Aromatic residues within the substrate-binding cleft of *Bacillus circulans* chitinase A1 are essential for hydrolysis of crystalline chitin

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Bacillus circulans chitinase A1 (ChiA1) has a deep substrate-binding cleft on top of its (β/α)₈-barrel catalytic domain and an interaction between the aromatic residues in this cleft and bound oligosaccharide has been suggested. To study the roles of these aromatic residues, especially in crystalline-chitin hydrolysis, site-directed mutagenesis of these residues was carried out. Y56A and W53A mutations at subsites –5 and –3, respectively, selectively decreased the hydrolysing activity against highly crystalline β -chitin. W164A and W285A mutations at subsites +1 and +2, respectively, decreased the hydrolysing activity against crystalline β -chitin and colloidal chitin, but enhanced the activities against soluble substrates. These mutations increased the K_m -value when reduced (GlcNAc)₅ (where GlcNAc is *N*-acetylglucosamine) was used as the substrate, but decreased substrate inhibition observed with wild-type ChiA1 at higher concentrations of this substrate. In

contrast with the selective effect of the other mutations, mutations of W433 and Y279 at subsite –1 decreased the hydrolysing activity drastically against all substrates and reduced the k_{cat} -value, measured with 4-methylumbelliferyl chitotrioside to 0.022 % and 0.59 % respectively. From these observations, it was concluded that residues Y56 and W53 are only essential for crystalline-chitin hydrolysis. W164 and W285 are very important for crystalline-chitin hydrolysis and also participate in hydrolysis of other substrates. W433 and Y279 are both essential for catalytic reaction as predicted from the structure.

Key words: aromatic amino acid, crystalline-chitin hydrolysis, catalytic domain, site-directed mutagenesis, substrate-binding cleft.

INTRODUCTION

In the classification system of glycoside hydrolases based on the amino-acid-sequence similarity established by Henrissat and co-workers [1–3], chitinases are classified into two different families: families 18 and 19. Many chitinolytic bacteria produce only family-18 chitinases, while other bacteria, such as *Streptomyces* species, produce both family-19 and family-18 chitinases [4,5]. Bacterial family-18 chitinases are further classified into three subfamilies: subfamilies A, B and C [6]. Chitinases in subfamily A have an insertion domain between the seventh and eighth β -strands of the (β/α)₈ barrel basic structure, while chitinases in subfamilies B and C do not have such an insertion domain. The three-dimensional (3D) structures of three bacterial family-18 chitinases, all of which belong to subfamily A, have been determined; these are chitinase A (ChiA) and chitinase B (ChiB) from *Serratia marcescens* QMB1466 [7,8] and chitinase A1 (ChiA1) from *Bacillus circulans* WL-12 [9].

B. circulans WL-12 produces ChiA1, ChiC1 and ChiD1 as the initial products of the three chitinase genes [10]. ChiA1 has the highest hydrolysing activity against insoluble chitin. This chitinase comprises the catalytic domain (CatD), two fibronectin type III-like domains (FnIIID) and the C-terminal chitin-binding domain (ChBD) [11,12]. 3D structures of the domains of *B. circulans* ChiA1 were determined separately: CatD by X-ray crystallography and ChBD and FnIIID by NMR [9,13,14]. CatD_{ChiA1} consists of an (β/α)₈-(triosephosphate isomerase) TIM-barrel, and two small insertion domains, β -domain 1 and β -domain 2, that are attached on top of the TIM-barrel provide

a deep cleft for substrate binding [9]. The crystal structure of inactivated CatD_{ChiA1} complexed with (GlcNAc)₇ (where GlcNAc is *N*-acetylglucosamine) suggests that cleavage of the chitin chain occurs at the second linkage from the reducing end and the presence of seven subsites, numbered –5 to +2, in the substrate-binding cleft was deduced from the complexed structure. In addition, outside of the substrate-binding cleft, two exposed tryptophan residues (W122 and W134) were found to be aligned on the extension of the oligomer chain bound to the cleft. These two aromatic residues have been shown to be essential for hydrolysis of crystalline chitin and have been proposed to play an important role in guiding a chitin chain into the substrate-binding cleft during crystalline-chitin hydrolysis [15].

Unlike the *B. circulans* ChiA1, the 3D structures of the entire molecules of the *S. marcescens* ChiA and ChiB were determined by X-ray crystallography [7,8]. The 3D structure of CatD of *Serratia* ChiA is basically very similar to CatD_{ChiA1}. Two exposed aromatic residues outside of the substrate-binding cleft and four aromatic residues in the cleft of CatD_{ChiA1} are all conserved in *S. marcescens* ChiA. In addition, two additional aromatic residues were found on the surface of the N-terminal domain of *S. marcescens* ChiA. All four exposed aromatic residues outside of the cleft have been shown to be essential determinants for crystalline-chitin hydrolysis. Three of them, two in the N-terminal domain and one in the catalytic domain, play vital roles in chitin binding and the remaining residue appears to be important for guiding the chitin chain into the substrate-binding cleft. Based on these observations, a model for the processive hydrolysis of crystalline chitin by ChiA has been proposed [16].

Abbreviations used: CatD, catalytic domain; ChBD, chitin-binding domain; ChiA1, chitinase A1; CM, carboxymethyl; 3D, three-dimensional; FnIIID, fibronectin type III-like domain; GlcNAc, *N*-acetylglucosamine; 4MU, 4-methylumbelliferone; 4MU-(GlcNAc)₃, 4-methylumbelliferyl-tri-*N*-acetylchitotriose; TIM, triosephosphate isomerase.

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In the present study, to clarify the roles of the aromatic residues within the deep substrate-binding cleft of *B. circulans* ChiA1, especially in crystalline-chitin hydrolysis, site-directed mutagenesis of these residues was carried out and alteration of the hydrolysing activity against various chitinous substrates was studied. The ultimate goal of this study was to understand how soluble chitinases degrade insoluble/crystalline substrates.

EXPERIMENTAL

Bacterial strains and plasmids

Escherichia coli JM109 cells were used as the host strain throughout the construction of various recombinant plasmids. Recombinant plasmid pHT012 carries the entire *chiA* gene from *Bacillus circulans* WL-12 [12]. pCatD, which carries the truncated *chiA* gene encoding only CatD, was constructed by replacing the *ApaI*–*HindIII* DNA region in pHT012 by the DNA fragment amplified by PCR using primers 5'-CGCATATGATAAATTTAAATAACA-CACTGC-3' and 5'-AAGCTTACAGATCGGCCCTTCAGTTT-3'. pHT002mut carrying an approx. 800-bp *ApaI*–*Bam*HI fragment, pHT012mutA carrying an approx. 700-bp *NcoI*–*NcoI* fragment, and pHT012mutB carrying an approx. 1050-bp *Bam*HI–*HindIII* fragment from the *chiA* gene [15] were used as the templates for site-directed mutagenesis by PCR.

Chemicals

Colloidal chitin and glycol chitin were prepared by the methods of Jeuniaux [17] and Yamada and Imoto [18] respectively. Chitin EX (powdered prawn-shell chitin), used in the chitin affinity-column chromatography for purification of ChiA1, and carboxymethyl chitin (CM-chitin) were purchased from Funakoshi Chemical Co., Tokyo, Japan. Highly crystalline β -chitin microfibrils from vestimentiferan *Lamellibrachia satsuma* were prepared as described previously [19]. Reduction of (GlcNAc)₅ was carried out as described previously [20]. (GlcNAc)₅ and (GlcNAc)₆ were obtained from Yaizu Suisan Chemical Co. Ltd, Shizuoka, Japan. 4-Methylumbelliferyl-tri-*N*-acetylchitotriose [4MU-(GlcNAc)₃] was purchased from Sigma.

Site-directed mutagenesis

Site-directed mutagenesis was carried out by PCR using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Primers used for the mutagenesis are summarized in Table 1. The mutant clones were selected after sequencing using an automated laser fluorescence DNA sequencer (Model 4000L; LI-COR).

Production and purification of ChiA1, CatD_{ChiA1} and their mutants

Wild-type and mutant chitinases were produced in *E. coli* JM109 cells carrying pHT012, pCatD and their derivatives that encoded various mutant chitinases. *E. coli* JM109 cells carrying a plasmid were grown for 20–23 h at 30 °C in Luria–Bertani medium containing 100 µg/ml ampicillin. The cells were then collected by centrifugation, chitinases were extracted from the cells by a cold osmotic-shock procedure [21] and collected by ammonium sulphate precipitation at 40% saturation for wild-type and mutant ChiA1, and at 60% saturation for wild-type and mutant CatD_{ChiA1}. ChiA1 and its mutants were then purified by chitin affinity column chromatography [22], and wild-type CatD_{ChiA1} and its mutants were purified by HPLC with a Poros HS/M column (PerSeptive Biosystems, Framingham, MA, U.S.A.).

SDS/PAGE analysis of purified chitinases was conducted by the method of Laemmli [23].

Table 1 Primers used for site-directed mutagenesis

Mutant	Primer
W53A	Forward 5'-CTATCCGTCGCGGCTGCGTACGG-3' Reverse 5'-CCGTACGCAGCCGCGGACGGATAG-3'
Y56A	Forward 5'-CCTGGGCTGCGGCCGCGAGAAC-3' Reverse 5'-GTTTCTGCGGCGCCAGCCAGG-3'
Y56A/W53A	Forward 5'-CTATCCGTCGCGGCTGCGGCCGCGAGAACATAATGTAG-3' Reverse 5'-CTACATTATAGTTTCTGCCGCGCCAGCCGCGGACGGATAG-3'
W164A	Forward 5'-CCGTTGGAGGAGCGACGTGGTCC-3' Reverse 5'-GGACCACGTCGCTCCTCCAACGG-3'
W285A	Forward 5'-CGGGGCTGCGCAAAAATCAGCGC-3' Reverse 5'-GCGCTGATTTTTGCGCAGCCCCG-3'
W164A/W285A	This mutant was made by connecting two DNA fragments, one carrying the W164A mutation and the other carrying the W285A mutation.
W433A	Forward 5'-GCGATGTCGCGGAGCTCAGCGG-3' Reverse 5'-CCGCTGAGCTCCGCAACATCGC-3'
Y279F	Forward 5'-GGATTAAACATTATGACATTCGATTTTAACG-3' Reverse 5'-CGTTAAATCGAATGTCATAATGTTAATCC-3'

Chitin-binding assay

Binding-assay mixtures in 1-ml glass microtubes containing various concentrations of chitinase protein and 0.5 mg of β -chitin microfibrils in 0.5 ml of 20 mM sodium phosphate buffer (pH 6.0) were incubated on ice with occasional mixing. Each mixture was centrifuged at 4 °C for 20 min at 10400 g to separate the supernatant from β -chitin microfibrils with bound protein. The supernatant containing free protein was collected, and the protein concentration was determined. The amount of bound protein was calculated from the difference between the initial protein concentration and the free protein concentration after binding.

Enzyme and protein assay

Reducing sugars generated by the degradation of β -chitin microfibrils, colloidal chitin, glycol chitin, CM-chitin and reduced (GlcNAc)₅ were measured by a modification of Schales' procedure [24] using (GlcNAc)₂ as the standard. Each assay mixture (total volume, 350 µl) contained 150 µg (dry weight) of substrate and various concentrations of enzyme in 0.1 M sodium phosphate buffer (pH 6.0).

Kinetic studies of wild-type and mutant chitinases were carried out using 4MU-(GlcNAc)₃ as the substrate. Reaction mixtures containing purified chitinase and various concentrations of 4MU-(GlcNAc)₃ in 0.1 M sodium phosphate buffer (pH 6.0) were incubated at 37 °C. The reactions were terminated by the addition of 0.4 M phosphate buffer (pH 11.9), and the amount of released 4-methylumbelliferone (4MU) moiety was measured spectrofluorimetrically with excitation at 360 nm and emission at 450 nm using a Hitachi spectrofluorometer F-3010 (Hitachi, Tokyo, Japan).

The protein concentration was estimated either by measuring the UV absorbance at 280 nm or by spectrofluorimetry at an excitation wavelength of 280 nm and an emission wavelength of 342 nm. The molar absorption coefficients used for the UV method were calculated from the amino-acid compositions of wild-type and mutant chitinases [25].

Analysis of hydrolysis products from (GlcNAc)₆

Hydrolysis of (GlcNAc)₆ by wild-type and mutant chitinases was carried out in 10 mM sodium phosphate buffer (pH 6.0) at 37 °C; the concentrations of the enzyme and substrate were 25 nM and 1.0 mM respectively. After incubation for a given period, the enzymic reaction was terminated by boiling the reaction mixture

Figure 1 Targets for site-directed mutagenesis

The relative positions of Y56, W53, W433, W164, W285, and Y279 are shown in dark grey, with the bound (GlcNAc)₇ in light grey. W134 and W122 are the residues on the surface of the catalytic domain and have been shown to be essential for crystalline-chitin hydrolysis. Q204, the substitute for the catalytic residue E204, is shown in black. Dotted line represents hydrogen bonding.

for 5 min. The mixture was then filtered through a cellulose acetate membrane (pore size, 0.45 μm). HPLC analysis of the hydrolysis products in the filtrate was carried out with an Ultron-NH₂ column (0.45 cm $\times$ 25 cm) in a liquid chromatograph (LC-6A system; Shimadzu Corporation, Kyoto, Japan) with water/acetonitrile as the eluent. Elution was monitored by measuring absorbance at 200 nm with a Shimadzu SPD-6A spectrophotometer.

RESULTS

Target amino acids for site-directed mutagenesis

Structural study of the inactivated catalytic domain of *B. circulans* ChiA1 (E204Q) complexed with (GlcNAc)₇ suggested that five aromatic residues, Y56, W53, W433, W164 and W285, interact with the bound oligomer chain in the deep substrate-binding cleft through hydrophobic stacking interaction, as illustrated in Figure 1 [9]. Seven subsites, from -5 to +2, were deduced from the complex structure, and Y56, W53, W433, W164 and W285 interact with the sugar rings at subsites -5, -3, -1, +1 and +2 respectively. These aromatic residues stack against the faces of pyranoside rings on the side opposite to which carbonyl oxygens of acetyl groups are extended. The oligomer chain is bent and twisted at subsite -1 and it was suggested that W433 at this subsite plays a major role in the catalytic reaction by holding the GlcNAc residues at subsite -1, which is in the boat form. Another aromatic residue, Y279, is in the position that interacts with the acetyl group of the GlcNAc residue at the subsite through hydrogen bonding between the hydroxyl group of Y279 and the carbonyl oxygen of the acetyl group. In addition, one water molecule was found to link the hydroxyl group of Y279 and the NH group of -1 GlcNAc. Such interactions suggest that this residue assists in the formation of oxazolinium-ion intermediates during the catalytic reaction by 'substrate-assisted catalysis'. W285 at subsite +2 and W164 at subsite +1 face the first and

second GlcNAc residues, respectively, starting from the reducing end of the oligomer chain that lies between the two tryptophan residues.

To clarify the roles of these aromatic residues, Y56, W53, W433, W164 and W285 were replaced by alanine, and Y279 was replaced by phenylalanine, and alterations in the enzymic properties caused by these mutations were studied. Wild-type and mutant chitinases were produced in *E. coli* cells and purified either by chitin affinity-column chromatography or by HPLC. The purified chitinases exhibited a substantial single protein band in SDS/PAGE analysis, as shown in Figure 2. Biochemical characterization was carried out using these purified preparations.

The effects of mutations on the hydrolysis of various chitinous substrates, including crystalline chitin, amorphous chitin and soluble chitin derivatives, were studied and are shown in Table 2. Figure 3 shows time courses of hydrolysis of crystalline β -chitin microfibrils by wild-type and mutant ChiA1s. When 12.6 pmol of ChiA1 was used, production of reducing sugar increased almost linearly for 10 min (up to a concentration of approx. 0.015 μmol of reducing sugar) and then gradually lost linearity. Therefore, the relative specific activities of β -chitin hydrolysis shown in Table 2 were determined by a 10-min reaction using amounts of various chitinases that give a reducing-sugar yield of less than 0.015 μmol .

Effect of mutations of Y56 and W53

Mutation of Y56 at subsite -5 or W53 at subsite -3 selectively decreased the specific hydrolysing activity against highly crystalline β -chitin microfibrils. The hydrolysing activities against the other substrates were not significantly affected, except for the effect of the W53A mutation on the activity on CM-chitin. This mutation increased the activity against CM-chitin by approx. 80%. The double mutation of the two aromatic residues almost abolished the hydrolysing activity against β -chitin microfibrils

Figure 2 SDS/PAGE analysis of purified ChiA1 and its mutants to show the purities of preparations used in the various experiments

(Top panels) Protein staining of the polyacrylamide gel with Coomassie Brilliant Blue R-250. (Lower panels) Chitinase activity detected on an agar replica of the polyacrylamide gel. Lane 1, wild-type ChiA1; lane 2, Y279F mutant; lane 3, W53A mutant; lane 4, Y56A mutant; lane 5, W164A mutant; lane 6, W285A mutant; lane 7, W164A/W285A mutant; lane 8, W433A mutant; lane 9, Y56A/W53A mutant. The amount of protein applied to each lane was 5 μ g.

Table 2 Relative specific hydrolysing activity (%) of ChiA1 and its mutants against various chitinous substrates

Chitinase/mutant	β -Chitin microfibrils	Colloidal chitin	CM-chitin	Reduced (GlcNAc) ₅
ChiA1	100.0	100.0	100.0	100.0
Y56A	7.4	105.3	108.8	105.9
W53A	3.5	92.0	177.9	97.2
Y56A/W53A	0.2	85.3	126.1	83.1
W164A	7.0	50.3	224.3	248.1
W285A	17.7	61.3	151.3	241.4
W164A/W285A	0.6	4.9	38.0	4.4
W433A	0.5	1.2	3.1	1.4
Y279F	3.2	3.8	6.8	1.6

and slightly decreased the activity against colloidal chitin and reduced (GlcNAc)₅. Double mutations increased the activity against CM-chitin, but it was significantly smaller than that by W53A mutation. These results demonstrated clearly that Y56 and W53 are required only for the hydrolysis of crystalline chitin, but not for soluble substrates. Furthermore, the presence of W53 appeared to obstruct the hydrolysis of CM-chitin.

To rule out the possibility that the observed defects in crystalline-chitin hydrolysis by mutant chitinase with either Y56A or W53A mutations are somehow related to their ability to bind to chitin, the chitin-binding activities of wild-type and mutant chitinases were compared using β -chitin microfibrils as the binding substrate. Figure 4 shows the binding isotherm of Y56A, W53A, wild-type ChiA1 and their catalytic domains. The binding activities of Y56A and W53A are indistinguishable from that of wild-type ChiA1, in clear contrast with the great loss of binding activity achieved by losing the chitin-binding domain, as

Figure 3 Hydrolysis of β -chitin microfibrils by wild-type and mutant ChiA1s

The amounts of chitinase used in the experiment were 12.6 pmol (ChiA1), 51.2 pmol (Y56A, W53A, W164A, W285A) or 204.8 pmol (W433A, Y279F, W164A/W285A and Y56A/W53A). The data shown in this Figure are standardized to the amount of reducing sugar produced by 12.6 pmol of each chitinase. $\blacklozenge$, ChiA1; $\bullet$, Y56A; $\blacktriangle$, W53A; $\blacksquare$, Y56A/W53A; $\diamond$, W433A; $\circ$, Y279F; $\triangle$, W164A; $\square$, W285A; $+$, W164A/W285A.

Figure 4 Equilibrium isotherms for the binding of wild-type and mutant chitinases to β -chitin microfibrils

$\bullet$, Wild-type ChiA1; $\blacktriangle$, ChiA1 Y56A mutant; $\blacksquare$, ChiA1 W53A mutant; $\circ$, wild-type CatD_{ChiA1}; $\triangle$, CatD_{ChiA1} Y56A mutant; $\square$, CatD_{ChiA1} W53A mutant.

demonstrated by the catalytic domains of wild-type and mutant chitinases. Therefore, it was confirmed that the drastically reduced β -chitin-hydrolysing activity caused by the Y56A and W53A mutations is not due to a change in their binding activity.

Effect of mutations of W164 and W285

Mutations of W164 at subsite +1 and W285 at subsite +2 greatly decreased the specific hydrolysing activity against highly crystalline β -chitin microfibrils (Table 2). In contrast with Y56A and W53A mutations, W164A and W285A mutations also decreased the activity against colloidal chitin by 40–50%. On the other hand, the hydrolysing activities against soluble substrates were increased by the W164A or W285A mutations, without exception. The hydrolysing activities of W164A and W285A against CM-chitin were approx. 1.5- and 2.2-fold higher, respectively, and those against reduced (GlcNAc)₅ were 2.5-fold higher when compared with wild-type ChiA1. The double mutation of W164

Table 3 Kinetic parameters of ChiA1 and its mutants

Chitinase	Substrate	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m
ChiA1	Reduced (GlcNAc) ₅	80.5	26.9	335*
W164A		206	35.6	173*
W285A		177	45.1	255*
ChiA1	CM-chitin	0.586‡	9.55	16.3†
W164A		0.258‡	24.1	93.4†
W285A		0.309‡	25.2	81.8†
ChiA1	4MU-(GlcNAc) ₃	2.20	22.4	10.2§
W433A		3.30	0.0050	0.0015§
Y279F		0.468	0.137	0.292§

* Units of k_{cat}/K_m in $mM^{-1} \cdot s^{-1}$.† Units of k_{cat}/K_m in $mg^{-1} \cdot ml \cdot s^{-1}$.‡ Units of K_m in mg/ml .§ Units of k_{cat}/K_m in $\mu M^{-1} \cdot s^{-1}$.

and W285 (W164A/W285A) greatly decreased the hydrolysing activity against all substrates tested, including reduced (GlcNAc)₅. This could be partly due to the structural effect of the double mutation, although CD spectra of the mutant chitinases are similar to that of wild-type ChiA1 (results not shown).

To study further the effect of mutations on the hydrolysis of soluble substrates, kinetic analyses using reduced (GlcNAc)₅ and CM-chitin were carried out. The amino-acid residues W164 and W285 are supposed to interact with GlcNAc residues placed at subsites +1 and +2 respectively. 4MU oligosaccharides, which are often used in the kinetic analyses of chitinases, are not suitable for kinetic study of W164A and W285A mutants because the 4MU moiety must be at the +1 position to be hydrolysed and liberate 4MU molecules. When hydrolysis of reduced (GlcNAc)₅ by wild-type ChiA1 was tested, substrate inhibition was observed at higher substrate concentrations, i.e. > 0.3 mM. Kinetic parameters were calculated from the data set below 0.3 mM and are shown in Table 3. Interestingly, such substrate inhibition was not observed when either the W164A or W285A mutants were tested. Mutation of either W164 or W285 increased both the k_{cat} and K_m values significantly. The k_{cat}/K_m values of the W164A and W285A mutants were approx. 50% and 66% those of ChiA1 respectively. These results suggest that the increase in hydrolysing activity against reduced (GlcNAc)₅ by the W164A or W285A mutations, shown in Table 2, is probably due to the diminution of substrate inhibition. In addition, the interaction of the GlcNAc residue(s) in the oligomer with either W164 or W285 was suggested to be, at least partially, a reason for the substrate inhibition observed with wild-type ChiA1. In contrast with oligomer hydrolysis, substrate inhibition was not observed in the hydrolysis of CM-chitin by wild-type ChiA1. Both W164A and W285A mutations decreased the K_m value and increased k_{cat} . Therefore, the presence of W164 or W285 simply interferes with hydrolysis of this soluble, long-chain substrate.

Since W164A and W285A mutations increased the hydrolysing activity against reduced (GlcNAc)₅, the products of (GlcNAc)₆ hydrolysis by W164A and W285A mutant chitinases were monitored to see whether these mutations affect the frequency of bond cleavage in the oligosaccharide (Figure 5). Wild-type ChiA1 generated a tetramer and dimer as the major products from (GlcNAc)₆ and the amount of (GlcNAc)₂ increased continuously during the reaction. (GlcNAc)₄ reached a plateau approx. 20 min after the beginning of the reaction and remained at the same level during the incubation period. This means that the cleavage of the second linkage from the reducing end of (GlcNAc)₆ occurred first, generating (GlcNAc)₂ and (GlcNAc)₄, and that (GlcNAc)₄ was

then cleaved into two (GlcNAc)₂ molecules. On the other hand, both W164A and W285A mutants produced (GlcNAc)₃ as the major product in addition to (GlcNAc)₂ and (GlcNAc)₄. Since the GlcNAc monomer was not produced in substantial amounts, (GlcNAc)₃ was not produced by the cleavage of (GlcNAc)₄. Therefore, cleavage at the middle of (GlcNAc)₆ by W164A and W285A mutant chitinases occurred frequently. Interestingly, the hydrolysis products from insoluble chitin were not affected by either W164A or W285A mutation and (GlcNAc)₂ was produced exclusively from β -chitin microfibrils and colloidal chitin by these mutant chitinases.

Effect of mutations of W433 and Y279

In contrast with the selective effect observed by the mutations of Y56, W53, W285 and W164, mutation of W433 at subsite -1 reduced drastically the hydrolysing activity against all substrates tested (Table 2). W433 is considered to interact with -1 GlcNAc through a hydrophobic stacking interaction, as mentioned above, holding this moiety at this position during the catalytic reaction. This result is consistent with the proposed role of this aromatic residue, showing that it is indispensable in the catalytic reaction. Mutation of Y279 also severely impaired the hydrolysing activity against all substrates. However, the effect was significantly smaller than that caused by the W433 mutation on all substrates. Y279 has been assumed to assist the formation of an oxazolinium ion intermediate. The fact that the tryptophan residue corresponding to W433 and the tyrosine residue corresponding to Y279 are conserved in almost all family-18 chitinases is consistent with the critical roles in the catalytic reaction.

To study further the effect of the Y279F and W433A mutations on the catalytic reaction, kinetic analysis using 4MU-(GlcNAc)₃ as the assay substrate was performed and the results are shown in Table 3. The k_{cat} -value of the W433A mutant was 0.022% of wild-type ChiA1's k_{cat} . On the other hand, the K_m -value of the W433A mutant was only slightly larger than that of wild-type ChiA1 and, as a consequence, its k_{cat}/K_m was decreased to 0.0147% of the wild-type's. Thus, it was confirmed that this residue is very important for the catalytic reaction of this enzyme. The k_{cat} - and K_m -values of the Y279F mutant were 0.61% and 21%, respectively, of the wild-type and consequently, the k_{cat}/K_m value was 2.86% that of wild-type ChiA1. Although the effect of substituting Y279 was not as great as that of substituting W433, these results demonstrate clearly that this residue is also important for the catalytic activity of this enzyme and support the presumed role of this residue in the catalytic reaction described above.

DISCUSSION

With regard to the previously proposed mechanism of hydrolysis of crystalline chitin by ChiA1, a chitin chain from crystalline chitin is introduced into the substrate-binding cleft of the catalytic domain and interacts with the W134 and W122 residues that are located on the surface of the catalytic domain. Within the substrate-binding cleft, a chitin chain slides through the cleft towards the catalytic site, interacting with Y56 and W53 [15,16]. Selective decreases of the specific hydrolysing activity against β -chitin microfibrils by Y56A and W53A mutations are consistent with this model. On the other hand, no significant effect was observed by Y56A and W53A mutations on the hydrolysis of reduced (GlcNAc)₅, which supports the idea that oligomers enter the substrate-binding cleft in a different fashion from a chitin chain derived from crystalline chitin [26]. Soluble substrates are assumed to enter the substrate-binding cleft from various

Figure 5 Time-course of (GlcNAc)₆ hydrolysis by the W164A and W285A mutants and wild-type ChiA1

Concentrations of the enzyme and substrate were 25 nM and 1.0 mM respectively. ●, (GlcNAc)₆; ○, (GlcNAc)₅; ▲, (GlcNAc)₄; △, (GlcNAc)₃; ■, (GlcNAc)₂; □, GlcNAc.

directions, while a chitin chain from crystalline chitin enters the cleft only from the edge of the cleft near subsite -5 by interaction with linearly aligned aromatic residues (W134 and W122) on the surface of the catalytic domain. This may explain the disagreement regarding the direction of hydrolysis obtained by 3D structural analysis of the catalytic domain complexed with (GlcNAc)₇ and that deduced from GlcNAc oligomer hydrolysis. Structural studies suggested that hydrolysis occurs at the second linkage, starting from the 'reducing end' of the bound oligomer chain. However, hydrolysis of the GlcNAc oligomer occurred most frequently at the second linkage from the 'non-reducing end', as demonstrated by the analysis of *p*-nitrophenyl-(GlcNAc)₃₋₆ hydrolysis [19] and analysis of the anomeric configuration of hydrolysis products generated from GlcNAc oligomers [27]. This means that information obtained from the analysis of oligomer hydrolysis is not directly applicable to hydrolysis of insoluble/crystalline chitin, the natural substrate for chitinases.

The W164A and W285A mutations altered the products obtained from the hydrolysis of (GlcNAc)₆. On the other hand, the types of hydrolysis products that were obtained from β -chitin microfibrils were not affected by the mutations. These results support the above-mentioned idea that soluble substrates and a chitin chain from crystalline chitin enter the substrate-binding cleft in different ways. According to our model of crystalline-chitin

hydrolysis, after the first hydrolysis at the second linkage from the reducing end, the chitin chain in the substrate-binding cleft slides towards the + subsites and fills +1 and +2 subsites (see Figure 6A). Two GlcNAc units is the minimum length of sliding required to allow the next hydrolysis. Sliding by two GlcNAc units is determined by the structure of the chitin chain itself, rather than the protein structure around the catalytic site. This seems to be the reason that hydrolysis products from β -chitin microfibrils remained unaltered by the mutations. Although W164A and W285A mutations did not affect the hydrolysis products from β -chitin microfibrils, these mutations greatly decreased the hydrolysing activity towards this substrate. Therefore, these two aromatic residues also constitute essential components for hydrolysis of crystalline chitin. We assume that W164 and W285 play important roles in crystalline-chitin hydrolysis; for example, in releasing (GlcNAc)₂ molecules from +1 and +2 subsites and/or in pulling the chitin chain for the next catalytic reaction. Both are essential for processive hydrolysis of the chitin chain from the crystalline chitin. W164A and W285A mutations increased the hydrolysing activity against soluble substrates and kinetic studies that attempted to clarify the reason for this unexpected observation revealed the involvement of these residues in the substrate inhibition observed in the hydrolysis of reduced (GlcNAc)₅ (Figure 6B). The presence of the two residues is important for hydrolysis of crystalline chitin, but interferes in the hydrolysis of

Figure 6 Possible interaction of a chitin chain from β -chitin microfibrils (A) and reduced (GlcNAc)₅ (B) with the catalytic site of wild-type and mutant ChiA1s

The dotted line indicates the cleavage site. A chitin chain derived from the surface of the β -chitin microfibrils enters the substrate-binding cleft from the edge of the cleft through interaction with W134 and W122 on the surface of the catalytic domain. On the other hand, oligomers can enter the substrate-binding cleft from various directions. Non-productive binding of reduced (GlcNAc)₅ interacting with W164 and W285 may be a reason for the observed substrate inhibition. Binding of the oligomer occupying -3 to +2 subsites of W285A was deduced from the results of (GlcNAc)₆ hydrolysis shown in Figure 5.

soluble substrates. Since a double mutation decreased the activity, the presence of either one of the residues may be essential for oligomer hydrolysis, although the decrease in activity could be due partly to a structural effect of the double mutation.

The aromatic residues corresponding to W53, W164 and W285 are conserved in essentially all chitinases in subfamily A and the residue that corresponds to Y56 is conserved in most of this subfamily. A structural feature that is characteristic of subfamily A chitinases is the presence of an insertion domain between the seventh and eighth β -strands of the $(\beta/\alpha)_8$ -TIM-barrel catalytic domains [6]. It forms a deep substrate-binding cleft that is assumed to be important in processive hydrolysis of the chitin chain. Conservation of the aromatic residues corresponding to Y56, W53, W164 and W285 in subfamily A chitinases, but not in subfamily B and C chitinases, would be rational, if the deep substrate-binding cleft and these aromatic residues are both required for processive hydrolysis. W122 and W134, which were shown previously to be essential for crystalline-chitin hydrolysis, are only conserved in some members of subfamily A, which suggests a specialized role for this type of chitinase in crystalline-chitin hydrolysis. Such chitinases include *B. circulans* ChiA1, *S. marcescens* ChiA, *Streptomyces coelicolor* ChiD, *Alteromonas* sp. O7 ChiA.

In contrast with the limited conservation of the aromatic amino-acid residues corresponding to Y56, W53, W164 and W285 in family-18 chitinases, tryptophan and tyrosine residues corresponding to W433 and Y279 are conserved in essentially all family-18 chitinases. This observation is in good agreement with the essential role of the two residues in the catalytic reaction. Indeed, the W433A mutation decreased the k_{cat} -value drastically. The effect of mutation was less severe than that of W433, and our results suggested that Y279 plays an important role in the catalytic reaction. Site-directed mutagenesis of the tyrosine residues corresponding to Y279 has been reported with heveamine (chitinase/lysozyme from the rubber tree, *Hevea brasiliensis*) by Bokma et al. [28] and *Serratia marcescens* ChiA by Papanikolaou et al. [29]. Mutation of Y390 in *S. marcescens* ChiA decreased k_{cat} to 0.42 % that of the wild-type. This result is in good agreement with the results that we have presented here. On the other hand, mutation of Y183 of heveamine decreased k_{cat} by 80 %. The effect was less significant than in the other two cases; however, the contribution of Y183 to the catalytic reaction was deduced from the complete loss of the catalytic activity by the double mutation of Y183 and D125. Taken together with the perfect conservation of the corresponding tyrosine residues in the family-18 chitinases and the structural data reported so far, these observations

indicate the essential role of the corresponding tyrosine in substrate-assisted catalysis by assisting the formation of oxazolinium ion intermediate.

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