Carbohydrate Research 343 (2008) 2754–2762

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

The dual *exo*/*endo*-type mode and the effect of ionic strength on the mode of catalysis of chitinase 60 (CHI60) from *Serratia* sp. TU09 and its mutants

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ARTICLE INFO

Article history:
Received 31 March 2008
Received in revised form 19 May 2008
Accepted 24 May 2008
Available online 17 June 2008

Keywords:
Chitinase
Chitin
exo/*endo*-Type
Mutation
Tryptophan

ABSTRACT

Mutations of the tryptophan residues in the tryptophan-track of the N-terminal domain (W33F/Y and W69F/Y) and in the catalytic domain (W245F/Y) of *Serratia* sp. TU09 Chitinase 60 (CHI60) were constructed, as single and double point substitutions to either phenylalanine or tyrosine. The enzyme–substrate interaction and mode of catalysis, *exo*/*endo*-type, of wild type CHI60 and mutant enzymes on soluble (partially N-acetylated chitin), amorphous (colloidal chitin), and crystalline (β-chitin) substrates were studied. All CHI60 mutants exhibited a reduced substrate binding activity on colloidal chitin. CHI60 possesses a dual mode of catalysis with both *exo*- and *endo*-type activities allowing the enzyme to work efficiently on various substrate types. CHI60 preferentially uses the *endo*-type mode on soluble and amorphous substrates and the *exo*-type mode on crystalline substrate. However, the prevalent mode of hydrolysis mediated by CHI60 is regulated by ionic strength. Slightly elevated ionic strength, 0.1–0.2 M NaCl, which promotes enzyme–substrate interactions, enhances CHI60 hydrolytic activity on amorphous substrate and, interestingly, on partially N-acetylated chitin. High ionic strength, 0.5–2.0 M NaCl, prevents the enzyme from dissociating from amorphous substrate, occupying the enzyme in an enzyme–substrate non-productive complex. However, on crystalline substrates, the activity of CHI60 was only inhibited approximately 50% at high ionic strength, suggesting that the enzyme hydrolyzes crystalline substrates with an *exo*-type mode processively while remaining tightly bound to the substrate. Moreover, substitution of Trp-33 to either phenylalanine or tyrosine reduced the activity of the enzyme at high ionic strength, suggesting an important role of Trp-33 on enzyme processivity.

1. Introduction

Chitin is a polysaccharide that is attracting significant and increasing levels of attention in the pharmacological, biomedical, agricultural, and biotechnological fields.[1–7] Chitinases are a group of enzymes that catalyze the hydrolysis of chitin and are categorized into two glycosylhydrolase families, 18 and 19, based on their structure and mechanism of catalysis.[8–10] Family 18 chitinases have the classical $(\beta/\alpha)_8$ barrel catalytic domain[11–14] and produce products that retain their anomeric configuration (β→β). On the other hand, family 19 chitinases have a lysozyme-like structure[15] and produce products with an inverted anomeric configuration (β→α). Chitinases are further classified according to their mode of catalysis into two groups, *exo*-chitinases, which hydrolyze either the reducing or the non-reducing end of chitin strands, and *endo*-chitinases which hydrolyze within the chitin strand. The mechanism of hydrolysis of family 18 chitinases has been studied in some detail,[16–19] and these chitinases hydrolyze chitin via a substrate assisted mechanism, through an oxazoline ion intermediate, involving the carbonyl group of the aceta amido group of chitin. Therefore, a single acidic residue is sufficient for catalysis.[16,17]

A previous study revealed that *Serratia marcescense* produces three distinct chitinase enzymes, ChiA, ChiB, and ChiC that favor different modes of catalysis in terms of *exo*- or *endo*-type (20). ChiA and ChiB are processive chitinases that degrade chitin chains in opposite directions, while ChiC is a non-processive endo chitinase.[20] ChiA, a family 18 chitinase, from *S. marcescense* has been extensively studied,[21] and exposed aromatic residues on the surface of ChiA has been identified and suggested to be involved in binding and guiding of the substrate into the catalytic cleft during the processive mode of catalysis.[22] Changing these Trp residues to Ala residues reduces the ability of ChiA to bind to its substrate, as well as the activity of the enzyme on insoluble substrates.[22] A proposed model of hydrolysis of chitin by ChiA suggests it is an *exo*-chitinase hydrolyzing chitin strand from the reducing end.[22]

Abbreviations: 3D, three-dimensional; CHI60, *Serratia* sp. TU09 chitinase 60; WT CHI60, wild type CHI60; Trp, W, tryptophan; Tyr, Y, Tyrosine; Phe, F, phenylalanine; PNAC, partially N-acetylated chitin; kDa, kilodalton

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doi:10.1016/j.carres.2008.05.020

However, ChiA was able to hydrolyze both amorphous and crystalline chitin equally well, indicating that it likely possesses both *exo*- and *endo*-type activities and thus the mode of ChiA catalysis is ambiguous with respect to *endo*- and/ or *exo*-chitinase activities.[22]

We have successfully cloned and characterized chitinase 60 (CHI60) from *Serratia* sp. TU09 (Unpublished data; GenBank AY040610) and found it to be almost identical to the *chiA* gene from *S. marcescense* (e.g., GenBank DQ165083). By homology, CHI60 thus comprises three domains: an N-terminal domain, a $(\beta/\alpha)_8$ barrel catalytic domain, and a small $(\alpha+\beta)$-fold domain, which is inserted in the catalytic $(\beta/\alpha)_8$ barrel domain (12). CHI60 produces *N*,*N*′-diacetyl-chitobiose as a major product.

Here, we address two main points: firstly to understand how the exposed Trp residues in the tryptophan-track function on substrate binding, and secondly, to study the possible role of these residues on the mode of catalysis, that is, the *endo*- or *exo*-type activities of CHI60. CHI60 derivatives with single and double point substitutions of the exposed Trp residues track to Phe or Tyr at Trp-33 and Trp-69 in the N-terminal domain, and Trp-245 at the far end of the substrate binding cleft in the catalytic domain were created and used to study the substrate binding, mode of catalysis, and to infer the potential role of these three Trp residues in binding and guiding the substrate during processive hydrolysis.

The activities of wtCHI60 and CHI60 derivatives were determined on PNAC, colloidal chitin, and β-crystalline chitin as soluble, amorphous, and crystalline substrates, respectively. The application of different substrates makes it possible to distinguish between *exo*- and *endo*-chitinase activities of each enzyme. Soluble substrates can freely associate and dissociate from the active site and, therefore, enzymic activity on this substrate will largely reflect the total amount of active catalytic domains present in the reaction. Additionally, this activity can indicate *endo*-chitinase activity that is independent of substrate binding to Trp residues in the N-terminal domain as it has been shown that mutations of the Trp residues that are involved in substrate binding outside of the catalytic cleft did not affect the activity of the enzyme on soluble substrates.[15,22,28] Colloidal chitin was used to determine the *endo*-chitinase activity of each enzyme. To hydrolyze this substrate efficiently the enzyme must associate, hydrolyze, then dissociate from the initial site to the next hydrolytic site, because it cannot move processively along the entangled chitin strands of amorphous chitin. Finally, β-crystalline chitin was used to evaluate the processive *exo*-chitinase activity of each enzyme as the chitin strands are arranged in a parallel unidirectional manner with very few intermolecular hydrogen bonds, making it a good substrate for *exo*-chitinase.

The amount of enzyme that yields an equal hydrolytic activity on PNAC was used to hydrolyze β-chitin and colloidal chitin to ensure that an equal quantity of active catalytic domains was present in each reaction. Thus, the differences in the observed activities would reflect the change in the interaction between the N-terminal tryptophan-track of CHI60 and each of the substrates. Our previous studies have shown that CHI60 can hydrolyze both amorphous and crystalline chitin well, suggesting that CHI60 should possess both an *endo*- and *exo*-mode of chitin hydrolysis depending on the type of substrate it hydrolyses (our unpublished data). Because the interaction between the enzyme and substrate is a hydrophobic interaction between the sugar ring and Trp residues, it should be possible to influence the mode of hydrolysis of CHI60 by modulating the binding activity of CHI60 to its substrate. In this study, we have accomplished this by two approaches. First, by changing the ionic strength of the environment, using a salt gradient derived by supplementation of the reaction buffer with 0–2 M NaCl, and secondly, by generating derivatives of CHI60 with substitutions of the Trp residues in the tryptophan-track, changing the Trp residues to Phe or Tyr to reduce the enzyme–substrate interactions.

In our study, the enzyme hydrolytic products were not used to determine the *exo*- versus *endo*-type mode of hydrolysis, as the ratio of monosaccharide, disaccharide, and trisaccharide products does not reflect the *endo*- versus *exo*-type chitinase activity on the true substrate chitin used in our experiments. In contrast to previous reports, where *N*-acetyl-chitooligosaccharides were used as substrate, the interpretation of the product ratio to reveal the mode of hydrolysis was possible.

2. Results

2.1. Site-directed mutagenesis

The exposed Trp residues in the tryptophan-track at Trp-33 and Trp-69 in the N-terminal domain, and Trp-245 in the catalytic domain of *Serratia* sp. TU09 chitinase 60 (Fig. 1) were subject to codon alteration by site-directed mutagenesis to form the six single point mutations, W33F, W33Y, W69F, W69Y, W245F, and W245Y, and the three double point mutations, W33F/W69Y, W33F/W245F, and W69Y/W245Y. The correct codon exchange was confirmed by complete clone sequencing (data not shown). Wild type CHI60 (wtCHI60) and these substitution derivatives were then used to investigate the role of Trp-33, Trp-69, and Trp-245 on the mode of catalysis, and how these three exposed Trp residues function in binding and guiding the substrate during processive hydrolysis.

2.2. Expression and purification of wtCHI60 and derivatives

WtCHI60 and the nine mutant chitinases were expressed in *E. coli* DH5α cells and purified from the culture supernatants. All mutant chitinases were expressed at a comparable level to wtCHI60 showing no reduced expression or translation rates, or protein stability due to the codon changes (data not shown). WtCHI60 purification was performed by ultrafiltration with a 30 kDa MW cut-off, followed by DEAE-cellulose column chromatography. Chitinase activity was found in the unbound fractions, which were pooled, and the enzyme was further purified by chitin affinity adsorption. WtCHI60 was purified to apparent homogeneity as determined by SDS–PAGE resolution with comassie R250 blue staining (Fig. 2). Purification of the single and double Trp substitution chitinases was done in the same manner, in all cases to apparent homogeneity (Fig. 3) with the correct molecular weight of 60 kDa.

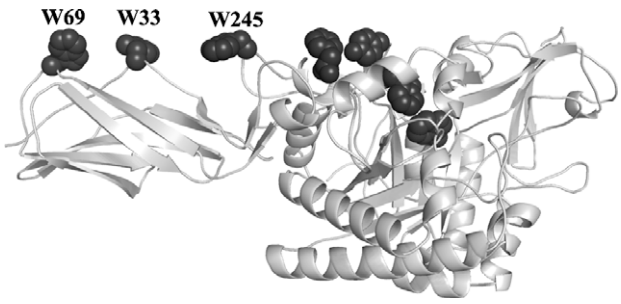

Figure 1. Positions of Trp-69, Trp-33, and Trp-245 in *Serratia* sp. TU09 CHI60. The α-carbon backbone of *Serratia* sp. TU09 CHI60, predicted by SWISS-MODEL version 36.0002[35–37] based on template, 1edqA, is shown as cartoon in *white*. The aromatic residues in the substrate binding track are shown as space filled model in *dark grey*, the exposed Trp-69 and Trp-33 residues in the N-terminal domain and Trp-245 in the catalytic domain are labeled. The three-dimensional structure representation was generated by PyMOL version 0.996rc6.

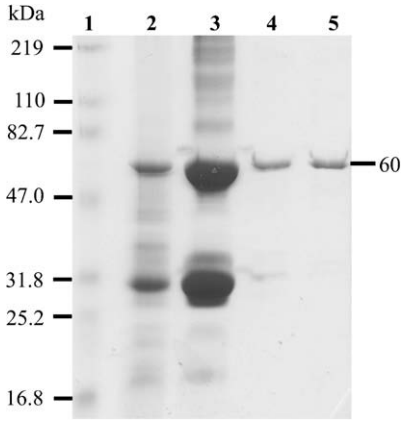

Figure 2. SDS–PAGE analysis of crude and purified wtCHI60. WtCHI60 expressed in *E. coli* DH5α cells was purified. Crude enzyme from the supernatant, partially purified protein from each purification step, and purified wtCHI60 were analyzed on SDS–PAGE. The gel was stained with Coomassie Brilliant Blue R-250. *Lane* 1, molecular mass standards; *lane* 2, crude CHI60; *lane* 3, partially purified wtCHI60 by ultrafiltration; *lane* 4, partially purified chitinases by DEAE-cellulose chromatography; and *lane* 5, purified chitinases by chitin affinity adsorption.

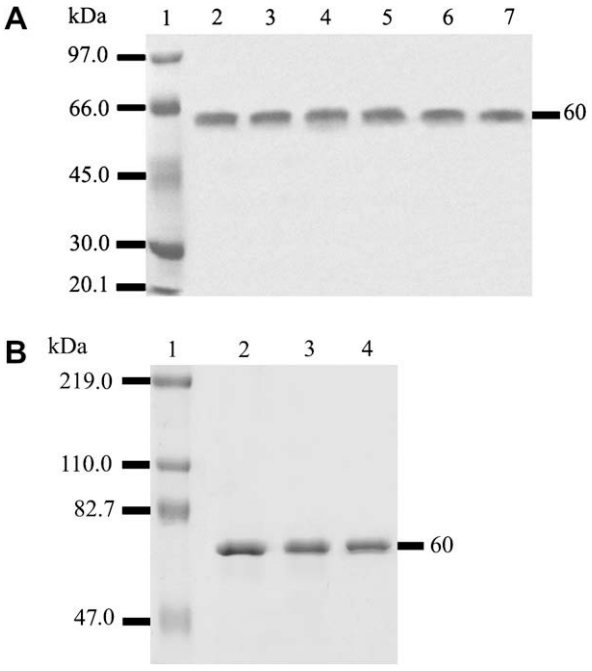

Figure 3. SDS–PAGE analysis of purified CHI60 derivatives. Chitinase derivatives expressed in *E. coli* DH5α cells were purified from the culture supernatant in the same manner as wtCHI60. The purified CHI60 derivatives were analyzed on SDS–PAGE. The protein gel was stained with Coomassie Brilliant Blue R-250. (A) *Lane 1*, molecular mass standards; *Lane* 2, W33F; *lane* 3, W33Y; *lane* 4, W69F; *lane* 5, W69Y; *lane* 6, W245F; and *lane* 7, W245Y and (B) *Lane* 1, molecular mass standards; *lane* 2, W33F/W69Y; *lane* 3, W33F/W245F, and *lane* 4, W69Y/W245Y.

2.3. The effect of substitution mutations on chitin binding activity

The chitin binding activities of wild type and mutant CHI60s were studied using colloidal chitin in 100 mM Tris–HCl buffer, pH 7.0 with an incubation time of 2 h. To minimize enzyme-mediated hydrolysis of the bound substrate, the binding assay mixture was maintained below 4 °C. When compared to wtCHI60, substitution of any one of the three aromatic residues, Trp-69, Trp-33, or Trp-245 with Phe or Tyr reduced the relative binding activity of the enzyme to 0.64, 0.69–0.77, and 0.82–0.84, respectively (Table 1). While the binding activity of mutants with substitution at Trp-69 and Trp-245 to either Phe or Tyr did not differ significantly, substitution of Trp33 to Tyr33 resulted in a much lower relative binding activity than Trp33 to Phe33 (0.69 compared to 0.77). Double mutations of either Trp-69 + Trp-33 or Trp-33 + Trp-245 further significantly reduced the observed binding activity of the enzyme to colloidal chitin to 0.46 and 0.59, respectively. However, the Trp-69 + Trp-245 double substitution displayed essentially the same relative binding value (0.63) as that seen in either single Trp substitution W69 F or W69Y.

Table 1
Effect of the Trp mutations on the chitin binding activity[a]

Chitinase	%Bound protein	Relative binding
WT CHI60	63.2 ± 1.8	1.0
W33F	48.2 ± 2.1	0.77
W33Y	43.7 ± 1.4	0.69
W69F	40.7 ± 3.2	0.64
W69Y	40.6 ± 2.9	0.64
W245F	51.9 ± 3.0	0.82
W245Y	53.4 ± 2.7	0.84
W33F/W69Y	29.0 ± 2.5	0.46
W33F/W245F	37.6 ± 1.9	0.59
W69Y/W245Y	39.6 ± 2.3	0.63

[a] The amount of each enzyme that bound to chitin in the 4 °C binding assay (see methods) displayed as the percentage of total enzyme that bound (% bound protein) and the relative amount bound compared to (normalized to) wtCHI60. Data are presented as the mean ± 1 SD derived from at least four independent repeats.

2.4. The effect of ionic strength on the hydrolytic activity of wild type and Trp substituted CHI60 derivatives

The effect of ionic strength on the activity of each CHI60 (mutant and wt) was determined using (i) soluble substrate, PNAC, (ii) amorphous substrate, colloidal chitin, and (iii) crystalline substrate, β-crystalline chitin. Although, the increase in ionic strength should not significantly affect the hydrolytic activity of wtCHI60 on PNAC, a 20% increase in the enzyme activity was observed when the concentration of NaCl was increased from 0 to 0.50 M (Fig. 4A). Further increases in the NaCl concentration reduced the relative activity of the enzyme down to its initial activity observed at 0 M NaCl. Likewise, with PNAC as substrate, the six single-point mutants and the three double-mutants all showed a similar dependence on the ionic strength as wtCHI60, with some minor shifts in the optimum activity of some derivatives (Fig. 4A and D). The activity of wtCHI60 on colloidal chitin was also enhanced, approximately 20%, as NaCl increased from 0 to 0.25 M NaCl. However, further increases in the ionic strength from 0.5 to 2.0 M NaCl significantly reduced the relative activity of the enzyme to lower than 30% of its initial activity (Fig. 4B). The inhibitory effect of increasing ionic strength on the enzymatic activity was also partially reduced as expected for all single (Fig. 4B) and double (Fig. 4E) point mutants, with no significant difference between double point mutants over the single point mutants, because the mutation of Trp residues to Phe or Tyr would reduce the enzyme–substrate hydrophobic interactions. Within single point mutations, W69F gave the highest relief of inhibition followed by mutations at W33 and W245. Surprisingly, W69Y gave a much lower relief on inhibition at 2.0 M NaCl. In contrast, the activity of wtCHI60 on β-crystalline chitin slowly decreased as the ionic strength increased from 0 to 2.0 M NaCl to approximately 50% of the initial activity (Fig. 4C), and a similar trend was seen with the W69F, W245F, and W245Y single point mutations. W69Y showed a better relief of inhibition than other derivatives between 0 and 1.0 M NaCl. Interestingly, the activity of W33F and W33Y was affected more than that of

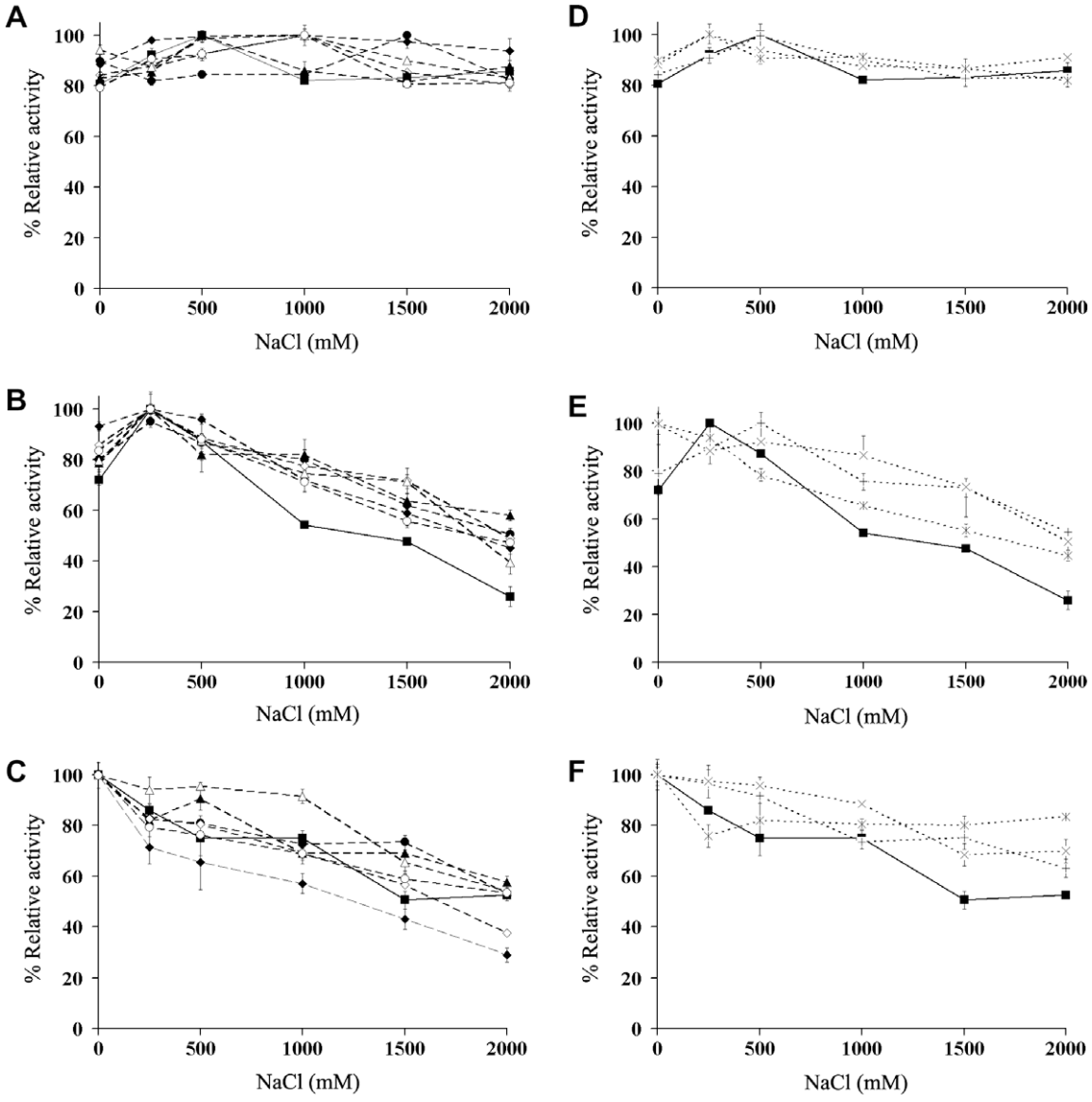

Figure 4. The relative activity of wtCHI60 and derivatives on soluble, amorphous, and β-crystalline substrates with increasing ionic strength. WtCHI60, single and double point mutation derivatives were used to hydrolyze PNAC, 0.5 mg/mL (A and D); colloidal chitin, 1 mg/ml (B and E); and β-chitin, 10 mg/mL (C and F). Reactions were performed in 20 mM citrate buffer pH 5.0, at 37 °C for 30 min. The ionic strength was modulated by the addition of NaCl from 0 to 2.0 M final concentration as indicated. Single point mutations are shown in panel (A), (B), and (C). Double point mutations are shown in panel (D), (E), and (F). Data are the mean ± 1 S.D derived from at least four independent repeats. The figure shows –■–, WT CHI60; –◇–, W33F; –♦–, W33Y; –▲–, W69F; – △–, W69Y; –●–, W245F; –○–, W245Y; ···+···, W33F/W69Y; ···×···, W33F/W245F and ···*···, W69Y/W245Y.

wtCHI60 as the ionic strength was increased from 0 to 2.0 M NaCl (Fig. 4C). However, the enzyme activity of all three double point mutants (Fig. 4F) was inhibited far less by increasing ionic strength compared to single point mutations and wtCHI60 (Fig. 4F).

In addition, we found that the activity of wtCHI60 on colloidal and β-crystalline chitin was not dramatically affected by most mutations, in the absence of NaCl (Table 2). The double mutation of W33F + W69Y had the highest impact on the activity of the enzyme, reducing its activity to approximately 0.86- and 0.53-fold on colloidal chitin and β-crystalline chitin, respectively. Interestingly, single mutations of Trp at positions 33 and 69 to Phe, and position 245 to Phe or Tyr, on the other hand, increased the activity of the enzyme on these substrates (Table 2). Curiously, the double mutation of W33F + W254F and W69Y + W245Y demonstrated a slight decrease in activity on β-crystalline chitin, while demonstrating a slight increase in activity on colloidal chitin.

2.5. The rate of enzymatic reaction of wtCHI60 and derivatives at low and high ionic strengths on amorphous and crystalline substrates

The rate of enzymatic hydrolysis of colloidal chitin by wtCHI60 and the W69F mutant was studied at low and high ionic strengths to determine if the enzyme forms a non-productive complex at high ionic strength conditions. Furthermore, the rate of enzymatic hydrolysis of β-crystalline chitin by wtCHI60 and the W69Y/W245Y was studied to confirm that the enzyme continues to work processively on β-crystalline chitin at high ionic strength conditions.

In the absence of NaCl, the hydrolysis reaction of colloidal chitin by wtCHI60 and the W69F mutant proceeded with both enzymes to substrate depletion within 50 min. However, in the presence of 2.0 M NaCl, the activity of wtCHI60 was abolished after

Table 2
Effect of the Trp mutations on chitinase activity[a]

Chitinase	Colloidal chitin relative activity	β-Crystalline chitin relative activity
WT CHI60	1.0	1.0
W33F	2.68	2.16
W33Y	0.98	1.05
W69F	2.11	3.42
W69Y	0.98	0.95
W245F	1.50	1.42
W245Y	1.30	1.26
W33F/W69Y	0.86	0.53
W33F/W245F	1.25	0.79
W69Y/W245Y	1.36	0.68

[a] The activity of wtCHI60 and derivatives, in the absence of NaCl, in the activity assay on colloidal and β-crystalline chitin (see methods) displayed as relative activity of each chitinase derivative compared to (normalized to) wtCHI60. The activity of wtCHI60 on colloidal and β-crystalline chitin was 44 and 19 mU, respectively. Data are presented as the mean derived from at least four independent repeats.

10 min, yielding approximately 30% of reducing sugar compared to that in the absence of NaCl addition (Fig. 5A). In contrast, although the W69F activity rate was reduced it was observed throughout the reaction period, yielding 65% of reducing sugar at 60 min compared to that in the absence of NaCl.

On β-crystalline chitin in the absence of NaCl, hydrolysis reaction of β-crystalline chitin by wtCHI60 and the W69Y/W245Y mutant proceeded with both enzymes to substrate depletion within 60 min. Interestingly, the activity rate of W69Y/W245Y mutant was significantly higher than that of wtCHI60 (Fig. 5B). In the presence of 2.0 M NaCl, although both the wtCHI60 and W69Y/W245Y mutant activity rates were reduced, it was observed throughout the reaction period. W69Y/W245Y mutant demonstrated a significantly higher activity rate than wtCHI60 after the first 25 min, yielding 90% and 74% of reducing sugar at 60 min compared to the enzymes in the absence of NaCl, respectively.

3. Discussion

3.1. The effect of mutations on substrate binding activity

Changing the exposed tryptophan-track, Trp-33 and Trp-69 in the N-terminal domain, and Trp-245 in the catalytic domain,

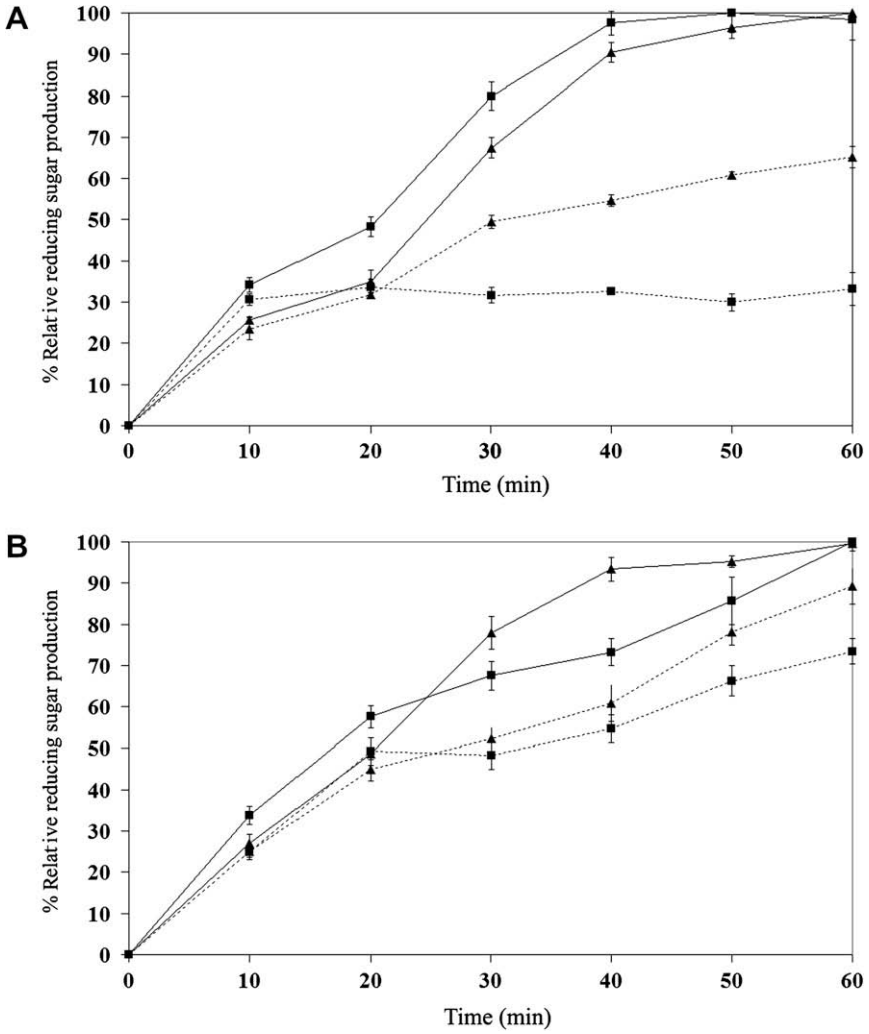

Figure 5. The rate of enzymatic hydrolysis of wtCHI60 and derivatives at low and high ionic strength conditions on amorphous substrate and crystalline substrate. The rate of hydrolysis of wtCHI60 and W69F on colloidal chitin, 2 mg/mL (A) and the rate of hydrolysis of wtCHI60 and W69Y/W245Y on β-crystalline chitin, 670 mg/mL (B) were determined at low and high ionic strength. Reactions were performed in 100 mM citrate buffer pH 5.0, at 37 °C for 60 min. For high ionic strength, NaCl was added to 2.0 M final concentration. Figure (A) shows –■–, WT CHI60 in the absence of NaCl; –▲–, W69F in the absence of NaCl; –■–, WT CHI60 in the presence of 2.0 M NaCl, and –▲–, W69F in the presence of 2.0 M NaCl. Figure (B) shows –■–, WT CHI60 in the absence of NaCl; –▲–, W69Y/W245Y in the absence of NaCl; –■–, WT CHI60 in the presence of 2.0 M NaCl, and –▲–, W69Y/W245Y in the presence of 2.0 M NaCl.

residues of CHI60 to Phe or Tyr reduced the interaction between CHI60 and its chitin substrate. Substrate binding was particularly affected by changes in Trp-69, which is located at the far end of the N-terminal domain, suggesting a particular importance of Trp-69 (compared to Trp-33 and Trp-245) for the substrate binding activity of CHI60, consistent with that previously observed when Trp-69 was changed to Ala.[22,29] We found that changing Trp-33 to Tyr had a greater effect on the binding activity than Trp-33 to Phe (relative binding activity of 0.69 compared to 0.77) as expected, because Phe has greater hydrophobicity than Tyr (−2.5 vs −2.3 kcal/mol).[33,34] However, we did not observe significant differences in binding activity between Tyr and Phe substitutions at Trp-69 and Trp-245, suggesting that not only is the hydrophobicity at these positions important for enzyme substrate interaction, but also that the position as well as the structure and conformation of the amino acid residue plays a role on the enzyme–substrate interaction.

3.2. CHI60 possesses both *exo*- and *endo*-type activities

CHI60 uses an *endo*-type mode when hydrolyzing soluble chitin (PNAC) involving mainly the catalytic domain, and this activity is independent of the Trp residues in the tryptophan-track that are involved in substrate binding. This is clearly supported by the observations that single and double substitution mutants did not significantly alter the observed enzyme kinetics on this substrate, and all mutants and wild type enzyme activities on this substrate were essentially independent of increases in the ionic strength of the reaction buffer (Fig. 4A and D). Interestingly, a slight increase in CHI60 activity on PNAC was observed as the ionic strength was increased from 0 to 0.5 M NaCl, which may be due to the enhancement of weak interactions of the tryptophan-track and/or the hydrophobic and aromatic residues within the substrate binding cleft to PNAC.

CHI60 also appears to use predominantly an *endo*-type mode when it hydrolyzes amorphous chitin as expected, as the enzyme cannot move processively along the entangled chitin strand, but can only move processively through short stretches of the exposed chitin chain on the substrate. Based on this scenario, the prediction that increased ionic strength buffers would decrease the observed *endo*-chitinase activity, due to increasingly more of the enzyme being unable to disassociate and thus remaining bound to the substrate in a non-productive complex, was supported by two sets of experimental data herein. Firstly, wtCHI60 displays high activity at low salt concentrations which decreases with increasing salt concentrations (Fig. 4B), and the rate of hydrolysis of wtCHI60 and W69F is similar at low ionic strength but at 2.0 M NaCl, wtCHI60 losses its activity while W69F remained active, albeit at a reduced rate (Fig. 5A). The activity, approximately 30%, observed in the first 10 min was probably a result of the enzyme hydrolyzing processively through short stretches of exposed chitin chain on the colloidal chitin substrate. Moreover, when the high salt buffer of the wtCHI60 was removed and replaced with salt-free buffer the activity of wtCHI60 was recovered to more than 80% (data not shown). Taken together, these results support that CHI60 performs an *endo*-chitinase activity on amorphous substrate, and at high ionic strength buffers the enzyme can be entrapped in an enzyme–substrate non-productive complex.

In contrast, CHI60 predominantly uses an *exo*-chitinase mode when it processively hydrolyzes β-crystalline chitin by entering the hydrolytic site at the amorphous region using the *endo*-type mode or the reducing ends of the substrate using the *exo*-type mode. The hydrolysis then proceeds processively along the chitin chain. Thus, as the ionic strength was increased from 0 to 2.0 M NaCl the activity of wtCHI60 decreased from 100%, using *exo*- and *endo*-type mode, down to approximately 50% (Fig. 4C), using mainly *exo*-type mode processively, where it continues to hydrolyze the substrate at high ionic strength (Fig. 5B). This reduction in activity may be caused by the forming of non-productive complexes at amorphous regions or by the slower processive rate of the enzyme in this mode.

S. marcescens produces three distinct chitinase enzymes, ChiA, ChiB, and ChiC, which favor different *exo*- or *endo*- modes of catalysis.[20,30] The *chi*60 gene is almost identical at the nucleotide sequence level to ChiA. Moreover, the deduced amino acid sequence of CHI60 is found to be identical to ChiA; therefore, CHI60 is also predicted to be an *exo*-chitinase, hydrolyzing chitin strands from the reducing end.[22] Nevertheless, the ability of CHI60 to readily hydrolyze both amorphous chitin and crystalline chitin suggests that CHI60 possesses a dual mode of hydrolysis, that is, both competent *endo*- and *exo*-chitinase activities, depending on the type of substrate it hydrolyzes and the ionic strength of the environment. A recent report has suggested that the *exo*-type of activity observed for ChiA during hydrolysis of crystalline chitin was due to the better accessibility of chain ends, rather than an intrinsic property of the enzyme.[30] In contrast, we have shown herein that mutations of the Trp residues involved in substrate binding of wtCHI60 can modulate the *exo*- and *endo*-type activities of the enzyme on insoluble substrates, clearly demonstrating that the *exo*- and *endo*-type modes of catalysis of CHI60 is an intrinsic property of the enzyme. However, this property can be modulated by the surrounding ionic strength and mutations, which reduce the enzyme–substrate interactions. Furthermore, other reports have suggested that some fungal β-1,4-glycosylhydrolase enzyme may also possess both *exo*- and *endo*-type activities.[31,32]

3.3. The effect of mutations of the Trp residue on enzyme substrate interaction and mode of hydrolysis

We used the single and double Trp substitution derivatives of CHI60 to evaluate further the role of Trp-33, Trp-69, and Trp-245 on binding and guiding the substrate during hydrolysis, and to confirm our hypothesis on the mode of catalysis. As already noted, the effect of ionic strength on the enzyme activity on PNAC as substrate of all six single point mutants was similar to wtCHI60 (Fig. 4A), indicating that the enzyme retains its activity throughout the ionic strength range (0–2 M NaCl) studied. Moreover, as expected, on colloidal chitin, substitutions of Trp to either Phe or Tyr that reduced the hydrophobic interaction between enzyme and substrate, shifted the enzyme activity profile above wtCHI60 as the ionic strength increased (Fig. 4B), demonstrating that the single point mutants were affected less by the high ionic strength than wtCHI60. We hypothesize that the weaker substrate binding activity of these mutants (Table 1) allows them to function better at high ionic strengths by allowing the enzyme to dissociate and then re-associate at a new substrate site and so not form locked enzyme–substrate complexes at elevated ionic strengths. This notion is further supported by the enhanced rate of hydrolytic activity at 2.0 M NaCl of W69F comparing to wtCHI60 (Fig. 5A). Interestingly, mutant W69Y which revealed a similar binding activity to W69F, demonstrated a much lower enzyme activity at 2.0 M NaCl, suggesting that the characteristics and the conformation of the amino acid residue are also important for the enzyme substrate interaction.

On β-crystalline chitin, the predicted reduction of enzyme–substrate interaction of the single point mutants to allow the enzyme to be more active than wtCHI60 was supported at slightly elevated ionic strengths (0–1.0 M NaCl), but only a minor improvement at 2.0 M NaCl was seen compared to wtCHI60 (Fig. 4C). The increase in enzymatic activity of the CHI60 derivative on β-crystalline chitin at elevated ionic strengths may possibly be caused by the reduced interaction strength between substrate and enzyme allowing the

enzyme either to be liberated from the amorphous regions of the substrate better or to move along the chitin chain more efficiently. Substitution of Trp-33 by either Phe or Tyr both reduced the activity of the enzymes compared to wtCHI60, as the ionic strength increased. Surprisingly, W33F displayed a lower activity than W33Y, suggesting that Trp-33, or any conformational change that W33F may also induce, may play an important role in the processivity of the enzyme. In this scenario, changing Trp-33 renders the enzyme to be less processive, either by reducing the ability of the enzyme to stay on the substrate or its ability to move along the chitin strand.

We hypothesized that double substitutions would further reduce the interaction between the enzyme and substrate and therefore enhance the effects already observed with the single point mutations. As expected, the activity of all three double mutations on PNAC was not affected by ionic strength (Fig. 4D). Curiously though, on colloidal chitin, all the double point mutants did not show increased enzyme activity compared to the single point mutants at elevated ionic strength (Fig. 4E), despite having a reduced binding ability in two out of the three cases (Table 1). Perhaps the remaining enzyme–substrate interaction is contributed by the aromatic residues within the catalytic domain of CHI60, although this remains to be verified by experimental and modeling approaches. Regardless, under this notion a further decrease in enzyme–substrate interactions contributed by a second point mutation of the Trp residues in the N-terminal tryptophan-track cannot further increase the activity of the enzyme at high ionic strength conditions. In some support for this we have also found that mutation of different Trp sites in CHI60 (Trp-275 and Trp-418 within the catalytic domain) to Ala can also relieve the inhibitory effect of high salt on the enzyme (data not shown). On the other hand, with β-crystalline chitin as substrate, the enzyme activity of all double point mutants was slightly higher than that of all the single point mutants at high ionic strength. Thus, although further reduction in the substrate-enzyme interaction does not affect the enzymatic activity on amorphous substrates, it does have a further positive effect on crystalline substrates in high ionic strength buffers. Perhaps double mutations increase the processively hydrolyzed β-crystalline chitin at high ionic strengths by allowing the enzyme to move along the substrate more efficiently given that the enzyme–substrate interaction of double mutants is lower than wtCHI60 and single mutants. This idea can be further supported by our finding that the double substitution mutant W69Y/W245Y gave a higher activity rate than wtCHI60 at 2 M NaCl, where it mainly hydrolyzed β-crystalline chitin processively using *exo*-type activity (Fig. 5B) The two outer Trp residues, Trp-69 and Trp-33, have impact on the activity of CHI60 more than Trp-245.

We conclude that CHI60 has a dual mode of catalysis, which can be regulated by the modulation of the enzyme–substrate interaction, either by point mutations of Trp residues in the tryptophan-track or by changing the surrounding ionic strength. A model mechanism of how CHI60 hydrolyses chitin is proposed based on the type of substrate and is shown in Figure 6. For soluble substrates, the substrate can diffuse into the catalytic site independent of the chitin binding domain and thus the activity of CHI60 is not affected by ionic strength (Fig. 6A).

For insoluble substrates, CHI60 uses the N-terminal domain to latch onto and bind to the substrate. On amorphous chitin, CHI60 cannot processively hydrolyze the chitin chain, instead CHI60 uses an *endo*-type mode of hydrolysis. CHI60 binds and hydrolyzed amorphous chitin chain then dissociates to the next cleavage site to continue the hydrolysis process. CHI60 can also be entrapped into an enzyme–substrate non-productive complex at elevated ionic strengths (Fig. 6B). However, on β-crystalline chitin the enzyme can processively hydrolyze the substrate and is only partially affected by high ionic strength (Fig. 6C).

4. Experimental

4.1. Bacterial strains and plasmids

Escherichia coli DH5α was the host strain used throughout the construction and expression of the *chi*60 gene and its derivatives. pKKCHI60 was generated from shotgun cloning of the wild type *chi*60 from *Serratia* sp. TU09 into pBluescript SK⁻. The *chi*60 gene was sequenced (Genbank accession number AY040610) and will be described elsewhere.

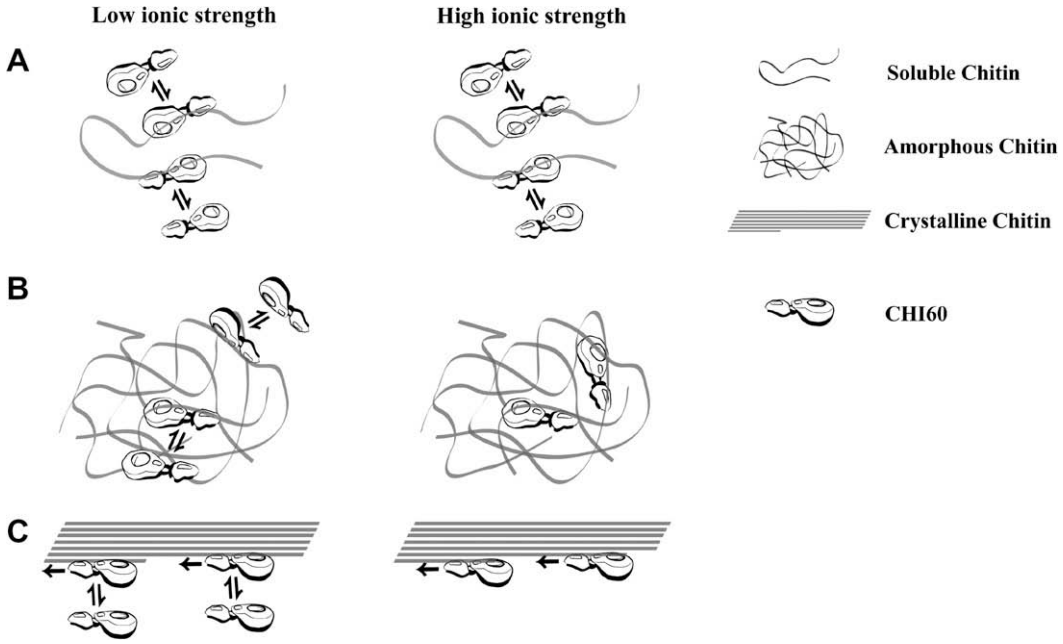

Figure 6. Model for hydrolysis of CHI60 on soluble, amorphous, and β-crystalline substrates.

4.2. Site-directed mutagenesis

Site-directed mutagenesis was carried out by polymerase chain reaction using a QuickChange site-directed mutagenesis kit (Stratagene) and pKKCHI60 as the template. The primers used for site-directed mutagenesis at residue Trp-33, Trp-69, and Trp-245 to Phe and Tyr were 5′-CGA CCA TCG CC*T (T/A)C*G GCA ATA CCA A-3′ and 5′-TAT TGC C*G(T/A) A*GG CGA TGG TCG GCT T-3′,5′-GGA ATT TA*T (A/T)C*A ATG GCG AC-3′ and 5′-CGC CAT TG*(A/T) A*TA AAT TCC AG-3′, and 5′-CGT TACCGC C*T(T/A) C*GA TGA CC-3′ and 5′-GGT CAT C*G(A/T) A*GG CGG TAA CG-3′, respectively. Following mutation and cloning in DH5α the respective clones were selected by DNA sequencing. The entire open reading frame of each derivative was sequenced to ensure that the desired mutation was the only mutation in each *chi*60 derivative. DNA sequencing was done using an automated laser fluorescence DNA sequencer (Model CEQ8000; Beckman) and the CEQ™ DTCS-Quick Start Kit (Beckman Coulter™). The sequence data were analyzed by GENETYX computer software. To construct the gene encoding CHI60 with double mutation (W33F/W69Y, W33F/W245F, and W69Y/W245Y), the second mutation was introduced by using the respective single point mutant as the template for each second site mutation.

4.3. Expression and purification of wtCHI60 and its mutants

Wild type CHI60 (wtCHI60) and its mutants were expressed in *E. coli* DH5α cells carrying plasmid pKKCHI60 or its derivatives, using the constitutively expressing endogenous promoter present in *chi60 gene*. CHI60 and derivatives were purified from the culture supernatant. For chitinase production, the culture was grown in LB medium containing 100 μg/mL ampicillin, at 37 °C. Chitinase purification was achieved by ultrafiltration (MW cut off 30 kDa) followed by DEAE-cellulose chromatography and chitin affinity adsorption, respectively. Crude chitinase was applied to a DEAE-cellulose column equilibrated with 20 mM Tris–HCl buffer pH 7.0. The enzyme was found in the unbound fractions, which were pooled, adjusted to 0.5 M Tris–HCl buffer pH 7.0 and adsorbed on colloidal chitin at 4 °C for 2 h. The chitinase-colloidal chitin complex was collected by centrifugation at 9800*g*, for 20 min at 4 °C. Chitinase was released by incubation in 50 mM Tris–HCl buffer pH 7.0 at 37 °C for 2 h and dialyzed against excess of the same buffer to remove the hydrolytic products. Purification was followed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using 10% (w/v) slab gels with comassie blue staining.[23]

4.4. Enzyme and protein assays

Chitinase activity was determined by measuring the amount of reducing end groups, generated by the degradation of various chitinous substrates, colorimetrically with ferric ferrocyanide reagent by a modified Schale's method, using *N*-acetyl-D-glucosamine as standard.[24] One unit (U) of chitinase activity was defined as the amount of enzyme able to liberate 1 μmol of *N*-acetyl-D-glucosamine per minute. Assays were done in triplicate. Protein concentration was determined by a Bradford assay,[25] using bovine serum albumin as standard.

4.5. Chitin binding assay

Binding assay was performed in 500 μL of 100 mM Tris–HCl buffer, pH 7.0 containing a total protein concentration of 0.5 μg. wtCHI60 or Trp substitution derivatives were incubated with 25 mg colloidal (excess amount) chitin on ice for 2 h. Each mixture was then centrifuged at 15,000*g*, for 20 min at 4 °C. The supernatant containing unbounded protein was collected, and the protein concentration was determined. The amount of bound protein was calculated by subtracting the amount of unbound protein from the total protein initially added. Each reaction was done independently and the data are presented as the mean ± 1 SD derived from at least four independent repeats.

4.6. Effect of ionic strength on the hydrolytic activity of wtCHI60 and derivatives

The hydrolytic activity of wtCHI60 and derivatives was studied on soluble (partially N-acetylated chitin (PNAC), 0.5 mg/mL) and insoluble substrates, amorphous (colloidal chitin, 1 mg/mL), and crystalline (β-crystalline chitin, 10 mg/mL), in 20 mM citrate buffer pH 5.0 with NaCl added from 0 to 2 M as indicated. The reaction was performed in a total volume of 750 μL at 37 °C for 30 min. An equal amount of enzymatic activity, as determined by assaying on PNAC as substrate, for wtCHI60 and each derivative was used on each type of substrate; 250 mU, 1200 mU, and 2400 mU of purified enzyme were used for soluble, amorphous and crystalline substrates, respectively. The different amount of enzyme used on each substrate was due to the difference in substrate specificity. Each reaction was repeated independently, and data are presented as the mean ± 1 SD derived from at least four independent repeats.

4.7. Rate of enzymatic reaction of wtCHI60 and derivatives at low and high ionic strengths on amorphous and crystalline substrates

Hydrolysis of colloidal chitin was carried out in a reaction mixture containing 200 mU/mL of purified wtCHI60 or W69F chitinase (assayed on colloidal chitin) and 2 mg/mL of colloidal chitin in 100 mM citrate buffer, pH 5.0. Hydrolysis of β-crystalline chitin was carried out in a reaction mixture containing 80 mU/mL of purified wtCHI60 or W69Y/W245Y chitinase (assayed on β-crystalline chitin) and 670 mg/mL of β-crystalline chitin in 100 mM citrate buffer, pH 5.0. For high ionic strength, NaCl was added to the reaction mixture to a final concentration of 2.0 M. Reactions proceeded at 37 °C for 60 min. Aliquots were taken every 10 min and stopped by immersing the sample in boiling water for 15 min. The amount of product at each time point was then determined as previously described.

4.8. Chemicals

Shrimp shell and squid pen chitin were purchased from Ta Ming Enterprises Co., Ltd (Samutsakon, Thailand). Partially N-acetylated chitin (PNAC) at 50% degree of deacetylation was prepared from squid pen chitin by homogenous deacetylation of alkaline chitin solution. Colloidal chitin was prepared from powdered shrimp shell chitin by the methods described by Jeuniaux[26] and Yamada and Imoto.[27] β-Crystalline chitin was prepared from squid pen chitin by grinding a 5% (w/v) suspension in water using a high speed blender at 10,000 rpm until well swollen.

Acknowledgments

The authors would like to thank Dr. Robert Douglas Butcher, Dr. Saowarath Jantaroa, and Dr. Kuakarun Krusong, Faculty of Science, Chulalongkorn University for critically reading the manuscript and providing valuable suggestions. We would also like to especially thank Mr. Thanakorn Theerapanon, for his assistance on the graphics presented in this work. This work was partially supported by the Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University and the Center for Chitin-Chitosan Biomaterials, Metallurgy and Material Science Research Institute, Chulalongkorn University.

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