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Importance of Exposed Aromatic Residues in Chitinase B from *Serratia marcescens* 2170 for Crystalline Chitin Hydrolysis

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Chitinase B (ChiB) of *S. marcescens* has five exposed aromatic residues linearly aligned toward the catalytic cleft, Tyr481 and Trp479 in the C-terminal domain, and Trp252, Tyr240 and Phe190 in the catalytic domain. To determine the contribution of these residues to the hydrolysis of crystalline β-chitin, site-directed mutagenesis, to replace them by alanine, was carried out. The Y481A, W479A, W252A, and Y240A mutations all decreased the binding activity and hydrolyzing activity toward β-chitin microfibrils. Substitution of Trp residues affected the binding activity more severely than that of Tyr residues. The F190A mutation decreased neither the binding activity nor the hydrolyzing activity. None of the mutations decreased the hydrolyzing activity toward soluble substrates. These results suggest that ChiB hydrolyzes crystalline β-chitin via a mechanism in which four exposed aromatic residues play important roles, similar to the mechanism of hydrolysis by ChiA of this bacterium, although the directions of hydrolysis of the two chitinases are opposite.

Key words: aromatic amino acid residues, chitinase, crystalline chitin, *Serratia marcescens*, site-directed mutagenesis.

Abbreviations: GlcNAc, *N*-acetylglucosamine; ChiA, *S. marcescens* chitinase A; ChiB, *S. marcescens* chitinase B; ChiA1, *B. circulans* WL-12 chitinase A1.

Serratia marcescens produces three chitinases (ChiA, ChiB and ChiC1) and a chitin binding protein (CBP21) of unknown function in the presence of chitin (*1–4*). All three chitinases belong to family 18 in the sequence-based classification system of glycoside hydrolases (*5–7*). ChiA consists of an all β-N-terminal domain similar to the fibronectin type III domain and a catalytic $(\beta/\alpha)_8$-barrel domain with a small α + β domain inserted between the seventh and eighth β-strands of the $(\beta/\alpha)_8$ barrel (Fig. 1) (*8*). ChiB consists of a catalytic domain, which has a fold similar to that in ChiA, and a small C-terminal domain (*9*). In spite of having catalytic domains with a similar fold, the two chitinases are assumed to digest chitin chains in opposite directions, that is, ChiA hydrolyzes the chitin chain from the reducing end and ChiB from the non-reducing end (*9*, *10*). The two chitinases act synergistically in the hydrolysis of insoluble and crystalline chitin (*2*, *3*).

In recent studies on ChiA from *Serratia marcescens* and a related chitinase, ChiA1, from *Bacillus circulans,* it was shown that exposed aromatic residues located inside and outside of the active site cleft on the catalytic domains and in the extra N-terminal domain in ChiA are important for the binding and hydrolysis of crystalline chitin. ChiA1 of *B. circulans* WL-12 has a catalytic domain very similar to that of *S. marcescens* ChiA. This chitinase comprises two fibronectin type III–like domains and a C-terminal chitin-binding domain in addition to a catalytic domain (*11*). The chitin-binding domain makes a major contribution to the chitin-binding activity of ChiA1, and is required for efficient hydrolysis of crystalline chitin (*12*). In addition to the importance of the chitin-binding domain, we recently demonstrated the importance in crystalline chitin hydrolysis of aromatic amino acid residues exposed on the catalytic domain and, later, ones in the deep catalytic cleft (*13*, *14*). Careful comparison of the catalytic domains of *B. circulans* ChiA1 and *S. marcescens* ChiA revealed the presence of four exposed aromatic residues linearly aligned toward the deep catalytic cleft of *S. marcescens* ChiA. A site-directed mutagenesis study revealed that all four residues are 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 the chitin binding. The remaining one, which is most proximal to the catalytic cleft, has been suggested to be important for guiding the chitin chain into the catalytic cleft. Based on these observations, a model for processive hydrolysis of crystalline chitin by ChiA has been proposed (*15*).

As shown in Fig. 1, ChiB from *S. marcescens* also contains a prominent stretch of surface-exposed aromatic residues linearly aligned toward the catalytic cleft, as demonstrated by van Aalten *et al.* (*9*). In the present study, the contribution of these exposed residues to crystalline chitin hydrolysis was examined and compared to that in the case of ChiA. Elucidation of the roles of these aromatic amino acid residues in crystalline chitin hydrolysis by ChiB is important for understanding the mecha-

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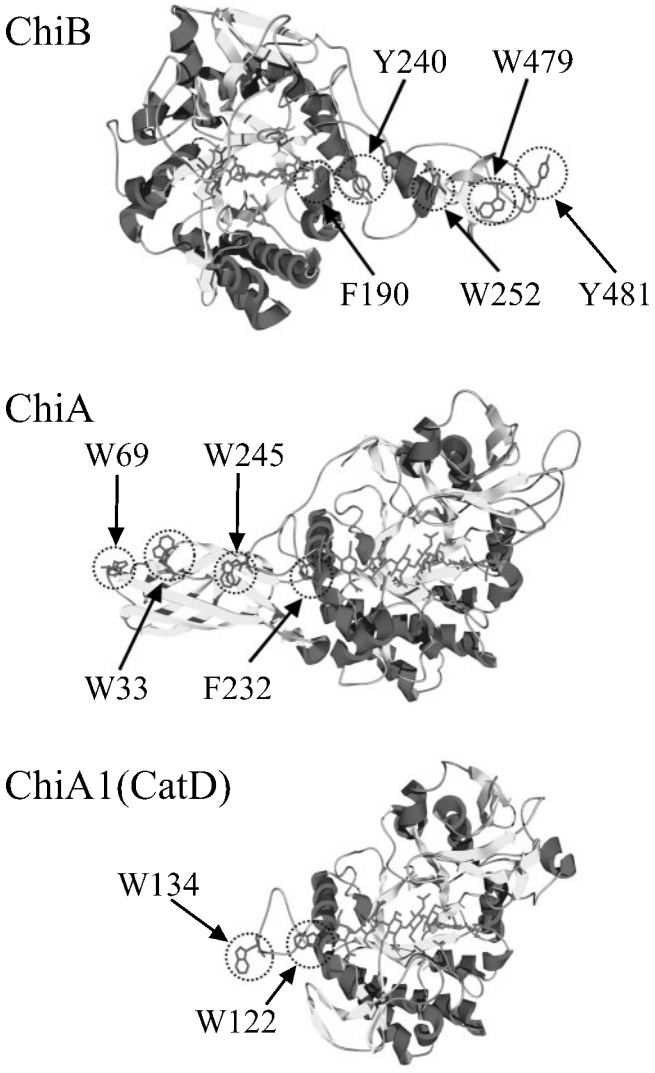

Fig. 1. **Exposed aromatic residues of ChiA, ChiB and ChiA1.** Exposed aromatic residues are shown on ribbon-drawings of the 3D structures of ChiA (PDB ID: 1EDQ) and ChiB (PDB ID: 1E15) from *S. marcescens*, and ChiA1(PDB ID: 1ITX) from *Bacillus circulans*. Green indicates the bound chitin oligomer.

nism of cooperative hydrolysis of crystalline chitin by ChiA of this bacterium.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—*Escherichia coli* JM109 was the host strain used throughout the construction of various recombinant plasmids. Recombinant plasmid pMCB7 (*3*) carries the *chiB* gene from *S. marcescens* 2170. *E. coli* DH5α cells harboring pMCB7 or derivatives of it was used for the production of ChiB or mutants of it. pMCB7a carrying the *Eco*RI–*Sal*I fragment of 971 bp corresponding to the N-terminal half and pMCB7b carrying the *Sal*I–*Kpn*I fragment of 1,331 bp corresponding to the C-terminal half of ChiB were used as templates for site-directed mutagenesis by PCR.

Chemicals—Colloidal chitin and glycol chitin were prepared by the methods of Jeuniaux (*16*), and Yamada and Imoto (*17*), respectively. Highly crystalline β-chitin microfibrils from a vestimentiferan, *Lamellibrachia satsuma*, were prepared as described previously (*18*). Reduction of $(GlcNAc)_5$ was carried out as described (*19*). Soluble chitin and $(GlcNAc)_5$ were obtained from Yaizu Suisan Chemical Co., Ltd. (Shizuoka, Japan). The degree of deacetylation and approximate molecular weight of the soluble chitin were 38.8% and from 200,000 to 300,000, respectively.

Site-Directed Mutagenesis—Site-directed mutagenesis was carried out by PCR using a QuikChange site–directed mutagenesis kit (Stratagene, CA). The primers used for the mutagenesis are summarized in Table 1. The mutant clones were selected after sequencing with an automated laser fluorescence DNA sequencer (Model 4000L; LI-COR).

Production and Purification of ChiB and Its Mutants—Wild-type and mutant chitinases were produced in *E. coli* DH5α cells carrying pMCB7 and its derivatives encoding various mutant chitinases. *E. coli* DH5α cells carrying a plasmid were grown in LB medium containing 100 μg/ml ampicillin and 0.4 mM isopropylthio-β-D-galactoside for 24 h at 33°C, collected by centrifugation, and then disrupted by sonication. After the removal of unbroken cells and debris by centrifugation, proteins in the soluble fraction were collected by ammonium sulfate precipitation (20–40% saturation). The precipitate was dissolved in a small volume of 20 mM Tris-HCl buffer (pH 8.0) containing 0.4 M ammonium sulfate and then applied to a Phenyl-Sepharose 6FF (Amersham Biosciences) column (1.5 × 20 cm) previously equilibrated with the same buffer, according to the method described by Brurberg *et al.* (*20*). After the column had been washed with 5 column volumes of the same buffer to remove unadsorbed proteins, the adsorbed proteins were eluted with 20 mM Tris-HCl containing 0.1 M ammonium sulfate. The peak fraction of ChiB or one of its mutants was collected and dialyzed against 1 mM sodium phosphate buffer (pH 6.0) and then applied to a hydroxyapatite column (1.5 × 20 cm) previously equilibrated with the same buffer and eluted with the same buffer. The unabsorbed protein fractions containing ChiB were collected and lyophilized. ChiB proteins were further purified by a second cycle of hydroxyapatite column chromatography. SDS-PAGE analysis of the purified chitinases was conducted by the method of Laemmli (*21*).

Chitin-Binding Assays—Binding assay mixtures in 1-ml glass microtubes containing various concentrations of protein and 0.5 mg of binding assay substrate in 500 μl 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 9,500 × *g* to separate the supernatant from the substrate with bound protein. The supernatant containing free protein was collected, and then 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 sugar generated on the degradation of various chitinous substrates was measured by a modification of Schale's procedure using *N*-acetyl glucosamine as a standard (*22*). The reaction mixture (total 750 μl) comprised purified chitinase

Table 1. **Primers used for site-directed mutagenesis.**

Mutant	Primer	Mutant
F190A	Forward	5′-CATCGCCGGCGCCGGCGGCGCTGCCTTCCTGTCGCGCTATTACAG-3′
	Reverse	5′-CTGTAATAGCGCGACAGGAAGGCAGCGCCGCCGGCGCCGGCGATG-3′
Y240A	Forward	5′-CGGGCCGACCTTCGCCAACGCGCTGCGCG-3′
	Reverse	5′-CGCGCAGCGCGTTGGCGAAGGTCGGCCCG-3′
W252A	Forward	5′-CTGGGCTGGAGCGCGGAAGAGCTGACC-3′
	Reverse	5′-GGTCAGCTCTTCCGCGCTCCAGCCCAG-3′
W479A	Forward	5′-TGGCAGACCAAGGCGGGTTACATCA-3′
	Reverse	5′-TGATGTAACCCGCCTTGGTCTGCCA-3′
Y481A	Forward	5′-GACCAAGTGGGGTGCCATCACCTC-3′
	Reverse	5′-GAGGTGATGGCACCCCACTTGGTC-3′

and 1 mg (dry weight) of each substrate in 0.1 M sodium phosphate buffer (pH 6.0).

The protein concentration was estimated from the absorbance at 280 nm using the molar extinction coefficients calculated from the amino acid compositions of the proteins (*23*). For the chitin binding assay, the protein concentration was estimated by spectrofluorometry (Hitachi F-3010 spectrofluorometer) with an excitation wavelength of 280 nm and an emission wavelength of 342 nm. A separate standard curve was prepared for each protein. The molar extinction coefficients (ε) used for the UV determination were calculated from the amino acid compositions of the wild-type and mutant chitinases (*23*).

RESULTS AND DISCUSSION

Positions of Exposed Aromatic Residues and Site-Directed Mutagenesis—ChiB of *S. marcescens* consists of a catalytic domain with a TIM barrel fold and a small C-terminal domain. In contrast, ChiA of this bacterium consists of an N-terminal domain and a catalytic domain with a TIM barrel fold, as illustrated in Fig. 1. Four aromatic amino acid residues, two in the N-terminal domain and two in the catalytic domain, were found on the surface of ChiA and were demonstrated to be essential determinants for crystalline chitin hydrolysis. Similar to the four exposed aromatic residues of ChiA, five aromatic residues linearly aligned on the surface of the ChiB molecule have been observed (*9*). Tyr481 and Trp479 are in the C-terminal domain, and Trp252, Tyr240 and Phe190 in the catalytic domain. Rotation of the side chain of Phe190 around χ_1 along with the binding of $(GlcNAc)_5$ to the catalytic cleft has been demonstrated by van Aalten *et al.* previously (*24*). In order to determine whether these aromatic residues are involved in the hydrolysis of crystalline chitin, site-directed mutagenesis, to replace them by alanine, was carried out and then the effects of the mutations on the hydrolysis of various chitinous substrates, including highly crystalline β-chitin, were studied.

Wild-type and mutant chitinases were produced in *E. coli* cells carrying an appropriate plasmid extracted from *E. coli* cells and purified by hydrophobic interaction chromatography followed by two cycles of hydroxyapatite column chromatography. The purified chitinases thus obtained gave single protein bands on SDS-PAGE analysis, as shown in Fig. 2. The subsequent experiments were performed with these purified preparations.

Effects of Mutations on the Hydrolyzing Activity and Binding Activity toward Crystalline β-Chitin—The effects of the mutations on the hydrolyzing activity and binding activity toward highly crystalline β-chitin microfibrils were first examined. Figure 3B shows the time course of hydrolysis of β-chitin microfibrils (1.0 mg dry weight) by 15 pmol of ChiB or one of its mutants. The Y240A, W479A and W252A mutations severely decreased the hydrolyzing activity toward β-chitin microfibrils. Mutation of Tyr481, which is located most distally to the catalytic cleft among the five aromatic residues, decreased the activity as well, although the effect was slightly weaker than those of the other three mutations. On the other hand, mutation of Phe190, which is located most proximally to the catalytic site, did not have any effect on the β-chitin hydrolyzing activity.

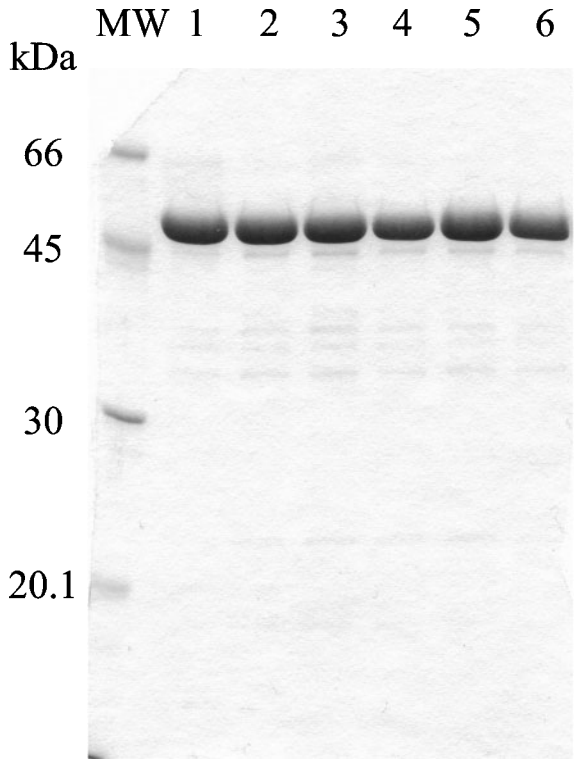

Fig. 2. **SDS-PAGE analysis of the wild-type and mutant chitinases.** Purified chitinases (10 μg each) were applied and protein bands were visualized by Coomassie Brilliant blue R-250 staining. Lane 1, wild-type; lane 2, F190A; lane 3, Y240A, lane 4, W252A; lane 5, W479A; lane 6, Y481A; lane MW, size markers.

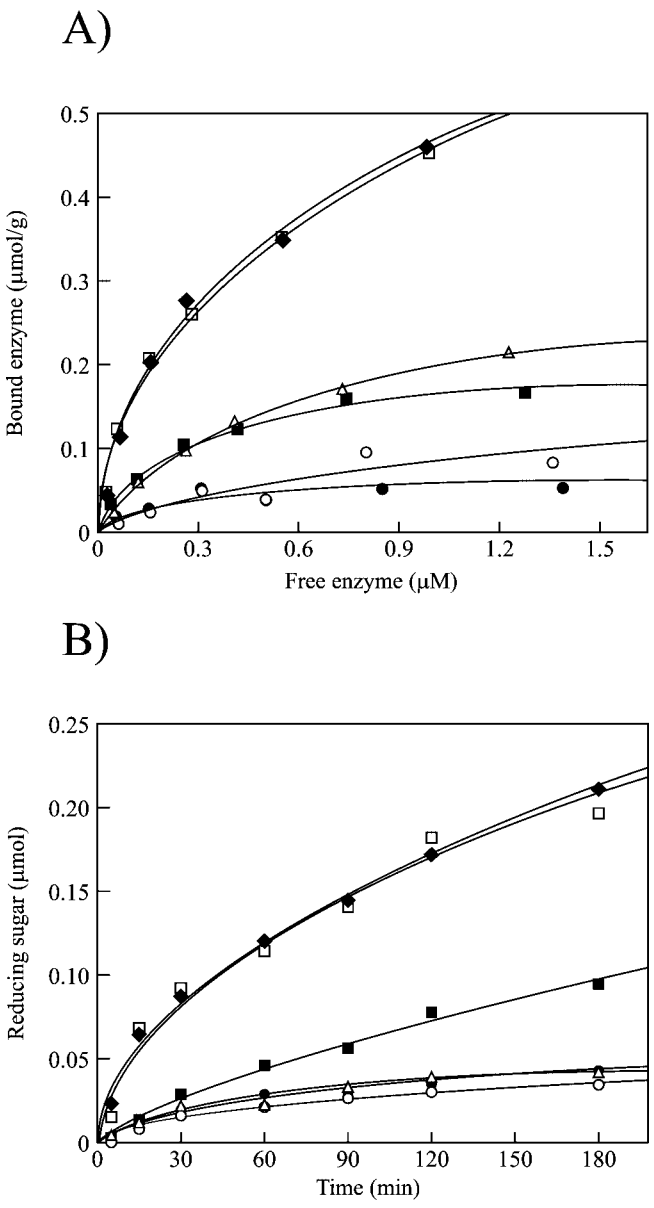

Fig. 3. **Time course of hydrolysis and the binding isotherms as to β-chitin microfibrils.** (A) Adsorption isotherms. Assay mixtures contained 0.5 mg (dry weight) of β-chitin microfibrils and 150 pmol of ChiB or one of its mutants. (B) Time course of hydrolysis. Reaction mixtures containing 1.0 mg (dry weight) of β-chitin microfibrils and 15 pmol of ChiB or one of its mutants were incubated at 37°C, and then the amount of released reducing sugar was measured. Symbols: closed diamonds, wild-type; open squares, F190A; open triangles, Y240A; open circles, W252A; closed circles, W479A; closed squares, Y481A.

To determine whether these exposed aromatic residues are involved in the chitin binding by this enzyme, the binding activities toward β-chitin microfibrils of wild-type and mutant ChiBs were compared. Figure 3A shows the binding isotherms of ChiB and its mutants as to β-chitin microfibrils. The F190A mutation did not have any effect on the binding activity of this chitinase. On the other hand, mutation of Trp252 and Trp479 severely and almost equally decreased the binding activity of this chitinase, respectively. Mutation of Tyr240 and Tyr481 also decreased the binding activity, but less severely than did the mutation of the two Trp residues. It is notable that the effect of the Y240A mutation on the hydrolyzing activity was significantly greater than that of the Y481A mutation, which decreased the binding activity almost equally.

Effects of the Mutations on the Hydrolyzing Activity toward Soluble Substrates—The hydrolyzing activity toward soluble and amorphous substrates of wild-type and mutant ChiBs was examined. Table 2 shows the specific hydrolyzing activity of the mutant and wild-type ChiBs. Substitution of exposed aromatic residues by Ala had no effect on the oligomer hydrolysis. On the other hand, a small increase in the activity toward soluble chitin was observed as a result of the W252A or W479A mutation. In addition, an approximately two-fold increase in the activity toward glycol chitin was observed as a result of mutation of Tyr240 or Trp252. An increase in the hydrolyzing activity toward glycol chitin as a result of mutation of exposed aromatic amino acid residues has also been observed for *S. marcescens* ChiA and *B. circulans* ChiA1, as we reported previously (*13*, *15*). None of the substitutions decreased the hydrolyzing activity toward soluble substrates.

Hydrolysis of colloidal chitin was not affected by these mutations under the assay conditions employed in Table 2. However, the mutant chitinases other than F190A produced lower amounts of reducing sugar than wild-type ChiB, as judged when assayed with a smaller amount of colloidal chitin and an extended incubation period (data not shown). This probably reflects the nature of this substrate, since colloidal chitin is considered to be a non-homogeneous substrate comprising an amorphous part and a relatively crystalline part. Hydrolysis of the relatively crystalline part would require participation of these aromatic amino acid residues.

From these results, it appeared that Tyr481, Trp252, Trp479, and Tyr240 are involved in chitin binding and play important roles in crystalline chitin hydrolysis. Phe190 is involved in neither chitin binding nor hydrolyzing activity toward crystalline chitin, despite the fact that structural comparison of ChiB and ChiA indicated that Phe190 is analogous to the important Phe232 in ChiA (Fig. 1). None of the five aromatic residues mutated in this study are required for the hydrolysis of soluble substrates, and some of them even seem to hamper the hydrolysis of some of the higher M_r soluble substrates (Table 2). The results clearly show that the affinity of ChiB for chitin not only depends on the C-terminal domain (containing Trp479 and Tyr481, and often referred to as the chitin-binding domain) but also on residues in the catalytic domain (Tyr240 and Trp252). Analogous observations have been made for ChiA (*15*). The importance of aromatic amino acid residues in insoluble substrate binding has been extensively studied for the cellulose binding modules (CBMs) of cellulases, which are often compared with chitinases. CBMs that bind to the crystalline region of cellulose, such as family 2a CBM of xylanase 10A from *Cellulomonas fimi* (*25*) and family 5 CBM of Cel5 from *Erwinia chrysanthemi* (*26*), generally have three solvent-exposed aromatic residues. These CBMs bind to crystalline cellulose in the absence of the other domain(s), and mutation of each of the three

Table 2. **Relative specific hydrolyzing activity of ChiB and its mutants toward various chitinous substrates.**

Chitinase	Specific hydrolyzing activity (units/nmol)[a]			
	Reduced $(GlcNAc)_5$	Soluble chitin	Glycol chitin	Colloidal chitin
ChiB	0.760 (100)	0.677 (100)	0.021 (100)	0.124 (100)
F190A	0.800 (105)	0.715 (106)	0.019 (91)	0.119 (96)
Y240A	0.686 (90)	0.650 (96)	0.044 (210)	0.121 (98)
W252A	0.742 (98)	0.856 (126)	0.038 (181)	0.121 (98)
W479A	0.820 (108)	0.789 (117)	0.021 (100)	0.119 (96)
Y481A	0.787 (104)	0.649 (96)	0.019 (91)	0.114 (92)

[a]Relative specific activity (%) is shown in parentheses.

exposed residues greatly reduces the binding affinity to crystalline cellulose. These observations are in consistent with the finding that ChiB (and ChiA) requires aromatic amino acid residue(s) in the catalytic domain in addition to the two aromatic residues in the C-terminal (or N-terminal) domain for binding.

The Roles of Exposed Aromatic Residues of ChiA and ChiB—The results described above clearly demonstrated functional similarity between the exposed aromatic residues of ChiB and those of ChiA, despite the striking differences in the domain organization and direction of hydrolysis of the two chitinases. In the case of ChiA, substitution of Trp69, Trp33 or Trp245 significantly reduced both the binding activity and hydrolyzing activity toward highly crystalline β-chitin. Substitution of Phe232 did not affect the binding activity but significantly reduced the hydrolyzing activity. Based on these observations and some other data, a model for the processive hydrolysis of crystalline β-chitin by ChiA has been proposed (*15*). It can be summarized as follows. ChiA binds to the crystalline chitin surface through interaction between three aromatic residues (Trp69, Trp33 and Trp245) and the GlcNAc residues in a single chitin chain on the crystalline chitin surface. The chitin chain is introduced into the catalytic cleft from the reducing end side through interaction with Phe232. In the catalytic cleft, the introduced chitin chain slides through the cleft to the catalytic site, and then second linkages from the reducing end are progressively cleaved, releasing $(GlcNAc)_2$ units continuously. Several aromatic residues within the cleft may play major roles in holding and also sliding of the chitin chain. As a result, the ChiA molecule proceeds on the crystalline chitin surface towards the non-reducing end side of the microfibrils with the N-terminal domain at the head, releasing $(GlcNAc)_2$ units from the reducing end of the chitin chain.

Hydrolysis from opposite ends of the chitin chain by ChiA and ChiB (from the reducing end by ChiA and from the non-reducing end by ChiB) was first suggested based on the 3D-structures of the two chitinases (*9*). Very recently, this was proved by combined analysis involving a reducing end labeling technique and a tilt-microdiffraction method to study the hydrolysis of β-chitin microfibrils by the two chitinases (unpublished results). We chose five aromatic residues as targets for site-directed mutagenesis, but found that Phe190, which was first compared with Phe232 as the residue located at the entrance of the catalytic cleft, was not important for crystalline chitin hydrolysis. Therefore, four of the exposed aromatic residues turned out to be involved in crystalline chitin hydrolysis, two in the catalytic domain and the other two in other domains, of both ChiA and ChiB.

Several interesting differences in the effect of mutations of the four aromatic residues between the two chitinases were observed. Two Tyr and two Trp residues are involved in the binding activity toward crystalline chitin of ChiB, while three Trp residues participate in the binding activity of ChiA. The contribution of Tyr residues to the binding activity of ChiB was significantly lower than that of Trp residues. Phe232 in ChiA does not contribute to the chitin binding activity, but substitution of this residue reduced the hydrolyzing activity significantly (*15*). This residue was assumed to contribute to crystalline chitin hydrolysis by guiding a chitin chain into the catalytic cleft, not via binding activity. No such residue was observed in ChiB. Tyr240, rather than Phe190, of ChiB may correspond to Phe232 of ChiA in the sense that this residue is located most proximally to the catalytic cleft among the four aromatic residues involved in crystalline chitin hydrolysis. Substitution of Tyr240 decreased the binding activity significantly, and therefore this residue is obviously involved in the chitin binding activity. However, this residue may play a role by guiding a chitin chain into the catalytic cleft in addition to its role in chitin binding, since the effect of the Y240A mutation on the hydrolyzing activity was greater than that of Y481A, despite the almost equal effects of the two mutations on the binding activity.

Although there are some differences in the details, the results obtained in this study suggest that ChiB hydrolyzes crystalline β-chitin in a similar manner to ChiA, via mechanisms in which exposed aromatic residues play very important roles. The physiological importance of the opposite chain end preference of the two chitinases remains to be clarified.

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