Roles of the Exposed Aromatic Residues in Crystalline Chitin Hydrolysis by Chitinase A from Serratia marcescens 2170*

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Four exposed aromatic residues, two in the N-terminal domain (Trp-69 and Trp-33) and two in the catalytic domain (Trp-245 and Phe-232) of Serratia marcescens chitinase A, are linearly aligned with the deep catalytic cleft. To investigate the importance of these residues in the binding activity and hydrolyzing activity against insoluble chitin, site-directed mutagenesis to alanine was carried out. The substitution of Trp-69, Trp-33, or Trp-245 significantly reduced the binding activity to both highly crystalline β-chitin and colloidal chitin. The substitution of Phe-232, which is located closest to the catalytic cleft, did not affect the binding activity. On the other hand, the hydrolyzing activity against β-chitin microfibrils was significantly reduced by the substitution of any one of the four aromatic residues including Phe-232. None of the mutations reduced the hydrolyzing activity against soluble substrates. These results clearly demonstrate that the four exposed aromatic 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. Phe-232 appeared 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 chitinase A is proposed.

The chitinases sequenced so far are classified into two different families, families 18 and 19, in the classification system of glycosyl hydrolases based on the amino acid sequence similarity of their catalytic domains (1–3). The catalytic domains of family 18 chitinases have (β/α)8 barrel folds (4–8), whereas those of family 19 chitinases have a high α-helical content and share a structural similarity with chitosanase and lysozyme (9, 10).

Chitinolytic bacteria generally produce multiple chitinases encoded by different genes. Many chitinolytic bacteria produce only family 18 chitinases, whereas some other bacteria such as Streptomyces species produce family 19 chitinases in addition to family 18 chitinases (11). Bacterial family 18 chitinases are further classified into three subfamilies, subfamilies A, B, and C (12). Chitinases in subfamily A have an insertion domain between the seventh and eighth β-strands of the (β/α)8 barrel basic structure, whereas chitinases in subfamilies B and C do not have such an insertion domain. The three-dimensional (3D)1 structure of the three-bacterial family 18 chitinases, all belonging to subfamily A, has been determined including those of chitinase A (ChiA) and chitinase B from Serratia marcescens QMB1466 and chitinase A1 (ChiA1) from Bacillus circulans WL-12 (5, 6, 8). Serratia ChiA comprises three domains: an N-terminal domain, a catalytic (β/α)8 barrel domain, and one (α + β) domain, which is inserted in the (β/α)8 barrel domain. Serratia ChiB comprises a catalytic (β/α)8 barrel domain and a C-terminal chitin-binding domain (ChBD). B. circulans ChiA1 comprises the catalytic domain (CatD), two fibrinectin type III-like domain, and the C-terminal ChBD. The 3D structures of the entire molecules of the former two chitinases were determined by x-ray crystallography. On the other hand, the 3D structures of the domains constituting B. circulans ChiA1 were determined separately. The structures of CatD were determined by x-ray crystallography, and those of ChBD and fibrinectin type III-like domain were determined by NMR (8, 13).2

The 3D structure of CatDChiA1 is basically very similar to that of Serratia ChiA. Two small insertion domains, β-domain 1 and β-domain 2, located on top of the (β/α)8 barrel provide a deep cleft for substrate binding (8). The crystal structure of inactivated CatDChiA1 complexed with (GlcNAc)3 suggested that the cleavage of the chitin chain occurs at the second linkage from the reducing end. Seven subsites numbered from −5 to +2 in the deep substrate-binding cleft were deduced. The oligomer chain bound to the cleft was bent and twisted at the third sugar ring at subsite −1. Four tryptophans, Trp-285, Trp-164, Trp-433, and Trp-53, in the substrate-binding cleft and one tyrosine, Tyr-56, at the edge of the substrate-binding cleft were identified as the residues interacting with the GlcNAc units of the bound oligomer through stacking interactions. In addition, two exposed Trp residues, Trp-122 and Trp-134, were found outside of the catalytic cleft 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 crystalline chitin hydrolysis and have been proposed to play an important role in guiding a chitin chain into the catalytic cleft during crystalline chitin hydrolysis (14).

The two exposed aromatic residues and the four aromatic residues in the catalytic cleft of CatDChiA1 are all conserved in

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1 The abbreviations used are: 3D, three-dimensional; ChiA, S. marcescens chitinase A; ChiA1, B. circulans WL-12 chitinase A1; ChBD, chitin-binding domain; CatD, catalytic domain; CatDChiA1, catalytic domain of B. circulans ChiA1; Cel6A, cellobiohydrolase II; Cel7A, cellobiohydrolase I.

2 M. Shirakawa, unpublished data.
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**Serratia** ChiA. In this study, the roles in chitin binding activity and hydrolyzing activity of the two exposed aromatic residues in the catalytic domain and the two additional aromatic residues found on the surface of the N-terminal domain of Serratia ChiA were studied.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—**Escherichia coli XLI-Blue (Stratagene, La Jolla, CA) was the host strain used throughout the construction of plasmids carrying the chiA genes with various mutations. *E. coli* DH5α was used as the host strain for the production of the wild-type and mutant chitinase proteins. Plasmid pNC112 carrying the wild-type chiA gene from *S. marcescens* 2170 was described previously (15). Site-directed Mutagenesis—Site-directed mutagenesis was carried out by polymerase chain reaction using a QuickChange site-directed mutagenesis kit (Stratagen) and pNC112 as a template. The primers used for site-directed mutagenesis were 5′-CGGCCATCCGCGCGGCACACC-3′ for Trp-33 → Ala mutation (W33A), 5′-CCTGGAAATTTAGCGAATGGCAGAC-3′ for Trp-69 → Ala mutation (W69A), 5′-GATCCAGATCCGCGCGGCCTG-3′ for Phe-232 → Ala mutation (F232A), and 5′-GGGGCTAAGCGCGGATGACCCC-3′ for Trp-245 → Ala mutation (W245A). The mutant clones were selected after sequencing the entire open reading frame to ensure that the desired mutation was the only mutation in each mutated gene. Sequencing was done by using an automated laser fluorescence DNA sequencer (Model 4000L, LI-COR) and the ThermoSequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Bio-tech) for sequencing reaction.

To create the gene encoding ChiA with a double mutation (W33A/W69A), the second mutation was introduced into the gene encoding W33A by using the primers for W69A mutation. The third mutation of triple mutant (W33A/W69A/W245A) was introduced into the gene encoding W33A/W69A by using the primers for W245A mutation.

**Purification and Characterization of ChiA and Its Mutants—**ChiA and its mutants were produced in *E. coli* DH5α cells carrying the plasmid pNC112 or its derivatives and purified from culture supernatants. After collecting chitinase proteins by ammonium sulfate precipitation (80% saturation), wild-type ChiA, W33A, W69A, W245A, and F232A were purified by chitin affinity column chromatography (16) with some modifications as follows. Crude chitinase was applied to a chitin affinity column previously equilibrated with 20 mM phosphate buffer, pH 6.0. Peak fractions containing purified chitinase were collected for SDS-polyacrylamide gel electrophoresis of the purified chitinases in 12.5% slab gels was conducted by the method of Laemmli (17).

**Enzyme and Protein Assays—**Reducing sugar generated by the degradation of various chitosan substrates was measured by a modification of Schales’ procedure using N-acetylglucosamine as a standard (18). The reaction mixture (total 750 μl) contained purified chitinase 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 each protein (19). For the chitin binding assay, the protein concentration was estimated by spectrophotometry (Hitachi F-3010 Spectrophotometer) at an excitation wavelength of 280 nm and an emission wavelength of 342 nm. A standard linear curve was prepared for each protein.

**Chitin Binding Assay—**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 9500 × g to separate the supernatant from substrate 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.

**Electron Microscopy—**Enzyme-treated β-chitin microfibrils were deposited on carbon-coated grids and were allowed to dry. All of the electron micrographs were taken with a JEOL 2000EXII electron microscope operated at 100 kV and recorded on Mitsubishi electron microscope film. Diffraction contrast imaging in the bright field mode was used to visualize the sample without further contrast enhancement. The images were taken at magnifications of × 1000 to × 6000 under low dose exposure with the use of a minimum dose system (JEOL).

**Chemicals—**Chitin EX (powdered raw shell chitin) and carbamoyl chitin were purchased from Funakoshi Chemical Co. (Tokyo, Japan). Soluble chitin (GlcNAc) was obtained from Yaizu Suisan Chemical Co., Ltd. (Shizuoka, Japan). The degree of deacetylation was 38.8%, and approximate molecular weight of the soluble chitin was from 200,000 to 300,000. Colloidal chitin and glycol chitin were prepared from powdered crab shell chitin purchased from Funakoshi Chemical Co. by the methods described by Jeaniaux (20) and Yamada and Imoto (21), respectively. Reduction of (GlcNAc), was carried out as described by Yanase et al. (22). Microcrystalline β-chitin from vestimentiferan (Lamellibrachia satsuma) was prepared as described previously (23).

**RESULTS**

**Four Aromatic Residues Linearly Aligned on the Surface of ChiA Molecule—**ChiA from *S. marcescens* QMB1466 comprises three domains: an N-terminal domain with an immunoglobulin-like fold, a catalytic (βα)8 barrel domain, and a small (α + β) fold domain, which is inserted in the catalytic (βα)8 barrel domain (5). The role of the N-terminal domain has not yet been clarified, although involvement in interactions with the chitin chain has been suggested (24). However, the following two observations strongly suggested that the N-terminal domain of Serratia ChiA participates in the chitin binding. First, ChiA from *S. marcescens* 2170, which has 99.3% amino acid identity with ChiA from QMB1466, has significant chitin binding activity (15). Second, the catalytic domains of *B. circulans* ChiA1 and *Serratia* ChiA are structurally very similar as shown in Fig. 1, and CDWChia does not have significant chitin binding activity (25). The chitin binding activity of *B. circulans* ChiA1 depends on the C-terminal ChBD (26).

To examine chitin binding activity of the N-terminal domain itself, the N-terminal domain of ChiA from *S. marcescens* 2170 was produced by using an *E. coli* expression system. However, isolated N-terminal domain did not show significant chitin binding activity (26). Therefore, we examined the 3D structure of ChiA reported by Perrakis et al. (5, 24) and found that Trp-33 and Trp-69 in the N-terminal domain and Trp-245 in the catalytic domain are linearly aligned on the surface of the ChiA molecule (Fig. 1A). These three aromatic residues are assumed to be involved in the chitin binding of ChiA based on the analogy to the cellulose-binding domains of several cellulases. It is well known that these three aromatic residues linearly aligned on the surface of cellulose-binding domains play major roles in cellulose binding (27–30). In addition, when the 3D structure of CDWChiaA complexed with (GlcNAc) was superimposed on the structure of Serratia ChiA, two exposed aromatic residues, Phe-232 and Trp-245, corresponding to Trp-122 and Trp-134 of *B. circulans* ChiA1 (Fig. 1B) that are exclusively required for crystalline chitin hydrolysis, were identified (Fig. 1C). In summary, a total of four linearly aligned aromatic amino acids, two in the N-terminal domain and two in the catalytic domain of Serratia ChiA, thus were identified. Some of these residues must be identical to the residues in the N-terminal domain, which were proposed by Perrakis et al. (24) to be in ideal positions to facilitate an interaction with an extended sugar chain.

From the above observations and the structural description described by Perrakis et al. (24), it is hypothesized that Serra-
ChiA binds chitin mainly through the interaction between GlcNAc residues in the chitin chain and the exposed aromatic residues in the N-terminal and catalytic domains, and that this chitin chain is introduced into the catalytic cleft to be hydrolyzed at the catalytic site located at the bottom of the deep substrate-binding cleft.

**Production and Purification of the Enzymes**—To investigate the possible importance of the Trp-69, Trp-33, Trp-245, and Phe-232 in the binding activity and hydrolyzing activity against insoluble chitin, site-directed mutagenesis of the four aromatic residues to alanine was carried out. The wild-type and six mutant chitinases, W69A, W33A, W245A, F232A, W33A/W69A, and W33A/W69A/W245A, were produced in E. coli cells carrying a plasmid encoding either a wild-type or mutated chiA gene and purified from the culture supernatants. All mutant chitinases were produced at a level similar to that at which wild-type ChiA was produced. Purification of chitinase proteins was carried out either by chitin affinity column chromatography or by hydroxyapatite column chromatography. F232A behaved like ChiA did on the chitin column; i.e., the two proteins were eluted from the column with 20 mM sodium acetate buffer, pH 4.0, after the column was washed with the same buffer at pH 5.0. On the other hand, W69A, W33A, and W245A appeared to bind more weakly than wild-type ChiA did and were eluted from the chitin column at the washing step with 20 mM sodium acetate buffer, pH 5.0, along with many other contaminating proteins. Therefore, 0.5 M NaCl was included in the buffer to prevent elution of these mutant chitinases at the washing step, and purification was finally achieved by elution with 20 mM sodium acetate buffer, pH 4.0. Double and triple mutants of ChiA, W33A/W69A and W33A/W245A, did not bind chitin column even in the presence of 0.5 M NaCl and, therefore, were purified by hydroxyapatite column chromatography.

The purified chitinases were essentially homogeneous as judged by SDS-polyacrylamide gel electrophoresis analysis (Fig. 2). Approximately 14 mg of purified chitinase was obtained from a 1-liter culture of E. coli cells carrying each chitinase gene.

**Effect of the Mutations on the Chitin Binding Activity**—The chitin binding activities of wild-type and mutant ChiAs were studied using colloidal chitin and highly crystalline \( eta \)-chitin microfibrils isolated from L. satsuma. The binding assay was carried out at pH 6.0 since the optimum pH for the hydrolysis reaction of ChiA is 6.0 (31). To minimize hydrolysis of the binding substrates, the binding assay mixture was maintained below 4°C. The binding reaction times were 3 h for \( \beta \)-chitin microfibrils and 1 h for colloidal chitin since preliminary experiments indicated that the binding to \( \beta \)-chitin microfibrils requires approximately 3 h, and that binding to colloidal chitin requires less than 1 h to reach equilibrium (data not shown).

Fig. 3 shows binding isotherms of the wild-type and mutant ChiAs to \( \beta \)-chitin microfibrils and colloidal chitin. Wild-type ChiA did not bind to the \( \beta \)-chitin microfibrils drastically (Fig. 3A). On the other hand, mutation of Phe-232 did not affect the binding activity significantly. The mutations of Trp-69, Trp-33, and Trp-245 also reduced the binding activity to colloidal \( \beta \)-chitin but less significantly than the cases of \( \beta \)-chitin microfibrils. The effect of mutation of Trp-69, which is located in...
the N-terminal domain and is most distal to the catalytic cleft among the four aromatic residues, was more severe than the effects of the other two mutations, suggesting special importance of this residues in the binding activity (Fig. 3B). Double and triple mutations of the three residues deprived binding activity to colloidal chitin almost completely. Mutation of Phe-232 did not affect the binding activity to colloidal chitin and to β-chitin microfibrils.

These results clearly demonstrated that Trp-69 and Trp-33 in the N-terminal domain and Trp-245 in the catalytic domain are essential for the chitin binding activity of ChiA. Therefore, the chitin binding activity of this enzyme depends not only on the N-terminal domain but also on the catalytic domain. The N-terminal domain is important for the chitin binding activity of this enzyme, but the N-terminal domain alone is not sufficient to confer full binding activity.

**Effect of the Mutations on the Hydrolyzing Activity**—The effect of the mutations on the hydrolyzing activity against colloidal chitin and β-chitin microfibrils was examined. The hydrolysis of β-chitin microfibrils by 50 pmol of wild-type or mutant ChiA was monitored by measuring the amount of reducing sugar released from the substrates. As shown in Fig. 4A, the hydrolyzing activity against β-chitin microfibrils was significantly reduced by all four single mutations including F232A, which did not affect the binding activity, although some hydrolysis was still observed. The effect of F232A was even more severe than that caused by W33A. This observation suggests that the defect in hydrolyzing activity of F232A is not attributed to a defect in chitin binding activity, whereas the defect in the hydrolyzing activity of W69A, W33A, and W245A
may be due to, at least in part, a defect in chitin binding activity. Double and triple mutations of the three residues completely impaired hydrolyzing activity against β-chitin microfibrils.

Fig. 4B shows the time course of hydrolysis of colloidal chitin by 10 pmol of ChiA and its mutants. Because ChiA hydrolyzes colloidal chitin much faster than β-chitin microfibrils, smaller amounts of the enzymes were used in these experiments. The effects of four single mutations were much less than those observed in the hydrolysis of β-chitin microfibrils. Interestingly, W69A, which caused the most severe defect among four single mutants in the binding activity to colloidal chitin, did not reduce the hydrolyzing activity at all. W33A and W245A, which also reduced the binding activity to colloidal chitin, only slightly reduced the hydrolyzing activity. F232A, which had the same level of binding activity as wild-type ChiA, exhibited the lowest hydrolyzing activity among the four mutant chitinases. On the other hand, double and triple mutations again reduced hydrolyzing activity drastically, although a slight hydrolysis was still observed.

The effects of mutations on the hydrolyzing activity against various soluble substrates were also examined. The substrates tested include reduced (GlcNAc)_5, soluble chitin, carboxymethyl chitin, and glycol chitin. As shown in Table I, none of the mutations including double and triple mutations reduced the activities against all soluble substrates. The hydrolyzing activities of the six mutant chitinases were essentially the same as that of wild-type ChiA against reduced (GlcNAc)_5, soluble chitin and carboxymethyl chitin. Interestingly, F232A exhibited increased activity against the glycol chitin. The specific hydrolyzing activity of F232A against glycol chitin was 2.5-fold higher than that of wild-type ChiA. Both glycol chitin and carboxymethyl chitin are derivatives of chitin chemically modified at the C6 position of GlcNAc residues. In this sense, the two soluble substrates are structurally similar; however, the hydrolyzing activity against carboxymethyl chitin was not affected by the F232A mutation. The reason for this difference is unclear.

**Unidirectional Processive Action of ChiA on β-chitin Microfibrils**—The β-chitin microfibrils isolated from the protective tubes of *L. satsuma* are highly crystalline β-chitin as demonstrated by electron microdiffraction (23). The microfibrils were treated with intact ChiA and its mutants, and the morphological change was examined by electron microscopy. As shown in Fig. 5A, the initial microfibrils displayed a uniform and well-defined microfibrillary form. On the other hand, after treatment with ChiA, the microfibrils were primarily shortened, and the tips of the microfibrils were gradually narrowed and sharpened at one end very similar to needles (Fig. 5B). The morphology of the microfibrils treated with ChiA are compatible with the notion that the hydrolysis of β-chitin microfibrils by ChiA occurs unidirectionally and processively. β-chitin microfibrils treated with W33A and F232A are shown in Fig. 5C and D. W33A and F232A also formed needle tips at one end of the microfibrils clearly demonstrating that the two mutant chitinases are able to hydrolyze crystalline microfibril in part by a manner similar to intact ChiA. The needle tips formed by F232A appeared to be sharpest among three chitinases. This observation seems to be consistent with the idea that this mutant chitinase has a significant defect in introducing chitin chain into the catalytic cleft, and thus the starting of processive hydrolysis of chitin chains occurs less efficiently than do the other chitinases.

The shape of the needle tips generated by ChiA is very similar to those observed after treatment with ChiA1 from *B. circulans* (23, 26). Hydrolysis of the β-chitin microfibrils by *B. circulans* ChiA1 was experimentally demonstrated very recently to occur from the reducing end side of the microfibrils as suggested from the structural study of CatD_ChiA1.4 Because the catalytic domains of the two chitinases are very similar as mentioned already, the pointed tips formed by the ChiA must be at the reducing end side of the microfibrils.

**DISCUSSION**

Studies on the chitin binding activities of mutant ChiAs that have substitutions of the four exposed aromatic residues demonstrated that the three of them, Trp-69, Trp-33, and Trp-245, are essential for the binding activity of ChiA to insoluble chitin substrates. On the other hand, F232A, which is closest to the catalytic cleft among the four mutated aromatic residues, did not affect the binding activity. Because the substitution of any one of the three residues reduced the chitin binding activity, all three residues are indispensable to express full binding activity and, therefore, act cooperatively in the chitin binding of this enzyme. The shape of the needle tips generated by ChiA is very similar to those observed after treatment with ChiA1 from *B. circulans* WL-12 (23, 26). Hydrolysis of the β-chitin microfibrils by *B. circulans* ChiA1 was experimentally demonstrated very recently to occur from the reducing end side of the microfibrils as suggested from the structural study of CatD_ChiA1.4 Because the catalytic domains of the two chitinases are very similar as mentioned already, the pointed tips formed by the ChiA must be at the reducing end side of the microfibrils.

### TABLE I

| Enzymes     | Reduced (GlcNAc)_5 | Soluble chitin | Carboxymethyl chitin | Glycol chitin |
|-------------|--------------------|----------------|----------------------|--------------|
| ChiA        | 77.22 (100)        | 2.16 (100)     | 1.04 (100)           | 0.11 (100)   |
| W33A        | 76.81 (100)        | 2.06 (95)      | 1.11 (107)           | 0.12 (109)   |
| W69A        | 83.73 (108)        | 2.16 (100)     | 1.17 (113)           | 0.11 (100)   |
| F232A       | 71.03 (92)         | 2.56 (119)     | 1.03 (99)            | 0.28 (255)   |
| W245A       | 87.24 (113)        | 2.22 (103)     | 1.15 (111)           | 0.12 (109)   |
| Double      | 82.92 (107)        | 1.99 (92)      | 1.18 (114)           | 0.10 (91)    |
| Triple      | 88.54 (115)        | 1.95 (90)      | 1.01 (97)            | 0.12 (109)   |

* One unit of chitinase activity was defined as the amount of enzyme, which produces 1 μmol of reducing sugar/min.

* Values in parentheses represent relative specific activities (%).

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4 T. Imai, T. Watanabe, T. Yui, and J. Sugiyama, submitted for publication.
However, isolated ChBD exhibited significant chitin binding activity by itself, and this domain played a major role in the chitin binding activity of this enzyme (26). In contrast to the contribution to chitin binding activity, all four aromatic amino acids replaced in this study appeared to be essential for efficient hydrolysis of highly crystalline \( \beta \)-chitin microfibrils. Substitution of any one of the four aromatic residues decreased the hydrolyzing activity significantly. The defect in crystalline chitin hydrolysis of W69A, W33A, and W245A may be explained, at least in part, by the defect in the binding activity of these mutant chitinases. On the other hand, the reduced hydrolyzing activity of F232A is not related to its binding activity since F232A retained full binding activity. These results clearly demonstrated that the Phe-232 exposed on the surface of the catalytic domain is essential for the hydrolysis of crystalline chitin in a manner not related to chitin binding activity. Koivula et al. (32) reported that Trp-272 of Trichoderma reesei Cel6A is an essential determinant of crystalline cellulose hydrolysis. Mutagenesis of this residue selectively impaired crystalline cellulose hydrolysis but not hydrolysis of soluble or amorphous substrates. Because Trp-272 is located close to the entrance of the enclosed catalytic tunnel, the situation of Trp-272 seems to be rather similar to that of Tyr-170 of ChiA, which is located at the edge of the entrance of the substrate-binding cleft. However, although Phe-232 is located outside of the substrate-binding cleft, the effect of the substitution of Phe-232 is similar to that caused by the substitution of Trp-272 of Cel6A. Koivula et al. (32) suggested that Trp-272 has a specialized role in crystalline cellulose hydrolysis, possibly in guiding the glucan chain into the catalytic tunnel of Cel6A. These observations and the relative location of Phe-232 to the substrate-binding cleft strongly suggest that Phe-232 plays an important role in guiding and introducing the chitin chain into the catalytic cleft.

We suppose that the other aromatic residues, W69A, W33A, and W245A, are also involved in part in guiding the chitin

![Fig. 5. Bright-field diffraction contrast micrographs of L. satsuma \( \beta \)-chitin microfibrils. A, no enzyme treatment (control). B, after treatment with ChiA. C, after treatment with W33A. D, after treatment with F232A.](image)

![Fig. 6. Model for crystalline \( \beta \)-chitin hydrolysis by ChiA.](image)
chain into the catalytic cleft, although the extent of their participation is different depending on the position of each aromatic residue. This means that Trp-69, Trp-33, and Trp-245 may have dual roles in crystalline (or insoluble) chitin hydrolysis. Mutations of any one of these three residues reduced the binding activity to colloidal chitin, and W69A affected this binding most severely. On the other hand, hydrolyzing activity of W69A was indistinguishable from that of wild-type ChiA, whereas W33A and W245A exhibited slightly lower activity than W69A, and F232A was the lowest among four single mutants. Although the difference in the hydrolyzing activities between W69A and either W33A or W245A is very small, W33A and W245A always gave slightly lower activities than W69A in several independent experiments. The slightly lower hydrolyzing activities of W33A and W245A as compared with W69A and wild-type ChiA could be explained by the involvement of these residues in guiding a chitin chain into the catalytic cleft since the binding activities of W33A and W245A were significantly higher than that of W69A. In addition, the possible involvement of three residues in guiding the chitin chain may be partly responsible for the drastic reduction in the hydrolyzing activity against both β-chitin microfibrils and colloidal chitin by double and triple mutations. Of course, a complete loss of the binding activity must be another reason for the drastic reduction of hydrolyzing activity by these mutations.

Processive hydrolysis of crystalline chitin was suggested by electron microscopy of the β-chitin microfibrils treated with ChiA and its mutants. Processive hydrolysis by polysaccharide-degrading enzymes has been most extensively studied with Cel6A and Cel7A from *Serratia* (33, 34). The term “processivity” is used to indicate that an enzyme degrades a polymer chain without dissociating from it between successive catalytic events. Both Cel6A and Cel7A have an active site cleft with tunnel morphology. According to a recent hypothesis, a single glucan chain is introduced at the tunnel entrance and threads through the entire tunnel with eventual release of the product, cellobiose, from the end of the tunnel (32). The mechanisms of crystalline chitin hydrolysis that were proposed based on the structure of ChiA, especially from the positions of the aromatic residues, and the results obtained in this study, are consistent with the processive action of this enzyme, although ChiA does not have a tunnel-shaped catalytic cleft (see Fig. 6). Binding of ChiA to the crystalline chitin surface is achieved through the interaction among three aromatic residues (Trp-69, Trp-33, and Trp-245) and the GlcNAc residues in a single chitin chain on the crystalline chitin surface. The chitin chain interacting with the three aromatic residues is introduced into the catalytic cleft from the reducing end side of the chain through the interaction with Phe-232. In the catalytic cleft, the introduced chitin chain slides through the cleft to the catalytic site, and the second linkages from the reducing end are progressively cleaved, releasing (GlcNAc)\(_n\) units continuously. Tyr-170 at subite -5, Trp-167 at subite -3, Trp-539 at subite -1, Trp-275 at subite +1, and Phe-396 at subite +2 in the catalytic cleft play major roles in holding and also in sliding the chitin chain. The Trp residues around the catalytic site, such as Trp-167, Trp-539, Trp-275, and Phe-396, may be mainly responsible for bending and twisting the chitin chain at the subite -1. The interaction with these multiple aromatic residues, which are located not only within the catalytic cleft but also on the surface of the ChiA molecule, must result in a tight holding of a chitin chain by the enzyme during a number of hydrolyzing reactions, which occurs at the second linkage from the reducing end, thus resulting in the processive action of this enzyme. As a result of the processive hydrolysis of a single chitin chain in the crystalline β-chitin microfibrils, the ChiA molecule proceeds on the crystalline chitin surface toward the non-reducing end side of the microfibrils with the N-terminal domain at the head, releasing (GlcNAc)\(_n\) units from the reducing end of the chitin chain.

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