Processive glycoside hydrolases are the key components of enzymatic machineries that decompose recalcitrant polysaccharides, such as cellulose and chitin. The intrinsic processivity ($p_{\text{intr}}$) of cellulosomes has been shown to be governed by the rate constant of dissociation from polymer chain ($k_{\text{off}}$). However, the reported $k_{\text{off}}$ values of cellulosomes are strongly dependent on the method used for their measurement. Here, we developed a new method for determining $k_{\text{off}}$, based on measuring the exchange rate of the enzyme between a non-labeled and a $^{14}$C-labeled polymeric substrate. The method was applied to the study of the processive chitinase Cha from Serratia marcescens. In parallel, Cha variants with weaker binding of the N-acetylglucosamine unit either in substrate-binding site $-3$ (Cha-W167A) or the product-binding site $+1$ (Cha-W275A) were studied. Both Cha variants showed increased off-rates and lower apparent processivity on $\alpha$-chitin. The rate of the production of insoluble reducing groups on the reduced $\alpha$-chitin was an order of magnitude higher than $k_{\text{off}}$, suggesting that the enzyme can initiate several processive runs without leaving the substrate. On crystalline chitin, the general activity of the wild type enzyme was higher, and the difference was magnifying with hydrolysis time.

On amorphous chitin, the variants clearly outperformed the wild type. A model is proposed whereby strong interactions with the substrate-binding region is extended to the carbohydrate binding module (CBM), resulting in a total of 13 substrate-binding sites (high pushing potential) are required for the removal of obstacles, like disintegration of chitin microfibrils.

Structural polysaccharides such as cellulose (the homopolymer of $\beta$-1,4-linked glucose units) and chitin (the homopolymer of $\beta$-1,4-linked N-acetylglucosamine (NAG) units) are abundant sources of renewable carbon. Their enzymatic depolymerization is an important route to products of high value, which is important for chemical industry. Both cellulose and chitin have evolved to crystalline structures that make them recalcitrant to enzymatic degradation. Likewise, there are remarkable structural and functional similarities in enzymatic machineries employed in degradation of cellulose and chitin in nature. Therefore, the lessons learned from chitinase research have turned out to be useful in dissecting the mechanism of cellulose hydrolysis and vice versa. The best characterized enzymatic machineries of recalcitrant polysaccharide degradation are the cellulolytic system of the fungus Trichoderma reesei (1) and the chitinolytic system of the bacterium Serratia marcescens (4). The key components of both enzyme systems, the cellobiohydrolase TrCel7A of T. reesei and the chitinase Cha of S. marcescens, are processive enzymes that move toward the non-reducing end of the polymer and produce disaccharides as major products. Their processive abilities are governed by multiple interactions with consecutive monomer units along polymer chains and more or less closed architectures of active sites. The active site of Cha rests in a deep cleft containing four substrate ($-4$ to $-1$) and three product ($+1$ to $+3$) NAG unit binding sites in the catalytic domain ($5$–$8$). Furthermore, the substrate binding region is extended to the carbohydrate binding module (CBM), resulting in a total of 13 substrate-binding sites ($6$, $9$). The long chitin binding cleft is lined with aromatic residues, mostly Trp and Phe (Fig. 1). The hydrophobic interactions form a flexible sheath necessary for substrate recognition and sliding of the polymer chain between successive glycosidic bond cleavages. It has been shown that the replacement of the single Trp in the binding site $-3$ to Ala (W167A) drastically reduces the processivity of Cha in the hydrolysis of chitosan, a soluble derivative of chitin (7). The same has been demonstrated with another chitinase of S. marcescens, ChaB (10).
Off-rates and Processivity of Chitinases

Materials—Crab chitin (Sigma C7170), chitobiase (Sigma D1523), chitosan, 4-methylumbelliferyl β-diacylchitobioside (MU-NAG₂) hydrate (Sigma M9763), anthranilic acid (AA), sodium cyanoborohydride, sodium borohydride, and bovine serum albumin (BSA) were purchased from Sigma. The scintillation mixture was purchased from Merck. All chemicals were used as purchased.

Enzymes—S. marcescens ChiA and its variants ChiA-W167A and ChiA-W275A were produced and purified as described before (7, 32). Chitobiase was expressed in Escherichia coli and purified by ammonium sulfate precipitation and ion-exchange chromatography as described previously (33).

Chitin Substrates—Crystalline α-chitin was prepared from crab chitin. The chitin was suspended in water and incubated in 0.55 M HCl for 2 h at room temperature with three changes. After three washes with water, the chitin was incubated with 0.3 M NaOH at 70 °C for 3 h with three changes followed by several washes with water. Next, the chitin was washed with ethanol and incubated in acetone for 1 h with two changes at room temperature. Finally, the purified chitin was washed repeatedly with water and ground in a mortar. The purified chitin was N-acetylated with acetic anhydride. For that, chitin was washed three times with methanol and finally resuspended in methanol to give the chitin a concentration of 20 mg ml⁻¹. 1 ml of acetic anhydride was added per 1 g of chitin, and the mixture was incubated overnight at room temperature, with stirring. Next, O-deacetylation was carried out by adding 100 mm KOH in methanol and incubating for 4 h at room temperature, with

Reduced processivity of the ChiA variants was accompanied by better performance on chitosan. In contrast, the activity on crystalline chitin was poor, indicating the importance of processivity in the crystalline substrate degradation (7, 11). The reduction of processivity upon changing Trp to Ala in sub-
stirring. After that, the chitin was washed repeatedly with water and 50 mM sodium acetate, pH 6.1. Finally, 0.01% NaN₃ was added, and the chitin was stored at 4 °C.

Chitin nanowhiskers (CNWs) and 14C-labeled chitin nanowhiskers (14C-CNWs) were prepared by HCl treatment of crab chitin as described previously (34). The specific radioactivity of the 14C-CNW preparation was 4.18 × 10⁶ dpm mg⁻¹.

Amorphous chitin was prepared by the acetylation of chitosan. Chitosan was suspended in water; an equal volume of 20% acetic acid was slowly added, with stirring, and the mixture was diluted five times by slowly adding methanol, with stirring. 1 ml of acetic anhydride was added per 1 g of chitin, with stirring, and the mixture was incubated overnight at room temperature, without stirring. Next, the mixture was diluted five times by slowly adding water and stirring. The acetic acid was neutralized, and the O-deacetylation was carried out by adding NaOH to a final concentration of 50 mM and incubated overnight at room temperature, with stirring. The amorphous chitin was repeatedly washed with water and 50 mM sodium acetate, pH 6.1. Finally, 0.01% NaN₃ was added, and the chitin was stored at 4 °C.

Reduced chitin was prepared of α-chitin by NaBH₄ treatment. The chitin was washed twice with 0.25 M NaHCO₃/Na₂CO₃, pH 10, and resuspended in the same buffer to give the chitin a concentration of 2 mg ml⁻¹. The mixture was heated to 80 °C, and 5 mM sodium borohydride in 0.1 M NaOH was added to give a final concentration of 25 mM sodium borohydride followed by 1 h of incubation. The same amount of 5 mM sodium borohydride in 0.1 M NaOH was added four more times with 1 h of incubation at 80 °C after each addition. Next, an equal volume of 0.5 M acetic acid was added, and the mixture was incubated overnight at room temperature, with stirring. The reduced chitin was washed repeatedly with water, and 50 mM sodium acetate, pH 6.1, 0.01% NaN₃ was added and stored at 4 °C.

**Substrate Exchange Experiment—CNWs (2 mg ml⁻¹) or α-chitin (4 mg ml⁻¹) was incubated with 20 nm ChiA or its variants and 20 mM chitobiose, in 50 mM sodium acetate, pH 6.1, supplemented with BSA (0.2 mg ml⁻¹) at 25 °C, with stirring in the case of α-chitin. After the desired time, an equal volume of 14C-CNWs (2 mg ml⁻¹) in 50 mM sodium acetate, pH 6.1, was added and further incubated at 25 °C. After the selected times, aliquots were withdrawn and stopped by adding NaOH to 0.2 M. 3 g liter⁻¹ CNWs was added; chitin was sedimented by centrifugation (5 min at 10⁴ × g) and the amount of radioactivity in the supernatant was measured using a liquid scintillation counter. To make the reference curves, 14C-CNWs were mixed with CNWs or α-chitin, and the reaction was started by adding ChiA or its mutants. For t = 0, CNWs or α-chitin and 14C-CNWs were mixed, and NaOH was added before ChiA. The released radioactivity was converted to the concentration of 14C-chitobiose using a previously constructed calibration curve (34).

**Chitin Hydrolysis and Binding of Chitinases—**α-Chitin (4 mg ml⁻¹) was incubated with 20 nm ChiA or its variants and 20 mM chitobiose in 50 mM sodium acetate, pH 6.1, supplemented with BSA (0.2 mg ml⁻¹) at 25 °C, with stirring. After the selected times, aliquots were withdrawn, filtered through Whatman GF-D filters, and centrifuged for 5 min at 10⁴ × g. The amount of reducing groups in the supernatant was measured using the 3-methyl-2-benzothiazoline hydrzone hydrochloride (MBTH) method, as described previously (35). The amount of free chitinase was measured by MU-NAG₂ hydrolysis assay. For that, 100 μl of supernatant was incubated with 5 μM MU-NAG₂ at 25 °C. After the selected times, the reaction was stopped by adding NaOH to the final concentration of 10 mM. The reaction was diluted with 0.1 M ammonium hydroxide, and the concentration of released 4-methylumbelliferone was determined by fluorescence. The excitation and emission wavelengths were set to 360 and 450 nm, respectively. The concentration of free chitinase was calculated from the rate of MU-NAG₂ hydrolysis using appropriate reference curves. The MU-NAG₂ (5 μM) hydrolyzing activity (v/E₀) of ChiAWT, ChiA-W167A, and ChiA-W275A was 1.34 ± 0.03, 0.050 ± 0.005, and 0.0044 ± 0.0003 s⁻¹, respectively. The concentration of chitin-bound chitinase was found as the difference between the concentration of total and free chitinase.

**Chitobiose Inhibition of Chitinases—**The chitobiose inhibition of chitinase variants ChiA-W167A and ChiA W275A was measured on 14C-CNWs essentially as described previously (34). Inhibition was tested at 14C-CNW concentrations of 0.1, 0.25, and 1.0 mg ml⁻¹. Concentration of chitobiose varied between 0 and 5 mM.

**Measuring Initial Rates—**α-Chitin, CNWs, or amorphous chitin (0.05–3 mg ml⁻¹) were incubated with 20 nm ChiA or its variants in 50 mM sodium acetate, pH 6.1, supplemented with BSA (0.1 mg ml⁻¹) at 25 °C for 1 min, without stirring. The reaction was stopped by adding NaOH up to 0.2 M. For t = 0, NaOH was added before ChiA. Chitin was sedimented by centrifugation (5 min at 10⁴ × g) after which 500 μl of the supernatant was used for measuring the reducing groups with the MBTH method.

**Measuring Apparent Processivity—**Reduced chitin (1 mg ml⁻¹) was incubated with 10 nm ChiA or its variants in 50 mM sodium acetate, pH 6.1, supplemented with BSA (0.1 mg ml⁻¹) at 25 °C with stirring. At the desired times, aliquots were withdrawn, and the reaction was stopped by adding 0.2 M NaOH. The chitin was sedimented by centrifugation (2 min at 10⁴ × g), and the amount of soluble reducing groups in the supernatant was measured using the MBTH method. The amount of insoluble reducing groups was determined by fluorescence labeling of the chitin with anthranilic acid (AA). For that, the chitin pellet was washed twice with water, once with 50 mM sodium acetate, pH 6.1, and twice with water. The chitin was resuspended in 200 μl of water, and the AA labeling was carried out in 80% buffered methanol at 80 °C for 2 h as described previously (36). The concentrations of sodium cyanoborohydride and AA were 0.5 M and 50 mM, respectively. The labeled chitin was washed three times with water and three times with 50 mM sodium acetate, pH 6.1. Finally, the chitin was resuspended in 50 mM sodium acetate, pH 6.1, to the final concentration of 0.5 mg ml⁻¹, and the fluorescence of the suspension was measured using excitation and emission wavelengths set to 330 and 425 nm, respectively. Relative fluorescence of 310 intensity units μM⁻¹ determined for AA-labeled NAG in 0.5 mg ml⁻¹ chitin suspension was used for calibration. AA labeling of NAG was done according to the protocol of the preparation of AA/glu-
cose in water (36), and the relative fluorescence of AA-labeled NAG was 450 intensity units \( \text{mM}^{-1} \).

**Measuring the Probability of Endo-mode Initiation**—First, AA-labeled chitin was prepared from \( \alpha \)-chitin, using the above described protocol. Prior to enzymatic hydrolysis, the AA-labeled \( \alpha \)-chitin was incubated in 0.2 M NaOH for 15 min at room temperature to remove the nonspecific label, followed by three washes with 50 mM sodium acetate, pH 6.1. 1 mg ml\(^{-1} \) AA-labeled \( \alpha \)-chitin was incubated with 10 nM ChiA or its variants in 50 mM sodium acetate, pH 6.1, supplemented with BSA (0.1 mg ml\(^{-1} \)) at 25 °C, with stirring. At defined times, the reaction was stopped by adding 0.2 M NaOH, and the chitin was pelleted by centrifugation (2 min at \( 10^4 \times g \)). The released AA-sugars were determined by measuring the fluorescence in the supernatant using excitation and emission wavelengths set to 330 and 425 nm, respectively. The concentration of the reducing groups in the supernatant was measured in the MBTH method. The probability of the endo-mode initiation was calculated from the concentration of insoluble reducing groups measured in the apparent processivity experiment and the concentration of the released AA-sugars as described previously (17).

**Results**

**Measuring Off-rates of Enzymes in Substrate Exchange Experiments, Theoretical Considerations**—In the substrate exchange experiment (SEE), an enzyme (\( E \)) was incubated with a polymerizable substrate (\( S \)) of interest, and after the predefined time, the reaction was supplemented with \( ^{14} \text{C} \)-labeled polymerizable substrate (\( ^{14} \text{C}-S \)). Upon the addition of \( ^{14} \text{C}-S \), the release of \( ^{14} \text{C} \)-labeled product (\( ^{14} \text{C}-P \)) in time was followed. If the concentration of \( S \) is saturating for the enzyme, a lag phase in the formation of \( ^{14} \text{C}-P \) in time was expected, because the enzyme must release from \( S \) before it is available for \( ^{14} \text{C}-S \). For simplicity, we used a simple two-step reaction mechanism for the formation of \( ^{14} \text{C}-P \). According to Scheme 1, the rate of \( ^{14} \text{C}-P \) formation is proportional to the concentration of enzyme in complex with \( ^{14} \text{C}-S \) (\( [^{14} \text{C}-S]^E \)).

It can be shown that the time dependence of \( [^{14} \text{C}-S]^E \) is governed by the exponent in the form of \( (1 - \exp(-k_{\text{off}}t)) \). We note that this simple time dependence of \( [^{14} \text{C}-S]^E \) is valid only if \( k_{\text{off}} \), the rate of \( ^{14} \text{C}-S \) dissociation from \( E \), is high enough to ensure that the on-rate (\( k_{\text{on}}^{14} \text{C}[^{14} \text{C}-S] \)) is higher than the on-rate (\( k_{\text{off}}^{14} \text{C} \) and \( k_{\text{off}} \)). In other words, \( [^{14} \text{C}-S]^E \) must be saturating for the enzyme. The same is true for \( S \) before the addition of \( ^{14} \text{C}-S \). This ensures that before the addition of \( ^{14} \text{C}-S \), \( E \) is negligible, so that the dissociation from ES is a prerequisite for the reaction with \( ^{14} \text{C}-S \).

The analysis of SEE results is somewhat complicated by the absence of true steady state in the hydrolysis of heterogeneous polymeric substrates (37). The progress curves of cellulose hydrolysis seem to follow the fractal-like kinetics with gradual loss of activity in time. Because the complex system does not enable rigorous analytical treatment, empirical equations have been used to describe the progress curves. The simplest equation describing the hydrolysis of cellulose is the two-parameter equation introduced by Kostylev and Wilson (14). According to Equation 1,

\[
[^{14} \text{C}-P] = A t^b \tag{1}
\]

In Equation 1, \( [^{14} \text{C}-P] \) is the product concentration; \( A \) is the constant referred to as the enzyme activity, and the constant \( b \) is the hydrolysis power term (14, 38). The parameter \( A \) is a product of the concentration of productive enzyme-substrate complex and the catalytic constant. It has been shown that in cellulose hydrolysis, \( A \) scales with the total enzyme concentration \( (E_0) \), whereas \( b \) is independent on \( E_0 \) (14). Despite its simplicity, Equation 1 has been shown to describe the progress curves of cellulose hydrolysis over a large range of total conversions of the substrate (14). This encouraged us to use Equation 1 also in the analysis of SEE results. Assuming that at the moment of \( ^{14} \text{C}-S \) addition the concentration of the free enzyme is negligible, the availability of the enzyme for \( ^{14} \text{C}-S \) hydrolysis is governed by its release from the non-labeled \( S \). Thus, an apparent \( A \) is expected to increase in time analogously to \( [E^{14} \text{C}-S]^E \). Introducing the time dependence into Equation 1 results in Equation 2.

\[
[^{14} \text{C}-P] = A(1 - e^{-k_{\text{off}} t})^b \tag{2}
\]

To find numerical values for \( k_{\text{off}} \), the following approach was used. First, the reference time course, where \( S \) and \( ^{14} \text{C}-S \) were mixed together before the addition of the enzyme, was fitted to Equation 1 to find the values of parameters \( A \) and \( b \). Next, the time course of \( ^{14} \text{C}-P \) formation in the SEE experiment was fitted to Equation 2. In fitting the SEE results to Equation 2, the value of the parameter \( b \) was fixed to the value found from the analysis of the reference time course. This was necessary because of the interdependency between \( k_{\text{off}} \) and \( b \) in Equation 2. The average \( R \) values for fitting the reference time courses to Equation 1 and SEE results to Equation 2 were 0.9919 (\( n = 9 \)) and 0.9951 (\( n = 19 \)), respectively.

**Substrate Exchange Experiments with CNWs**—Recently, we have described the preparation and kinetic measurements with \( ^{14} \text{C} \)-CNWs (34). \( ^{14} \text{C} \)-CNWs were used as the labeled substrate throughout this study. In the first trials of measuring the off-rates of ChiA we used non-labeled CNWs as the substrate. The release of \( ^{14} \text{C} \)-soluble sugars from \( ^{14} \text{C} \)-CNWs upon hydrolysis by ChiA is shown in Fig. 2. For the reference curve of SEE, the \( ^{14} \text{C} \)-CNWs and CNWs were mixed together in equal amounts, and the reaction was started by the addition of ChiA. The presence of CNWs resulted in a 2-fold reduction in the release of \( ^{14} \text{C} \)-sugars, confirming the equivalence of CNWs and \( ^{14} \text{C} \)-CNWs (data not shown). In the SEE, the CNWs were first incubated with ChiA for 2 h before the addition of \( ^{14} \text{C} \)-CNWs. The release of \( ^{14} \text{C} \) in SEE revealed a transient lag phase followed by...
a near-linear increase of $[^{14}\text{C}]$ in time. However, the inspection of the reference curve reveals that there is no steady state as the rate of $[^{14}\text{C}]$ release gradually decreases with hydrolysis time (Fig. 2). These results suggest that the apparent linearity observed in SEE may be caused by the counterbalancing effects of the release of ChiA from CNWs and the decrease in hydrolysis rates of $[^{14}\text{C}]$-CNWs with hydrolysis time. Therefore, the results were analyzed using equations for fractal-like kinetics, Equation 1 for the reference curve, and Equation 2 for SEE experiments. The $k_{off}$ value of 0.012 ± 0.002 s$^{-1}$ for the dissociation from CNWs was found. Reference curves made using 5 and 10 nM ChiA resulted in the values of parameter $A$ (Equation 1) of 0.15 ± 0.04 and 0.29 ± 0.05, respectively. Corresponding values for the power term $b$ were 0.66 ± 0.06 and 0.63 ± 0.03. These results are in accord with the results of cellulose hydrolysis, demonstrating that $A$ scales with the enzyme concentration, whereas $b$ is independent (14). When the incubation time with CNWs was extended to 24 h, the lag phase in SEE was absent (Fig. 2). This suggests that changes in CNWs have occurred, resulting in either an increased $k_{off}$ value or the presence of a significant amount of free ChiA before the addition of $[^{14}\text{C}]$-CNWs. Because the suspension properties of CNWs did not permit the measurement of free ChiA, it was not possible to discriminate between these two possibilities.

Substrate Exchange Experiments with $\alpha$-Chitin—The $\alpha$-chitin used in SEE experiments was the parent substrate used for the preparation of CNWs by heterogeneous acid hydrolysis. Thus, the $\alpha$-chitin is expected to have lower crystallinity and a higher degree of polymerization compared with CNWs. Besides the wild type enzyme, two ChiA variants, ChiA-W167A and ChiA-W275A, were used in SEE experiments. First, we tested the product NAG$_2$’s inhibition of ChiA on $[^{14}\text{C}]$-CNWs. There was no difference in inhibition strengths measured at three different $[^{14}\text{C}]$-CNW concentrations, 0.1, 0.25, and 1.0 mg ml$^{-1}$, suggesting non-competitive type of inhibition (34). 23 ± 3 (data from Ref. 34), 40 ± 5, and 86 ± 8% of the activity of ChiAWT, ChiA-W167A, and ChiA-W275A, respectively, were retained in the presence of 5 mM NAG$_2$. To avoid possible complications because of the product inhibition in the long trials, the SEE reactions were supplemented with chitobiase to transform NAG$_2$ to two NAG moieties. Comparing the reference curves made with the mixture of $\alpha$-chitin and $[^{13}\text{C}]$-CNWs with those made with $[^{14}\text{C}]$-CNWs only revealed that $[^{13}\text{C}]$-CNWs effectively out-competed ChiA from $\alpha$-chitin (Fig. 3). During the first 5 min of hydrolysis of $\alpha$-chitin, the activities of both ChiA-W167A and ChiA-W275A were comparable with the activity of ChiAWT. However, with increasing hydrolysis times, ChiAWT clearly outperformed both variants (Fig. 4A). In SEE, the incubation time of 10 min with $\alpha$-chitin was first tested. The characteristic lag phase was seen with ChiAWT and ChiA-W275A but not with ChiA-W167A (Fig. 3). To test whether the absence of lag phase with ChiA-W167A was caused by high off-rates or a significant amount of free enzyme present before the addition of $[^{14}\text{C}]$-CNWs, we measured the binding to $\alpha$-chitin. The concentration of bound enzyme achieved the plateau value after 5–10 min in cases of all enzymes (Fig. 4B). At the $\alpha$-chitin concentration of 2 mg ml$^{-1}$, the concentration of free enzyme was less than 10%, suggesting that the absence of lag phase in SEE with ChiA-W167A was not caused by the presence of a high concentration of free enzyme. Using 2 h of incubation with $\alpha$-chitin before the addition of $[^{14}\text{C}]$-CNWs revealed a lag phase in case of all enzyme variants (Fig. 3). The $k_{off}$ values increased in the following order:
ChiAWT < ChiA-W275 < ChiA-W167A (Table 1). The apparent time dependence of \( k_{off} \) prompted us to measure the \( k_{off} \) values at longer pre-incubation times with \( \alpha \)-chitin. In the case of wild type ChiA, the same \( k_{off} \) values were measured for the series with pre-incubation times between 24 and 360 h. For ChiAWT, the average \( k_{off} \) value for 24–360-h pre-incubation times was within the error limits with the \( k_{off} \) value measured using 2 h of pre-incubation time (Table 1). Contrary to ChiAWT, no lag phase was observed with ChiA-W275A and ChiA-W275A in the series with pre-incubation times between 24 and 360 h (Fig. 3). Binding measurements revealed that for all enzymes the concentration of free enzyme measured after 24–360 h of incubation with \( \alpha \)-chitin was within a few percent of the total enzyme concentration (data not shown). This suggests that it is the apparent value of \( k_{off} \) of ChiA-W275A and ChiA-W275A that has increased with pre-incubation time. The general activity of ChiA-W167A was within the error limits with that of ChiA-W275A. However, both variants were ~3-fold less active than ChiAWT (Fig. 4C). The extent of \( \alpha \)-chitin degradation after 360 h of incubation was 18, 5, and 4.5% for ChiAWT, ChiA-W275A, and ChiA-W167A, respectively.

**Initial Rates at Different Chitin Substrates**—Progressing difference in the activity of the variants and ChiAWT with hydrolysis time suggests that the variants may be deficient in the hydrolysis of the more recalcitrant portion of \( \alpha \)-chitin. This prompted us to measure the initial rates with different chitin substrates. Initial rates were measured after 1 min of hydrolysis. In this time scale, the progress curves were near-linear. We note that the linearity of progress curves does not necessarily imply that the reaction is in true steady state. With all the enzymes and substrates tested, the initial rates measured at different substrate concentration followed Michaelis-Menten saturation kinetics (data not shown). Although originally derived for enzymes acting on soluble substrates, it has been shown that the Michaelis-Menten equation is applicable also for the processive enzymes acting on insoluble substrates (25, 26, 39).

$$pV_{app} = \frac{pV_{max}S_0}{pK_m + S_0}$$

(Eq. 3)

In Equation 3, \( pV_{app} \) is the initial (steady state) rate of the processive hydrolysis; \( S_0 \) is the initial concentration of the substrate, and \( pV_{max} \) and \( pK_m \) are processive analogs of the maximum velocity and Michaelis constant, respectively (25, 39). To get meaningful estimates for \( pV_{max} \) and \( pK_m \), the substrate must be in excess (25). In practice, this condition can be met by using very low enzyme concentrations (\( E_0 \)) (25). Experiments made using 20 and 40 nm ChiA showed that activity was scaled proportionally with enzyme concentration in the case of all substrates and concentrations used (data not shown). This confirms that the substrate is in excess in our study conditions.

**Measuring Apparent Processivity with Reduced \( \alpha \)-Chitin—** Apparent processivity (\( P_{app} \)) is defined as the number of processive catalytic events (\( N_{catal} \)) divided by the number of the initiations of processive runs (\( N_{init} \)). It has been shown that if the hydrolysis of reduced cellulose is followed under single-hit conditions, the number of insoluble reducing groups (IRG) generated to the reduced cellulose equals the sum of the reducing-end-exo and endo-mode initiations. However, the total number of enzyme-generated reducing groups (RGtot) equals the number of insoluble reducing groups (SRGs) and IRGs. Therefore, for the enzymes that employ reducing-end-exo and/or endo-mode initiation, the slope of the plot in coordinates \( [\text{RG}_{\text{tot}}] \) versus \( [\text{IRG}] \) is equal to \( pV_{max}/E_0 \) (25, 17, 18, 40). Here, we followed the hydrolysis of reduced \( \alpha \)-chitin by ChiAWT and its variants ChiA-W275A and ChiA-W167A. The activity on reduced \( \alpha \)-chitin was within the error limits with the activity on \( \alpha \)-chitin. \( pV_{max}/E_0 \) values measured with reduced \( \alpha \)-chitin were 7.8 ± 0.4, 7.1 ± 0.3, and 7.0 ± 1.2 s⁻¹ for ChiAWT, ChiA-W275A, and ChiA-W167A, respectively. Correspondingly, \( pK_m \) values were 1.1 ± 0.2, 2.1 ± 0.4, and 1.4 ± 0.5 mg ml⁻¹. For parameter values with \( \alpha \)-chitin, see Table 2. The number of IRGs was measured by fluorescence labeling of chitinase-treated reduced \( \alpha \)-chitin. \( [\text{RG}_{\text{tot}}] \) versus \( [\text{IRG}] \) plots for \( \alpha \)-chitin are shown in Fig. 5. We note that the lines in coordinates \( [\text{RG}_{\text{tot}}] \) versus \( [\text{IRG}] \) became upward at longer incubation times (higher IRG concentrations). This is indicative of a deviation from single-hit conditions, because repeated hits on the reducing end of the same chain produce SRGs but not IRGs. However, because the deviation from the linearity was not prominent, considering the error limits, full datasets were used in linear regression analysis. The slope of the linear regression line equals the value of \( pV_{max}/E_0 \), and the corresponding figures for ChiAWT (Table 2).
Off-rates and Processivity of Chitinases

### TABLE 1

$k_{\text{off}}$ values measured at different pre-incubation times with α-chitin

| Enzyme     | $A$  | $b^e$ | $k_{\text{off}}$ ($10^{-3}$ s$^{-1}$) |
|------------|------|-------|--------------------------------------|
|            | μM s$^{-1}$ |       | 10 min$^c$ | 2 h$^b$ | 24–360 h$^d$ |
| ChiAWT     | 0.72 ± 0.06 (43)$^e$ | 0.561 ± 0.022 (11)$^e$ | 2.8 ± 0.3 (22)$^e$ | 1.5 ± 0.5 (31)$^e$ | 1.7 ± 0.3 (25)$^e$ |
| W275A      | 0.87 ± 0.20 (44)$^e$ | 0.392 ± 0.027 (17)$^e$ | 3.5 ± 1.2 (46)$^e$ | 2.2 ± 0.7 (41)$^e$ | fast$^f$ |
| W167A      | 0.61 ± 0.11 (43)$^e$ | 0.389 ± 0.030 (17)$^e$ | fast$^f$ | 6.4 ± 3.7 (49)$^e$ | fast$^f$ |

$^a$ The parameter values of reference curves were found by non-linear regression analysis of data in Fig. 3 (controls, where $^{14}$C-CNWs and α-chitin were mixed together before incubation with ChiA) according to Equation 1.

$^b$ The $k_{\text{on}}$ values were found by non-linear regression analysis of data in Fig. 3 (SEE data, where ChiA was pre-incubated with α-chitin before the addition of $^{14}$C-CNWs) according to Equation 2.

$^c$ The value of parameter $b$ was fixed to the value found for reference curves (listed in this table) while fitting the data to Equation 2.

$^d$ The dissociation was considered to be fast in the cases, when the lag phase in $^{14}$C-P formation in SEE was not detectable.

$^e$ Standard deviations are from at least three independent measurements.

$^f$ The average of experiments with the times of pre-incubation of ChiA with α-chitin before the addition of $^{14}$C-CNWs were as indicated.

### TABLE 2

Initial rates based Michaelis-Menten kinetics parameter values on different chitin substrates

| Enzyme     | CNWs | α-Chitin | Amorphous chitin | CNWs | α-Chitin | Amorphous chitin |
|------------|------|----------|------------------|------|----------|------------------|
| ChiAWT     | 11.7 ± 0.9 | 7.6 ± 1.8 | 2.1 ± 0.2 | 0.34 ± 0.07 | 1.5 ± 0.6 | 0.31 ± 0.28 |
| W275A      | 4.9 ± 0.7  | 7.5 ± 2.1 | 12.3 ± 0.9 | 0.55 ± 0.20 | 1.8 ± 0.5 | 0.83 ± 0.04 |
| W167A      | 5.2 ± 0.8  | 8.9 ± 0.9 | 10.7 ± 1.4 | 0.90 ± 0.52 | 2.3 ± 0.8 | 1.14 ± 0.18 |

$^a$ $V_{\text{max}}$ and $K_m$ values were found by non-linear regression analysis of $P_{\text{app}}$ versus $S$ curves according to Equation 3. Standard deviations are from three independent measurements.

### FIGURE 5

Apparent processivity ($P_{\text{app}}$) measured with reduced α-chitin. Wild type ChiA (⊥), ChiA-W275A (△), and ChiA-W167A (□) are shown. Reduced α-chitin (1 mg ml$^{-1}$) was incubated with 10 nM ChiA in 50 mM NaAc, pH 6.1, at 25 °C. SRGs were measured with the MBTH method, and the formation of IRGs was measured with fluorescence labeling. $P_{\text{app}}$ was found as the sum of IRGs and SRGs. Error bars show S.D. and are from three independent experiments. Solid lines represent the best fit of linear regression and the slope of the line equals the $P_{\text{app}}$.

### TABLE 3

$pV_{\text{max}}$ and $P_{\text{Endo}}$ measured with α-chitin

| Enzyme     | $pV_{\text{max}}$ (μM) | $P_{\text{Endo}}$ (μM) |
|------------|------------------------|------------------------|
| ChiAWT     | 36 ± 5$^e$             | 0.76 ± 0.11$^e$        |
| W275A      | 14 ± 3                 | 0.88 ± 0.18            |
| W167A      | 16 ± 2                 | 0.90 ± 0.13            |

$^a$ Standard deviations are from at least three independent measurements.

Min of hydrolysis and 0.02 s$^{-1}$ (measured after 1 h of hydrolysis). For ChiAWT, these figures are approximately an order of magnitude higher than $k_{\text{off}}$ values measured using SEE experiments (Table 1).

Measuring the Probability of Endo-mode Initiation—The results of previous studies have suggested that ChiA and an analogous enzyme CHI60 from *Serratia* sp. can, besides reducing-end-exo initiation, also employ endo-mode initiation (40, 41). Therefore, we estimated the probability of endo-initiation ($P_{\text{Endo}}$) of ChiA by measuring the release of AA-sugars from reducing end AA-labeled α-chitin and comparing it with the number of IRGs generated in the hydrolysis of reduced α-chitin under the same experiment conditions. The release of AA-sug-
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Discussion

Processivity is an important kinetic property of polymer active enzymes (42). Processive enzymes remain attached to the polymer chain after the catalytic event. Provided that processivity is high enough, the complete processing of a polymer chain can be achieved after the initial productive binding. It has been proposed that the main determinant of \( P_{\text{max}} \) of cellulases is \( k_{\text{off}} \) (17). Although the importance of the \( k_{\text{off}} \) value of the enzymes processing recalcitrant polysaccharides is widely recognized, the experimental approaches to measure this parameter are scarce.

Here, we developed a new method for measuring the off-rates of a chitinase from the crystalline polysaccharide chitin. We found that the observed off-rates were dependent on both the nature of the chitin substrate and the hydrolysis time. After the incubation of ChiAWT with the CNW substrate for 2 h, an apparent \( k_{\text{off}} \) of 0.012 ± 0.002 s\(^{-1}\) was found. The corresponding value for \( \alpha\)-chitin was an order of magnitude lower, 0.0015 ± 0.0005 s\(^{-1}\) (Table 1). Within the series with different incubation times with \( \alpha\)-chitin, there was an initial drop in the value of \( k_{\text{off}} \) of ChiAWT and its variants (Table 1). However, after 2 h of incubation, the \( k_{\text{off}} \) of ChiAWT achieved a constant value that did not change during further incubation up to 360 h studied. The situation was different with the ChiA variants, ChiA-W275A and ChiA-W167A, dissociation of which was too fast to be measured after the long term (24–360 h) incubation with \( \alpha\)-chitin. The fast dissociation of ChiA-W275A and ChiA-W167A was accompanied by inefficiency in \( \alpha\)-chitin degradation as evidenced by increasing deficiency of variants compared with ChiAWT with hydrolysis time (Fig. 4, A and C). This indicates that changes in \( \alpha\)-chitin structure have occurred during the hydrolysis and that the remaining chitin was more recalcitrant to enzymatic degradation. Inefficiency of ChiA-W275A and ChiA-W167A in degradation of recalcitrant crystalline chitin is also revealed in initial rates based on \( pV_{\text{max}}/E_0 \) values (Table 2). On the substrate with the highest crystallinity, CNWs, ChiAWT outperformed both variants. In contrast to CNWs, on amorphous chitin both variants clearly outperformed ChiAWT, indicating that strong interaction with the chitin chain comes as a penalty on amorphous substrate. Better performance of ChiA-W275A and ChiA-W167A compared with ChiAWT on partially acetylated chitosan, a soluble chitin derivative, has been demonstrated before (7). At the same time, the activity of ChiA-W275A and ChiA-W167A on crystalline \( \beta\)-chitin was reduced by 50 and 70%, respectively (7). Similar trends have also been observed with processivity-deficient variants of the other S. marcescens chitinase, ChiB (10). Studies of TrCel7A variants with lower processivity also revealed higher activity compared with the wild type enzyme on amorphous cellulose (12, 43). Thus, the negative effect of processivity (slow off-rate) in hydrolysis of amorphous substrates seems to be a general feature among glycoside hydrolases evolved to degrade recalcitrant polysaccharides (7, 43). In the study by Zakariassen et al. (7), the processivity of ChiAWT, ChiA-W275A, and ChiA-W167A was assessed by measuring the product profile of the hydrolysis of soluble chitosan. This method provides a qualitative rather than quantitative measure of processivity (18). It was found that the processivity of ChiA-W275A was reduced dramatically, whereas ChiA-W275A had a less prominent effect on processivity (7). This is in qualitative agreement with the \( k_{\text{off}} \) values measured here. The larger effect of Trp167 to Ala substitution in reducing \( k_{\text{off}} \) is expected, because Trp167 interacts with the dimeric product. The strong effect of Trp275 to Ala substitution in relieving chitobiose inhibition (see under “Results”) and increasing the \( K_m \) value for chitotetraose (44) is also in accord with the role of Trp275 in ligand binding at the product-binding site + 1.

When looking for correlations between the values of parameters of enzyme kinetics and the performance of enzymes in chitin degradation, we first note that there is no direct correlation between \( k_{\text{off}} \) and enzymes performance. This is because ChiA-W167A has higher off-rates than ChiA-W275A (Table 1) but similar hydrolytic activity on all the substrates and time frames tested (Fig. 4, A and C, and Table 2). Although partially acetylated soluble chitosan is not a homopolymer, it is expected to be more close to an ideal polymer than insoluble chitin. Therefore, the processivity on chitosan is expected to reflect the intrinsic processivity of the enzymes. Unfortunately, the \( k_{\text{off}} \) values on chitosan are not available. From the results of this study, we can estimate \( P_{\text{intr}} \) on \( \alpha\)-chitin. Using the \( pV_{\text{max}}/E_0 \) values listed in Table 2 as a measure of the \( k_{\text{cat}} \) and \( k_{\text{off}} \) values measured after 2 h of incubation with \( \alpha\)-chitin (Table 1), the \( P_{\text{intr}} \) values of 5000 ± 1600, 3500 ± 1000, and 1400 ± 800 can be calculated for ChiAWT, ChiA-W275A, and ChiA-W167A, respectively. It has been shown that \( P_{\text{intr}} \) is related to the free energy of the binding of the polymer chain to the enzyme’s active site (19, 20). We note that the \( P_{\text{intr}} \) value estimated here for ChiA is in the same order with the \( P_{\text{intr}} \) value found for TrCel7A from the rate of the production of IRGs on reduced bacterial cellulose (17). The active site of TrCel7A has a tunnel-shaped architecture, whereas the active site of ChiA resides in a deep opened groove. Thus, our data are in line with the general paradigm that the closed active site architecture is not a sole
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determinant of processivity (42). However, there are at least two reasons why $P_{\text{intr}}$ values reported here must be treated with caution. (i) The $V_{\text{max}}/E_0$ values were taken as estimates of the $k_{\text{cat}}$ values. On CNWs, we recently demonstrated that a significant fraction of ChiA is bound non-productively with the active site free from the chitin chain even at the substrate concentrations saturating for the activity (34). If the same is true for $\alpha$-chitin, $V_{\text{max}}/E_0$ understimates $k_{\text{cat}}$ and also $P_{\text{intr}}$. (ii) The $k_{\text{off}}$ values were measured from the rates of the total dissociation of enzymes (Table 1), but in order to be used in calculating $P_{\text{intr}}$, $k_{\text{off}}$ should represent the dissociation of the chitin chain from the active site. If the dissociation of the CBM is slow, the $k_{\text{off}}$ for the total dissociation of enzyme may underestimate the $k_{\text{off}}$ for the dissociation from the active site, and $P_{\text{intr}}$ is overestimated. The importance of aromatic residues in the CBM of ChiA (Trp33 and Trp69) was clearly demonstrated by Uchiyama et al. (9) as substitution to Ala drastically reduced binding to crystalline $\beta$-chitin. At the same time, the substitution of Trp167 or Trp272 to Ala had only moderate effects on binding to $\beta$-chitin (45). The rates of the production of IRGs on reduced $\alpha$-chitin measured here also suggest a significant contribution of the CBM in the total dissociation of the enzyme. For ChiA, the rate constant of the production of IRGs ($V_{\text{IRC}}/E_0$) varied between 0.057 ± 0.005 and 0.021 ± 0.003 $s^{-1}$ as measured after 5 min and 1 h of hydrolysis, respectively. These figures are about an order of magnitude higher than the $k_{\text{off}}$ values (Table 1), meaning that the frequency of the initiations of processive runs is much higher than the frequency of the total dissociation of the enzyme from chitin. This is indicative of “lateral processivity,” where, upon the dissociation of the chitin chain from the active site, an enzyme remains attached to the crystal surface and seeks a new site for initiation through lateral diffusion. Lateral diffusion of cellulases, as well as their isolated CBMs, has been demonstrated in several FRAP studies (46–49). To the best of our knowledge, such data are not available for chitinases. Recently, we proposed that the rate-limiting step for ChiA acting on CNWs is the complexation with polymer chain (34). Because the generation of IRGs to the reduced chitin cannot be slower than complexation, the results presented here suggest that the complexation must be at least an order of magnitude faster than full dissociation of the enzyme from $\alpha$-chitin. Unfortunately, it is not possible to decide which step, complexation with chitin chain or dissociation of chitin chain from the catalytic domain, is rate-limiting for the production of IRGs on the reduced $\alpha$-chitin.

Similarly to what has been observed with processive cellulases (17, 50–53), and for ChiA acting on soluble chitosan (40), we note that ChiA displayed high probability of endo-mode initiation (Table 3). If endo-mode initiation is included, the possible number of initiation sites is expected to be much higher than one can predict from the degree of polymerization of $\alpha$-chitin (1230 ± 110 in our study as judged by AA labeling). Increasing the possibility of productive complex formation on a polymer with a low number of reducing ends may be the reason why there seems to be no strict exo-acting enzymes among recalcitrant polysaccharide active enzymes.

Regardless of the mode of initiation, the question of why the processive enzymes have evolved to such low off-rates (high $P_{\text{intr}}$ values) remains. Measuring the processivity of glycoside hydrolases is not straightforward, and the results are dependent on the method used (18, 54). The lengths of the processive runs of $Tr$Cel7A measured on different celluloses using different methods for measuring are in the range of 10–70 (17, 23, 26, 29, 43, 55, 56). Clearly these figures are far lower than $P_{\text{intr}}$ values of 4000 and 560 measured on bacterial cellulose and amorphous cellulose, respectively (17). Experimentally measured $P_{\text{pp}}$ values for ChiA range between 5 and 30, depending on the substrate and the method used for measuring (40, 57–59). Therefore, the large discrepancy between $P_{\text{pp}}$ and $P_{\text{intr}}$ seems to hold also for ChiA. It has been postulated that $P_{\text{pp}}$ is limited by the length of the obstacle-free path on the polymer. Although the molecular nature of these obstacles has not been clearly identified, cellulose chains on eroded surface (60–62), non-productively bound enzymes (60, 61), lignin and hemicelluloses (21, 63), amorphous regions on cellulose (23), and aggregates of cellulose microfibrils (21) have been proposed to serve as obstacles. The encounter of an obstacle stops the processive run and leaves the enzyme in the non-productive complex with the polymer chain end in the substrate-binding site $-1$ (for enzymes moving toward the non-reducing end of the substrate). In the case of processive enzymes, the strong binding in the product-binding sites +1 and +2 creates a cumulative force to ensure directional progression of the enzyme from the non-productive complex (polymer chain end in $-1$) to the productive complex (polymer chain end in +2) (3, 19, 64, 65). Therefore, it is tempting to speculate that strong binding of the enzyme in the non-productive complex dictated by low $k_{\text{off}}$ values helps to hold the enzyme behind obstacles in a stand-by position, whereas the force directed to fill the product-binding sites generates a “pushing potential” that, if strong enough, can remove or displace obstacles (Fig. 7). In an elegant high speed atomic force microscopy study by Igarashi et al. (66), the authors demonstrated that traffic jams formed by multiple halted $Tr$Cel7A molecules on parallel cellulose chains were, through collective pushing, able to disintegrate whole cellulose microfibrils. Disintegration of cellulose microfibrils by cellulases has been reported in several other microscopy studies (48, 49, 67–74). Thus, the evolution of processive cellulases and chitinases toward low off-rates may be related to the need of removing obstacles and disintegrating fibril aggregates. Enzymes with high off-rates (weak binding in the substrate sites) or with low “pushing potential” (weak binding in the product sites) are not capable of removing obstacles and are limited to the hydrolysis of the easily accessible part of the substrate only.

It must be noted, however, that cellulases are not selected based on their individual activities. It is the performance of the whole synergistic enzyme mixture that is the subject of evolution. In the light of obstacle model, any auxiliary activity that is able to remove obstacles should work synergistically with processive enzymes. Indeed, the results of several studies have pointed to the fact that synergism between processive enzymes and non-processive endo-enzymes (23, 63, 75, 76) or lytic polysaccharide monoxygenases (77, 78) is mediated by the removal of obstacles. Apparently, the molecular nature of the obstacles depends on the source and pre-treatment conditions.
FIGURE 7. Possible mechanism of degradation of recalcitrant polysaccharide by processive enzyme. An enzyme progresses along polymer chain hydrolyzing it in parallel until it encounters an obstacle (represented by solitary chain on polymer surface). An enzyme halted by the obstacle is bound to the chitin chain with the reducing chain end in the substrate-binding site – 1, or + 1 if the glycosidic bond is not correctly positioned for hydrolysis. Strong interactions between enzyme and polymer chain in the substrate-binding sites ensure low off-rate and gives to an enzyme a stand by status. At the same time, strong binding of the chain end in the product sites provides the enzyme with pushing potential and forces it to move forward. If obstacle is attached to the polymer surface through non-covalent interactions, a part of it may occasionally “melt” from the polymer surface (represented by two-headed arrow). In such a case, a stand-by enzyme with high pushing potential can move forward and disintegrate the obstacle. Weak binding in substrate-binding sites (like ChiA-W167A) causes the enzyme to dissociate and does not provide sufficient population of stand-by enzymes. Weak binding in product sites (like ChiA-W275A) does not provide the enzyme with strong enough pushing potential to move forward and disintegrate the obstacle. Therefore, an apparent processivity of ChiA-W167A and ChiA-W275A is determined by the average length of obstacle-free bath on polymer. The wild type enzyme can increase the length of the processive runs by pushing away the obstacles.

of the chitin/cellulose substrate. Therefore, the costs and benefits of processivity are expected to depend on the nature of auxiliary activities in combination with the nature of the substrate.

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