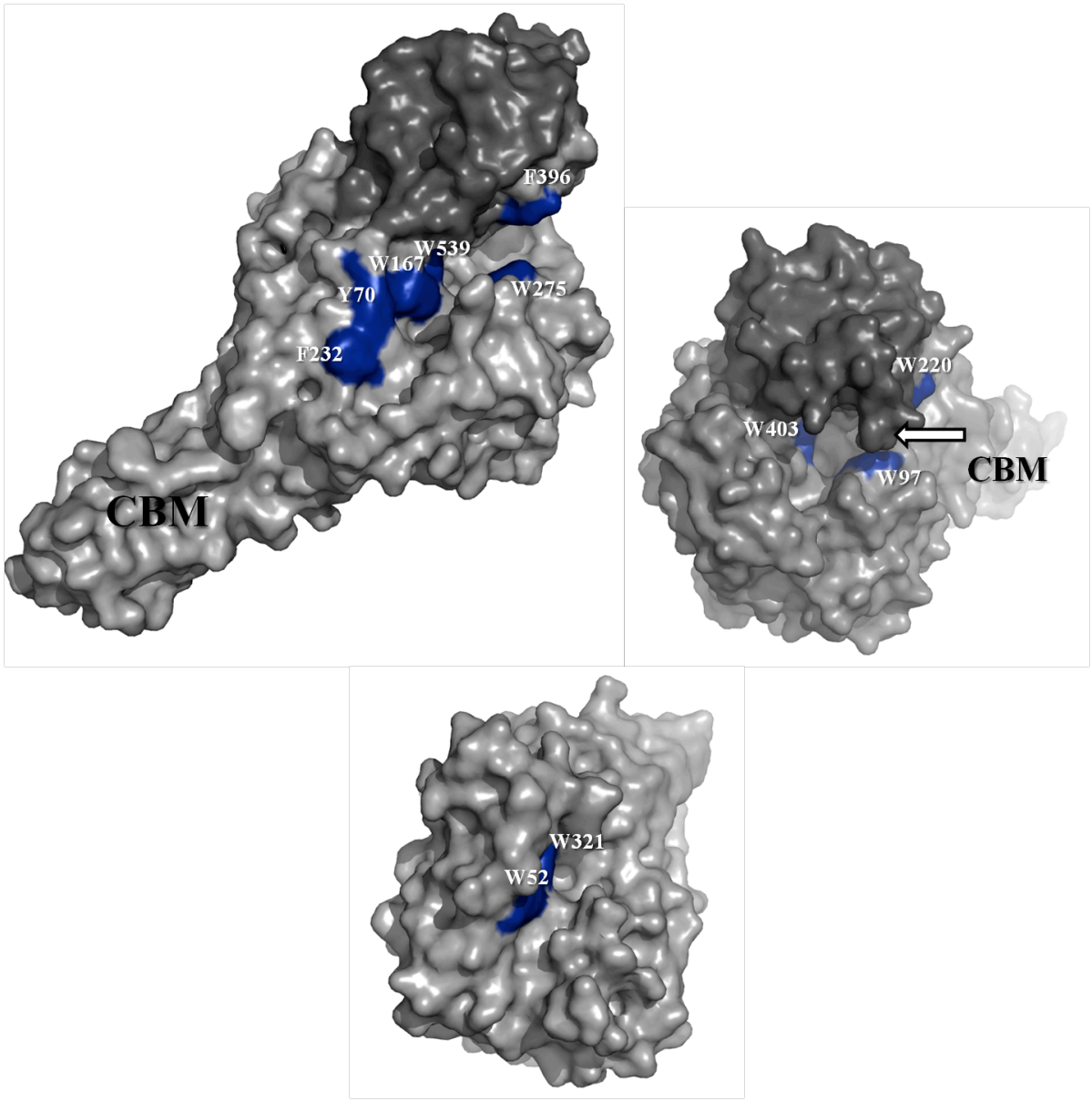

Figure 2. Crystal structures of ChiA from *S. marcescens* (top left; (Perrakis et al. 1994); pdb code 1ctn), ChiB from *S. marcescens* (top right; (van Aalten et al. 2000); pdb code 1e15), and the catalytic domain of ChiC from *L. lactis* (bottom; pdb code 3ian; this domain has 67 % sequence identity with ChiC from *S. marcescens*), The structures have been aligned by the position of their (conserved) catalytic centers, meaning that the substrate-binding clefts are shown in the same view. ChiA and ChiB contain the α+β insertion domain (a darker grey) and

have deep substrate binding clefts, while ChiC has a more shallow and open substrate-binding cleft. The side chains of up to six solvent exposed aromatic amino acids in equivalent structural positions are shown in blue. ChiA has an aromatic motif (Trp^{71}, Tyr^{34}, Trp^{31}) the in –6 to –3 glycon subsites and a Trp-Phe motif in the +1 and +2 aglycon subsites. ChiB has a Trp-Trp motif (Trp^{97}, Trp^{220}) in the +1 and +2 aglycon subsites. At the bottom of the –1 subsite, there is a fully conserved Trp in all family 18 chitinases (labeled W539 in ChiA, W403 in ChiB, and W321 in ChiC, respectively). Aromatic amino acids in the substrate-binding clefts are known to be important for substrate-binding (Uchiyama et al. 2001) and for a processive mode of action (Horn et al. 2006a; Zakariassen et al. 2009). Note the "roof" over that active site cleft in ChiB (indicated by an arrow). Both ChiA and ChiB contain a chitin-binding domain (indicated by "CBM"), with opposite orientations relative to the catalytic domain; this domain is clearly visible for ChiA but largely hidden for ChiB.

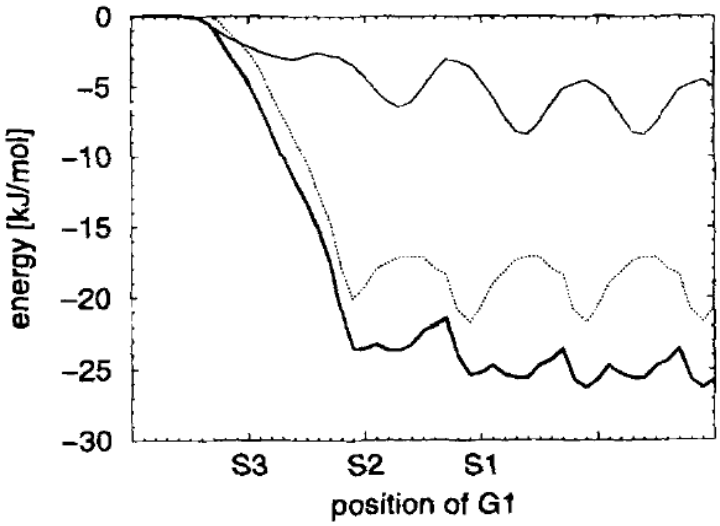

Figure 3. Energy profiles for a maltooligosaccharide sliding through the pore of maltoporin. The Figure is taken from Meyer and Schulz (1997). The hydrogen bonding energy profile is shown as a dotted line, the aromatic stacking energy profile is shown as a solid thin, and the total energy profile is shown as a solid thick line. S, observed binding site; G, glucose unit. Note that the energy minima of the aromatic stacking interactions tend to compensate the maxima of the polar interactions, resulting in a rather smooth total energy profile, which promotes processivity because high energy barriers are reduced (Meyer and Schulz 1997).

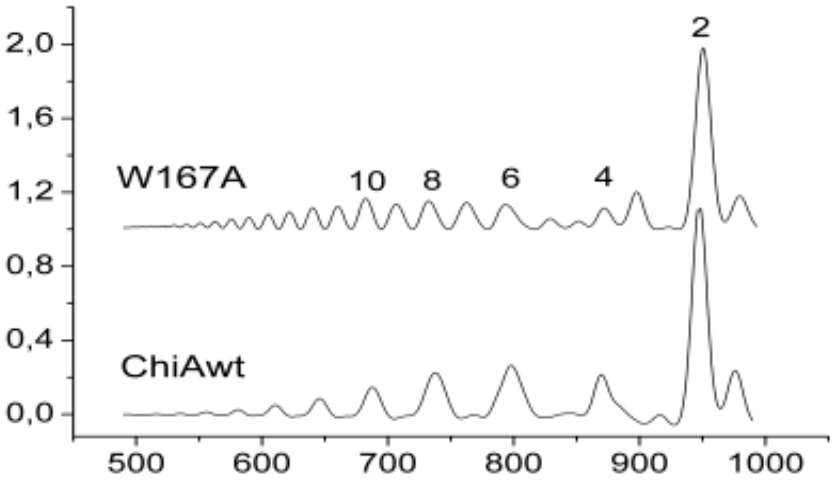

Figure 4. SEC analysis of products obtained after chitosan degradation by ChiA-WT and for ChiA-W167A. The product pattern for the wild-type indicates processivity; the pattern for W167A indicates loss of processivity.