Serratia marcescens Chitinases with Tunnel-Shaped Substrate-Binding Grooves Show Endo Activity and Different Degrees of Processivity during Enzymatic Hydrolysis of Chitosan†

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ABSTRACT: The modes of action of three family 18 chitinases (ChiA, ChiB, and ChiC) from *Serratia marcescens* during the degradation of a water-soluble polymeric substrate, chitosan, were investigated using a combination of viscosity measurements, reducing end assays, and characterization of the size-distribution of the oligomeric products. All three enzymes yielded a fast reduction in molecular weight of the chitosan substrate at a very early stage of hydrolysis, which is typical for endo-acting enzymes. For ChiA and ChiB, this is inconsistent with the previously proposed exo-attack mode of action. The main difference between ChiA, ChiB, and ChiC is the degree of processivity. ChiC is an endo enzyme with no apparent processivity. ChiA and ChiB are processive enzymes in which the substrate remains bound to the active cleft after successful hydrolysis and is moved along for the next hydrolysis to occur. ChiA and ChiB perform on average 9.1 and 3.4 cleavages, respectively, for the formation of each enzyme−substrate complex. ChiA and ChiB have deep, tunnel-like substrate-binding grooves. The demonstration of endo activity shows that substrate binding must involve the temporary restructuring of the loops that make up the roofs of the substrate-binding grooves, similar to what has been proposed for cellobiohydrolase Cel6A. The data suggest that the exo-type of activity observed for ChiA and ChiB during the degradation of solid crystalline chitin is due to the better accessibility of chain ends, rather than intrinsic enzyme properties.

Enzymatic depolymerization of soluble and crystalline polysaccharides is important both for living organisms and in industrial applications (*1*). The number of known genes thought to encode glycoside hydrolases currently exceeds 18 000 (*2*, *3*). Important depolymerizing glycoside hydrolases include cellulolytic enzymes capable of hydrolyzing both crystalline and amorphous cellulose (*4*, *5*), α-amylases (α-1,4-D-glucan glucanohydrolases), hydrolyzing starch and related polysaccharides (*6*), endopolygalacturonases, cleaving 1,4-α-D-galactosiduronic linkages in the smooth regions of pectin (*7*), agarases (*8*, *9*), carrageenases (*10*, *11*) and chitinases (see below). Generally, polysaccharide substrates can be degraded from one of the chain ends (exo attack) or from a random point along the polymer chain (endo attack). Each of these two mechanisms can occur in combination with a processive (multiple attack) (*4*, *12*, *13*) mode of action, meaning that the substrate is not released after successful cleavage but moves through the active site cleft for the next cleavage event to occur. Evidence for a sliding motion has recently been obtained for cellobiohydrolase Cel6A (*14*). It was shown that movement of the polymer chain is facilitated by extensive solvent-mediated interactions and through flexibility in hydrophobic surfaces provided by a sheath of tryptophan residues in the substrate-binding groove.

The endo-acting enzymes usually contain an open and extended substrate-binding cleft (*15*−*18*). Some exo-acting glycoside hydrolases bind the substrate to a well-defined pocket, where only binding involving the chain end is possible (*15*, *17*). A third category of depolymerizing glycoside hydrolases contains enzymes with deep active site grooves, which sometimes look like a tunnel (*19*). Enzymes with this architecture are usually thought to have an exo and/or processive mode of action (*20*) and include cellulases (*21*−*23*), carrageenases (*10*, *11*) and chitinases (see below).

Chitin, an insoluble and robust carbohydrate polymer of (1,4)-linked 2-acetamido-2-deoxy-β-D-glucose (GlcNAc or **A**-unit[1]), is an important structural component in a variety of organisms. By de-N-acetylation, chitin can be converted

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[1] Abbreviations: F_A, degree of acetylation; α, degree of scission; **A**, 2-acetamido-2-deoxy-β-D-glucose; **D**, 2-amino-2-deoxy-β-D-glucose; DP, degree of polymerisation; DP_n, number average degree of polymerisation; ChiA, chitinase A from *Serratia marcescens*; ChiB, chitinase B from *Serratia marcescens*; ChiC, chitinase C from *Serratia marcescens*; N_{cuts}, number of bonds cut for each enzyme substrate association; *pol*, polymer fraction; *olig*, oligomer fraction.

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to chitosan, a water-soluble copolymer of (1,4)-linked GlcNAc and 2-amino-2-deoxy-β-D-glucose (GlcN or **D**-unit). Chitosans are a family of copolymers that can be prepared with varying chemical compositions, that is, a fraction of **A**-units (F_A), with different chemical, physical and biological properties (*24*). F_A is defined as the molar concentration of acetylated units (**A**) divided by the total molar concentration of monomer units (**A** and **D**).

Microbes are capable of exploiting the chitin biomass through the production of chitinolytic enzymes as well as accessory chitin-disrupting proteins (*25*). For example, the Gram-negative soil bacterium *Serratia marcescens* produces three chitinases: ChiA, ChiB, and ChiC (*26*−*31*). All three chitinases belong to family 18 of glycoside hydrolases (*32*), which possess a $(\beta/\alpha)_8$ barrel catalytic domain with approximately six sugar-binding subsites (*33*−*37*). The enzyme hydrolyzes the glycosidic bond between sugar units bound to the −1 and +1 subsites. Hydrolysis by family 18 chitinases involves the *N*-acetyl group of the sugar located in the −1 subsite (substrate-assisted catalysis) (*38*−*41*), and as a consequence, productive substrate binding in all three enzymes requires an acetylated unit to be bound in the −1 subsite. Other subsites show less stringency in this respect, and it has been shown that ChiB from *S. marcescens* can degrade chitosans with F_A as low as 0.13 (*30*, *42*).

ChiA and ChiB both have deep tunnel-like active site grooves, which are extended by the surface of a chitin-binding domain located on the glycon and aglycon side of the catalytic center, respectively (*33*, *36*). Structural considerations (*15*, *30*) and various types of experiments have previously led to the suggestion that the two enzymes hydrolyze insoluble chitin in an exo-processive mode of action, in opposite directions (*42*−*44*). Experimental support for the exo mode of action comes from the results of microscopy studies of the degradation of β-chitin, which showed that chitin fibrils are degraded from the ends (*43*, *44*). It cannot be excluded, however, that the apparent exo action is due to the superior accessibility of the polymer chain ends rather than to intrinsic enzyme properties. Fiber shortening could also result from initial endo attacks near accessible ends, followed by processive action. Uchiyama et al. (*44*) noted that their observations were compatible with a processive mode of action. Using chitosans as a substrate, Sørbotten et al. (*42*) and Horn et al. (*30*) were able to show that, indeed, ChiA and ChiB act processively (Figure 1; see Figure legend for a detailed explanation of the chitosan experiments). Horn et al. (*30*) also showed that the degradation of chitosan with ChiA or ChiB resulted in the slow disappearance of the polymer fraction, whereas (nonprocessive) ChiC resulted in the fast disappearance of the polymer fraction (Figure 1), suggesting that ChiA and ChiB are exo-acting enzymes, whereas ChiC is an endo-acting enzyme. It was noted, however, that the results (Figure 1; (*30*)) were not conclusive because processive enzymes such as ChiA and ChiB can lead to a slow disappearance of the polymer fraction, regardless of whether the initial attack is endo or exo.

An intriguing question related to the mode of action of processive depolymerizing glycoside hydrolases with the tunnel-like architecture is how the enzymes initially bind to their substrates. One option, corresponding to an exo mode of action, is that the polymer is pulled into the active site groove/tunnel from one end. Another option, more compatible with an endo mode of action, is that the substrates enter the groove/tunnel from the top (that is, through the roof), which for some enzymes would involve temporary roof

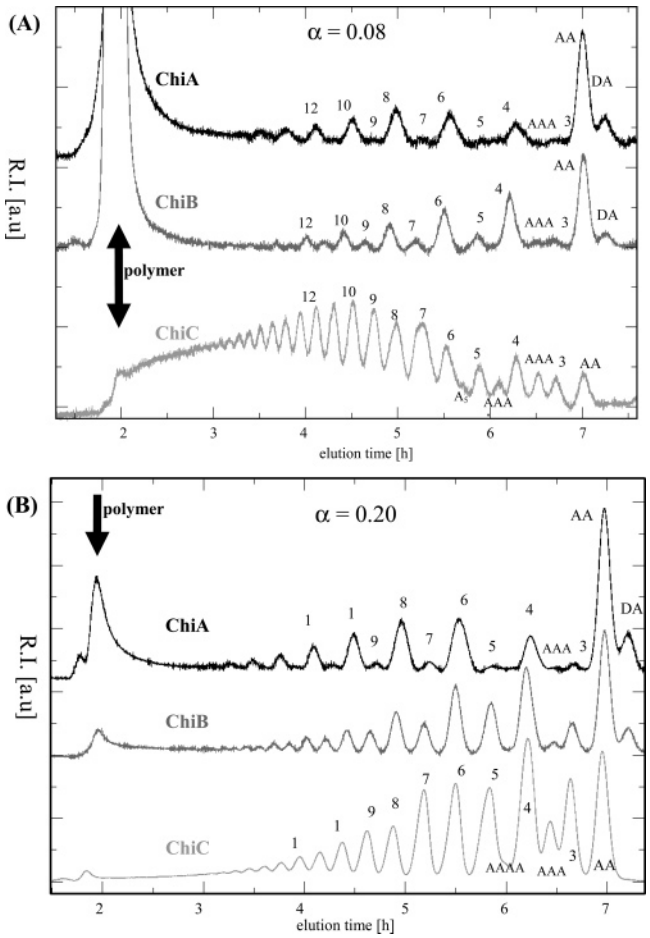

FIGURE 1: Size exclusion chromatography of products obtained after the degradation of chitosan (F_A = 0.65, DP_n = 800) with ChiA, ChiB, and ChiC. Data taken from ref 30 (*30*), where the experimental procedure is described in full (chitosan concentration 5 mg/ml; enzyme concentrations: 2.5μg/mL for ChiA, ChiB, and 1.5μg/mL for ChiC at pH 5.5; T = 37 °C). (A) Products at α = 0.08 (8% of all glycosidic bonds hydrolyzed). (B) Products at α = 0.20. The peaks are annotated according to their content, either by a sequence or by the length of the oligomer. The chromatograms for ChiA and ChiB show a slow disappearance of the polymer peak, which is often thought to be indicative of exo activity, but is shown here to be due to processivity. The dominance of even-numbered products show that ChiA and ChiB act processively, as discussed in detail in refs 30 and 42 (*30*, *42*). In summary, nonprocessive enzymes producing oligomers with DP >3−4 would produce oligomers of any given length, whereas the substrate in processive enzymes moves by 2 sugar units at the time because of the periodicity in the chitin/chitosan chain. Because some enzyme−substrate complexes formed along the processive pathway are nonproductive due to the binding of **D**-unit in the −1 subsite, longer oligomers, i.e., longer than two units, are formed and observed. In other words, the formation of nonproductive complexes does not necessarily lead to the dissociation of the enzyme−substrate complex; instead, processive movement continues and the next product to be cleaved off may thus be, e.g., 6, 8, or 10 sugar residues long. Note that these longer products still contain cleavable sequences, which are not explored during processive movement but which may be explored by rebinding of oligosaccharide products. The latter leads to the production of oligomers with an odd number of residues. The chromatograms for ChiC show the rapid disappearance of the polymer peak, relatively high content of longer oligomeric products, and no predominance of even-numbered products. This is diagnostic for a nonprocessive endo enyzme.

opening/restructuring. On the basis of structural data, the latter type of behavior has been suggested for cellobiohydrolase Ce16A from *Trichoderma reesei* (*45*, *46*) and for a carrageenase (*10*).

The extent of the hydrolysis reaction can be characterized by the degree of scission α, defined as the number of cleaved glycosidic linkages divided by the total number of glycosidic linkages. For example, $\alpha = 0.5$ for a sample containing only dimers, and $\alpha = 0.333$ for a sample containing only trimers. For polydisperse samples, $\alpha = DP_n^{-1}$, where DP_n is the number average degree of polymerization.

In this study, we have addressed the endo or exo character of the three *Serratia* chitinases by studying the degradation of water-soluble chitosan with a high F_A and a high molecular weight in the very early stages of the reaction (<1% of all bonds hydrolyzed, $\alpha < 0.01$). Thus, we could study the degradation of chitosan under conditions where the unhydrolyzed polymer chains still represent the dominating substrate and are not outnumbered by their degradation products. A careful analysis of the oligomeric products as well as changes in the molecular weight of the remaining polymeric substrate provides new insights into the mode of action of ChiA and ChiB, showing that these enzymes with their deep tunnel-like substrate binding grooves degrade the chitosan via an endo-processive mode of action.

MATERIALS AND METHODS

Preparation and Characterization of Chitosan. Chitin was isolated from shrimp shells by the method of Hackman (*47*) and milled in a hammer mill to pass through a 1.0-mm sieve. Chitosan was prepared by homogeneous de-N-acetylation of chitin (*48*). It has previously been shown that chitosans prepared in this way have a random distribution of **A** and **D** units (*49*−*51*). The chemical composition of chitosan, F_A, and the number average degree of polymerization DP_n were characterized by ^{1}H NMR spectroscopy (*49*) and by intrinsic viscosity measurements (*52*). DP_n was calculated from the Mark−Houwink−Sakurada (MHS) equation and measured intrinsic viscosity, $[\eta] = K(M_D(1 - F_A) + M_A F_A)\cdot DP_n)^a$, with $K = 1.23 \times 10^{-3}$, $a = 1.11$ (*53*), and the molecular weight of the monomer unit $M_A = 203$ gmol^{-1} (**A**-unit) and $M_D = 197$ gmol^{-1} (**D**-unit). As measured intrinsic viscosity depends on F_A, the values used for K and a had been previously determined for chitosan with $F_A = 0.65$ (*53*).

Size-Exclusion Chromatography (SEC). Details of the chromatographic separation of reaction products obtained upon the degradtion of chitosan have been described previously (*42*). In summary, the oligomers were separated on three columns in series, packed with Superdex 30 (Amersham Pharmacia Biotech; each with dimensions of 2.60 cm (i.d.) × 60 cm), using the previously described conditions (*42*). This allows for the separation of individual oligomers from the monomer up to 20 units, whereas oligomers with a DP larger than approximately 40 are eluted in the excluded (void) volume of the columns. Under these conditions, oligomers longer than 4 units elute in one peak, regardless of sugar composition, whereas shorter oligomers elute more than one peak, depending on sugar composition and sequence. (The peaks are appropriately labeled in the Figures; see ref 42 (*42*) for more information on peak assignment.)

Reducing End Assay. The concentration of the reducing ends in the reaction mixture was determined according to the method described by Horn and Eijsink (*54*). With this relatively new method, adapted from Anthon and Barrett (*55*), the signal per reducing end is independent of oligosaccharide length. Carefully dried samples of $(GlcNAc)_2$ were used as the standard.

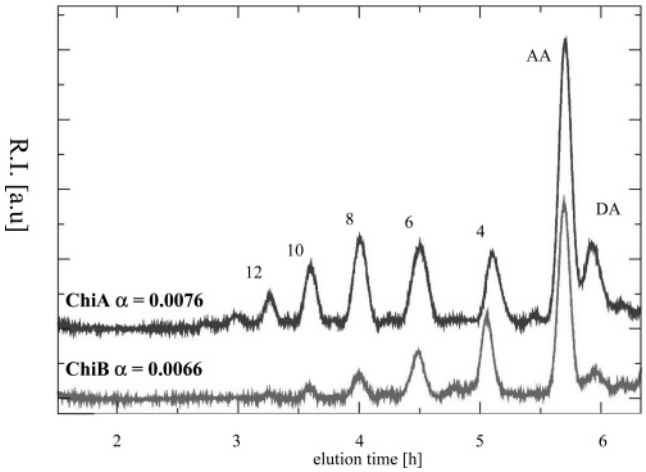

FIGURE 2: Size exclusion chromatography of low molecular weight products obtained after a very limited degradation of chitosan ($F_A = 0.65$, $DP_n = 800$) with ChiA or ChiB. Before analysis, the oligomer fraction was separated from the remaining polymeric material using dialysis. In similar reactions with ChiC, no low molecular weight products were found at all.

Preparation, Fractionation, and Characterization of Chitosan Hydrolyzed to a Very Low Extent. Chitosan (350 mg) ($[\eta] = 570$ mL/g for experiments with ChiA and ChiB and 660 mL/g for ChiC) with an F_A of 0.65 was dissolved to a concentration of 1 mg/mL in 8 mM HAc/NaAc at pH 5.5 containing 20mM NaCl. Chitosan was hydrolyzed by the addition of 17 ng/mL (experiments A1, B1, C1) or 34 ng/mL of enzyme (experiments A2, B2). After incubation at 37 °C for 45 min, the reactions were stopped by incubating at 90 °C for 5 min. The volume of the sample was then reduced to approximately 50 mL (from the starting volume of 350 mL) on a rotavapor and the pH lowered to 4.5. The sample was dialyzed (Medicell International Ltd., London U.K.; MWCO: 12−14000 Da) to separate the polymer and the oligomers for 6 days at 4 °C against distilled water (100 mL) that was changed every day (total volume of dialysates was 600 mL). The polymer fraction (contents inside the dialysis tubes) was lyophilized, and the oligomer fraction (the collected dialysates) were concentrated on a rotary evaporator, lyophilized, and stored for further analysis. The masses of the two fractions were used to calculate polymer and oligomer weight fractions w^{pol} and w^{olig}. Oligomer fractions (collected dialysates) were characterized by SEC chromatography, and the relative amounts of the oligomers (*DP* between 2 and 16; see Figure 2) were used to calculate the number average degree of polymerization (DP_n) of the oligomer fraction and the corresponding α^{olig}. The number average degree of polymerization of the polymer fraction and the corresponding α^{pol} were determined from measured intrinsic viscosities. The intrinsic viscosity was converted to the number average molecular weight (see above, (*53*)) from which the number average degree of polymerization (DP_n) of the polymer fraction (and corresponding α^{pol}) were determined.

Table 1: Analysis of Reaction Products Obtained upon the Degradation of Chitosan with ChiA, ChiB, and ChiC[a]

		before degradation		after degradation								
	c_{enz} [ng/mL]	$[\eta]$ (mL/g)	DP_n^{start}	$[\eta]$ (mL/g)	DP_n^{pol}	ΔDP_n	α^{pol} ($\times 10^{-3}$)	w^{pol}	α^{olig}	w^{olig} ($\times 10^{-2}$)	α ($\times 10^{-3}$)	N_{cuts}
A1	17	570	636	390	452	29%	2.2	0.990	0.30	1.0	5.2	6
A2	34	570	636	400	462	27%	2.2	0.983	0.33	1.7	7.7	10
B1	17	570	636	350	410	36%	2.4	0.995	0.35	0.5	4.2	3
B2	34	570	636	280	335	47%	3.0	0.989	0.34	1.1	6.7	4
C1	17	660	726	150	191	75%	5.3	1.00		0	5.2	1

[a] Reactions A1, B1, and C1 were performed with an enzyme concentration of $c_{enz} = 17$ ng/mL enzyme; reactions A2 and B2 were performed with $c_{enz} = 34$ ng/mL. Superscripts pol and olig indicate the polymer fraction and the oligomer fraction, respectively. $[\eta]$, measured intrinsic viscosity; DP_n^{pol}, number average degree of polymerization for the high molecular weight fraction (calculated from intrinsic viscosities); α, total extent of the reaction calculated using eq 1; w, mass fractions of the polymer and oligomer parts of the reaction mixture separated using dialysis; $w^{pol} + w^{olig} = 1$. N_{cuts}, number of cuts for each formation of the enzyme−substrate complex (calculated under the assumption that all attacks are endo).

Experiments with the Simultaneous Determination of Relative Viscosity and Reducing Ends. Chitosan ($F_A = 0.65$ and $DP_n = 700$) was dissolved in 40 mM acetate buffer (pH 5.5) containing 0.1 M NaCl (chitosan concentration: 1 mg/mL), to which the enzyme was added. The enzyme concentration was 66 ng/mL for ChiA and ChiB, and 6.6 ng/mL for ChiC. For the acid hydrolysis experiment, the same chitosan was dissolved in 0.14 M HCl. Hydrolysis reactions were performed as previously described (*57*) in a Schott-Gerate Ubbelohde capillary viscometer (type 531 01/Oa) immersed in a thermostated water bath, and the relative viscosities were monitored with an AVS-310 (Schott-Gerate) control unit at regular intervals. Samples from the viscosity experiment were withdrawn at regular intervals, and the concentration of reducing ends was determined using the reducing end assay (*54*). The number average degree of polymerization of the polymer fraction and the corresponding α^{pol} were determined from the decrease in relative viscosities that were converted to intrinsic viscosities assuming that reduced viscosity (i.e., the specific viscosity divided by the chitosan concentration) is equal to the intrinsic viscosity. The intrinsic viscosity was converted to the number average molecular weight (*53*) from which the number average degree of polymerization (DP_n) of the polymer fraction (and corresponding α^{pol}) was determined. The data presented below represent the average of three measurements.

RESULTS AND DISCUSSION

Products Formed at Very Low Extent of Hydrolysis: Endo versus Exo Activity. At the initial stage of degradation by a processive enzyme, the reaction mixture contains two fractions, one containing low molecular weight oligomers (mostly dimers) produced in the processive type of hydrolysis ($DP_n^{olig} \ll DP_n^{start}$) and the other containing the partially hydrolyzed high molecular weight substrate ($DP_n^{pol} \leq DP_n^{start}$).

To investigate the products formed at a very low degree of scission ($\alpha < 0.01$) by all three enzymes, chitosan was hydrolyzed to a very low extent (Table 1), and the reaction products were separated into high and low molecular weight fractions using dialysis. The oligomer size distribution was then analyzed using size exclusion chromatography, whereas the number average degree of polymerization of the high molecular weight fraction (DP_n^{pol}) was determined from viscosity measurements (*52*). Typical chromatograms of the low molecular weight fractions obtained for ChiA and ChiB are shown in Figure 2, whereas no oligomeric products could be detected with ChiC. (During the initial phase of the reaction, ChiC primarily produces very long oligomers that elute in or close to the polymer peak; see ref 30 (*30*).) The results are summarized in Table 1. The overall degree of scission (α) was calculated using eq 1 and the data in Table 1, where w^{pol} and α^{pol} are the weight of the fraction and the degree of scission for the high molecular weight products, and w^{olig} and α^{olig} are the weight of the fraction and the degree of scission for the low molecular weight products.

$$\alpha = \alpha^{olig} w^{olig} + \alpha^{pol} w^{pol} \quad (1)$$

The calculated degrees of scission are very low ($\alpha < 0.01$, Table 1). At these low α-values and for an exo-acting enzyme, one would expect only a very small reduction (<1%) in the number average degree of polymerization of the partially degraded polymeric substrate (*56*). For example, the formation of one dimer from each of the polymer chains would reduce DP_n^{pol} by 0.3% (starting $DP_n^{pol} = 600$, final $DP_n^{pol} = 598$). At the same time, the number of chain ends would be doubled, increasing the α-value from 1.7×10^{-3} ($= 1/DP_n = 1/600$) to 3.3×10^{-3} ($DP_n = 300$, meaning that $\alpha = 1/300$). However, as seen in Table 1, a sharp decrease in the chain length of the high molecular weight fraction (DP_n^{pol}) is observed for all three enzymes. This reduction is as high as 29−47% for ChiA and ChiB (depending on the experiment; Table 1) at time points during the reaction where the increase in α is as low as a factor five at most. The reduction in DP_n^{pol} for ChiA and ChiB is in fact in the same order of magnitude as that for the endo enzyme ChiC (75% reduction). These results are inconsistent with the previously proposed exo-processive mode of action for ChiA and ChiB and suggest that these two enzymes act in an endo fashion. Because of processivity, ChiA and ChiB produce significant amounts of oligomeric products at the initial stage of the reaction, whereas the nonprocessive endo enzyme ChiC does not. Thus, the results show that ChiA and ChiB act in an endo-processive fashion and confirm (see ref 30 (*30*)) that ChiC acts in an endo-nonprocessive fashion.

The data allow us to calculate the number of cuts for each enzyme substrate asociation N_{cuts}, representing the degree of processivity (eq 2, $\Delta\alpha$ and $\Delta\alpha^{pol}$ are changes in α and

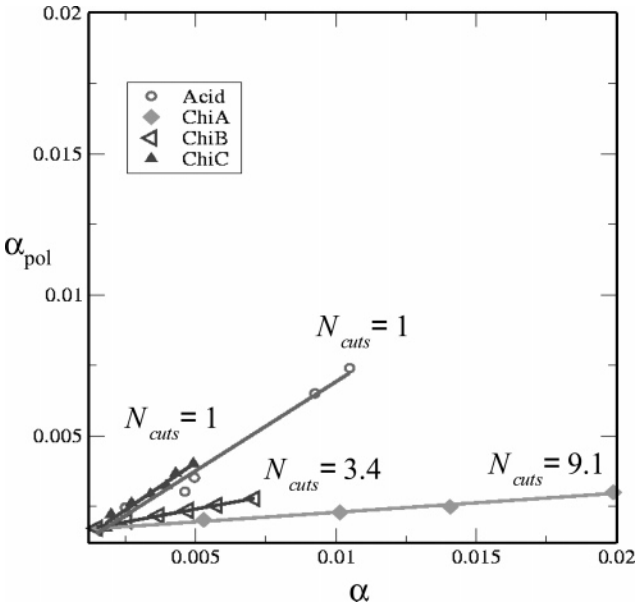

FIGURE 3: Changes in α^{pol} as a function of the reaction extent α. See text for a description and explanation of the calculation of the N_{cuts} values.

α^{pol}, respectively during the reaction[2]).

$$N_{cuts} = \frac{\Delta\alpha}{\Delta\alpha^{pol}} \quad (2)$$

The data obtained indicate that on this substrate ChiA is more processive with between 6 and 10 cuts for each enzyme−substrate formation compared to ChiB with between 3 and 4 cuts (Table 1). The degree of processivity was measured more accurately using a different experimental approach, which is described below.

Detailed Investigation of Processivity. To measure the amount of processive steps made by ChiA and ChiB more accurately, a series of independent experiments were performed. The total fraction of chain ends created in the enzymatic reaction (α) was monitored using a reducing end assay (*54*, *55*). An on line relative viscosity assay, sensitive only to changes in the high molecular weight fraction of the substrate, was used to measure α^{pol} during the course of the hydrolysis reaction.

For degradation reactions proceeding via a random nonprocessive endo mechanism, for ChiC (see above and ref 30 (*30*)) and for (nonenzymatic) acid hydrolysis (*57*), one would expect the α-values determined by the reducing end and the viscosity assays to be the same. However, if the polymer is degraded by a processive (endo or exo) mechanism or according to a nonprocessive exo mechanism, the total α-values from the reducing end assay should exceed the α-values determined from the viscosity assay (α^{pol}) because the cleavage of a small fragment (such as a dimer) from either end of a long molecule would only marginally reduce the viscosity of the solution. Figure 3 shows the degree of scission determined from the viscosity assay (α^{pol}) plotted against the degree of scission determined from the reducing end assay (α). For all experiments (ChiA, ChiB, ChiC, and acid hydrolysis), a linear relationship between α^{pol} and α was observed. The slopes (*s*) of the linear plots are directly related to the average number of cuts after the formation of an enzyme−substrate complex (eq 2). For acid hydrolysis, the slope is expected to be 1 ($s = 1$, no processivity). Assuming that ChiC is a nonprocessive endo enzyme, the slope for ChiC would also be expected to be 1. Indeed, data for the acid and ChiC hydrolysis show very similar slopes (0.63 and 0.67, respectively) of the linear relationship between α^{pol} and α but with a deviation from the expected value of 1. This discrepancy could be explained by an underestimation of the change in the molecular weight of the polymeric substrate using the viscosity assay. Also, the reduction in viscosity is converted to a reduction in DP_n using the MHS equation (see Materials and Methods), which might introduce further uncertainty in determined DP_n. It is also possible that the results from the reducing end assay used to determine the α-values of chitosan (with the chitin dimer as standard) deviate from the true reducing end concentrations. The fact that acid hydrolysis and hydrolysis by the nonprocessive endo enzyme ChiC yield almost identical slopes adds confidence to the data. Because acid hydrolysis by definition should give $s = 1$, we can apply a correction to the data obtained for ChiA and ChiB. Table 2 summarizes the experimental results and includes N_{cuts} values calculated both with and without slope correction. The data show that on average ChiB makes 3.4 cuts for each formation of the enzyme−substrate complex, whereas the experiment summarized in Table 1 suggested a similar value of 3−4. ChiA shows higher processivity and makes 9.1 cuts, whereas the experiment summarized in Table 1 suggests that this value lies between 6 and 10.

Table 2: Processivity as Derived from the Data Shown in Figure 3[a]

			corrected	
	s	N_{cuts}	*s*	N_{cuts}
acid hydrolysis	0.63	1.6	1.0	1.0
ChiA	0.07	14.3	0.11	9.1
ChiB	0.18	5.6	0.29	3.4
ChiC	0.67	1.5	1.1	0.9

[a] The table shows two datasets, one without and one with a correction based on the results of acid hydrolysis (see text). ***s***, slope of the curves shown in Figure 3; N_{cuts}, the average number of cuts per formation of an enzyme−substrate complex (1/*s*).

To fully illustrate that the observed changes in DP_n^{pol} are inconsistent with the exo mode of action, the experimental data was also plotted in a different way. Figure 4 shows changes in the DP_n^{pol} of the reaction mixture as a function of the reaction extent α. For ChiA and ChiB, the observed reduction in DP_n^{pol} is much larger than what is predicted for enzymes acting in an exo-processive mode of action, as predicted with a model for chitosan hydrolysis by ChiB (*58*); see below.

Mechanism of the Hydrolysis Reaction. The above results for ChiA and ChiB provide no support for an exo mode of action and show that when hydrolyzing soluble substrates ChiA and ChiB operate in a processive endo-attack mode of action. One remaining question then is how oligomers with odd number of residues (Figure 1) are produced. Three possible mechanisms are considered. (I) Both enzymes show a mixed exo/endo mode of action, with the exo attack creating oligomers with odd number of residues. This mecha-

[2] For each cut in the polymer part, the total number of chain ends will increase by $\Delta^{End} = N_{cuts}\,\Delta^{End}_{pol}$, where N_{cuts} is the average number of cuts for each enzyme−substrate complex formation. Therefore, $N_{cuts} = \Delta^{End}/\Delta^{End}_{pol} = \Delta\alpha\,/\Delta\alpha_{pol}$.

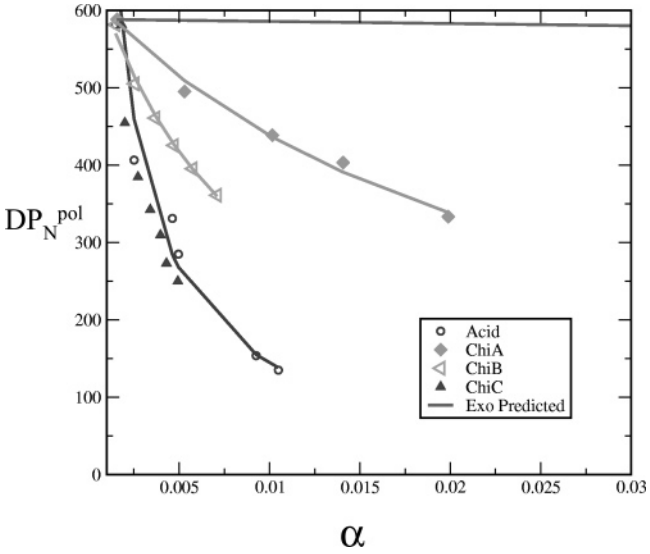

FIGURE 4: Changes in the DP_n^{pol} as a function of the reaction extent α. The changes observed for ChiA and ChiB are much larger than what is predicted for an exo-acting enzyme (prediction using the ChiB model described previously (*58*)).

nism is unlikely because the observed number of endo attacks (reduction in DP_n^{pol}, Table 1) combined with the degree of processivity and the observed total degree of hydrolysis (α) show that the number of exo attacks is much lower than what would be needed to produce the levels observed in Figure 1A (the results of additional experiments and modeling studies not shown). (II) In the processive mode of action, ChiA and ChiB can move along the polymer chain by an odd number of sugar units. This would, however, require a rotation of the substrate by 180° along the chain axis for the glycosidic bond (e.g., to bring the catalytically essential *N*-acetyl group of the −1 sugar to the correct position). As mentioned in the introduction and discussed in more detail elsewhere (*58*), this is unlikely for a high molecular weight substrate. In addition, if processive movement by an odd number of units is possible, this should occur at both low and high α-values. Consequently, the relative amounts of oligomers with an odd number of residues observed at a very early stage of the reaction (Figure 2, Figure 1a) should be similar to what is seen later on (Figure 1b; (*30*)), which clearly is not the case. (III) The oligomers with an odd number of residues are created by rehydrolysis of longer even-numbered oligomers created earlier on in the reaction. Even-numbered oligomers produced by processive enzyme action contain cleavable sequences inaccessible to the enzymes during processive action because of the inability of the enzymes to move between productive complexes by an odd number of sugar units. Thus, the rebinding of even-numbered oligomers may be productive, and this will predominately lead to the formation of oligomers with an odd number of residues. A detailed analysis of the hydrolysis products has shown that for ChiB, at $\alpha = 0.08$ (Figure 1A), already more than 10% of the polymer substrate (by weight) has been hydrolyzed into oligomers. The molar concentration of short chain products is already much higher than the concentration of the polymeric partially hydrolyzed substrate and therefore, the rehydrolysis of products may be expected. In dilute solution, the polymer chains will occupy a small fraction of the total volume; thus, the probability of a hydrolysis reaction will still be proportional to the molar concentration of

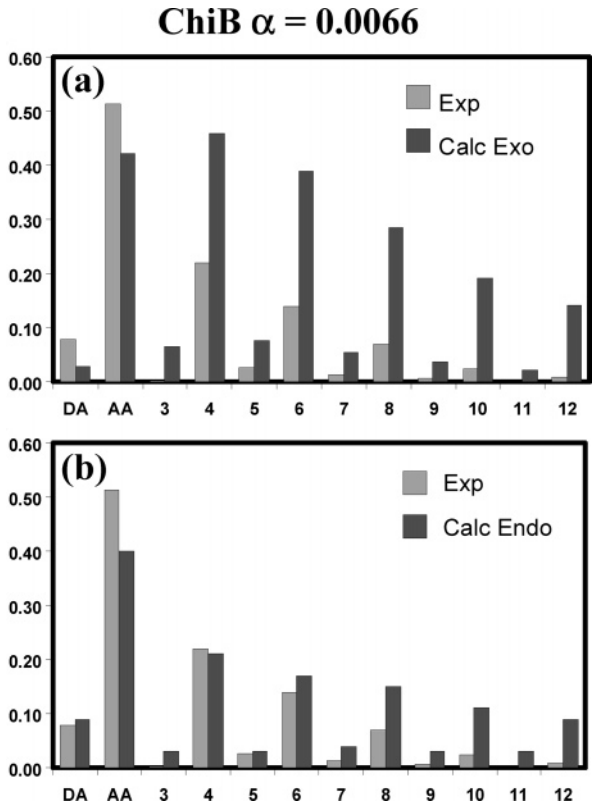

FIGURE 5: Comparison between observed and predicted reaction products for ChiB hydrolysis of chitosan with $F_A = 0.65$ and $\alpha = 0.0066$; (A) predicted by exo processive mode of action; (B) predicted by endo processive mode of action. Exp, observed; Calc Exo, predicted for the exo enzyme; Calc Endo, predicted for the endo enzyme.

polymer chains (as it is for an exo enzyme, where this probability is proportional to the concentration of chain ends) rather than to the molar concentration of monomers.

Modeling of the Hydrolysis Reaction. On the basis of the previously assumed exo-processive mode of action, we have previously constructed a model to predict oligomeric products (chain-length distribution and composition) from the hydrolysis of chitosan by ChiB (*58*). Using a Monte Carlo approach, the affinities of individual enzyme subsites for acetylated and deacetylated sugar units were refined by minimizing the difference between observed and predicted amounts of hydrolysis products obtained upon the degradation of chitosan with $F_A = 0.65$. The datasets used to develop and assess the model included data for α-values in the range from $\alpha = 0.05$ to $\alpha = 0.38$. In the model, the hydrolysis reaction could only start from a chain end. The final model yielded reasonable predictions of oligosaccharide production during the course of hydrolysis reactions with various chitosans as well as chito-oligosaccharides (*58*). Taking into account the results described above, we have now revised the model by removing the restrictions with respect to the initial binding mode (meaning that binding de facto becomes endo because there is a large surplus of endo-binding sites). Figure 5 shows that the refined model is much better than the old model in predicting the outcome of the reaction at very low α (<0.01), confirming that indeed the enzyme primarily binds in the endo mode. At higher α, the two models yield results that are similar (not shown), explaining why the old model was found to work well, despite the error

with respect to exo versus endo attack. The details of the updated model for ChiB as well as models for ChiA and ChiC that address subsite interaction parameters and the degree of processivity will be published elsewhere.

ChiA and ChiB and Chitin Hydrolysis. Our experimental data show that ChiA and ChiB act as processive endo enzymes when hydrolyzing water-soluble chitosan with a high F_A. However, there is evidence that these two enzymes, while acting on insoluble crystalline β-chitin, start hydrolysis from chain ends in the end face of the crystallite (*43*, *44*). It is thus possible that ChiA and ChiB behave differently with insoluble chitin than with water-soluble chitosan. However, it is not certain whether the action on the end faces of the crystallite (observed by electron microscopy of the crystallite) reflects the exo activity of the enzymes or whether it simply reflects the fact that the end face is much better accessible as a substrate to the enzyme. Interestingly, recent studies have shown that the disruption of the crystalline structure and (lack of) accessibility of the substrate are major limiting factors of the speed at which chitinases can degrade crystalline substrates (*25*, *59*).

CONCLUDING REMARKS

Using a water-soluble but highly acetylated high molecular weight chitosan and a combination of viscosity and reducing end assays, direct experimental data on the modes of action of three chitinases from *Serratia marcescens* have been obtained. Viscosity measurements show that all three enzymes significantly reduce the number average degree of polymerization of the soluble polymeric substrate at a very early stage of the hydrolysis reaction. This is consistent with an endo mode of action and not with an exo mode of action, which was previously suggested for ChiA and ChiB. Our data do not exclude the possibility that ChiA and ChiB also use some degree of exo binding. However, the main difference between ChiA, ChiB, and ChiC is the degree of processivity. With chitosan as the substrate, ChiA performs on average 9.1 cleavages and ChiB 3.4 cleavages for each enzyme−substrate complex formation. In the case of ChiC, the enzyme−substrate complex dissociates after each hydrolysis event. In the case of ChiA and ChiB, oligomers with an odd number of residues are produced by rehydrolysis of even-numbered oligomers produced in an earlier stage of the enzymatic reaction.

Detailed and quantitative descriptions of the processivity and binding modes of enzymes acting on insoluble carbohydrate polymers are challenging. For family 18 chitinases, the water-soluble chitosan substrate provides a unique possibility to directly observe and quantify the degree of processivity. In addition, our experiments show that, in the case of processive enzymes, the characterization of the changes in the high molecular weight fraction of the polymeric substrate is essential to delineate between exo and endo activity. An analysis of only the low molecular weight products as a function of reaction extent cannot distinguish between these two types of reaction mechanisms. It should be noted that the degree of processivity is likely to depend on the degree of acetylation of chitosan. Because acetylated units are important for catalysis, a lowering of F_A will result in increased average product length (*42*, *58*) and in an increased length of the path that needs to be traversed by a bound polymeric substrate between consecutive hydrolytic events. The longer the path, the larger the chance that the substrate dissociates from the enzyme somewhere along the way, which leads to reduced processivity. Further work is needed to analyze this in detail.

The results with chitosan clearly show that the polymeric substrate can enter the deep substrate clefts in ChiA and ChiB from the top, that is, through the roof of the tunnel. In ChiA, this roof is rather open, even in the enzyme−substrate complex (*60*), but ChiB, the free enzyme, and even more so the enzyme−substrate complex (*33*, *41*), show two long loops, which protrude from each side of the substrate-binding groove and which interact to close the roof of the tunnel. Therefore, in particular for ChiB, substrate binding must involve the temporary reorganization of roof structure, as has been suggested for processive cellulases (*18*, *61*) and carrageenases (*10*).

A single chitin binding domain (ChBD) is present both in ChiA and ChiB, and these domains are located on opposite sides of the active site. It has been suggested that the ChBDs contribute to exo binding, at the reducing and nonreducing ends (*33*, *43*) and that they may determine the direction of processivity (*43*). The results presented above indicate that the ChBDs are not involved in exo binding. These domains may, however, be involved in steering the direction of processivity. To date, no direct experimental data on the directionality of the processive motion for soluble substrates is available, and for an endo processive enzyme, this type of information cannot be obtained from simple end-labeling experiments.

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