Solution Structure of the Chitin-binding Domain of Bacillus circulans WL-12 Chitinase A1

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The three-dimensional structure of the chitin-binding domain (ChBD) of chitinase A1 (ChiA1) from a Gram-positive bacterium, Bacillus circulans WL-12, was determined by means of multidimensional heteronuclear NMR methods. ChiA1 is a glycosidase that hydrolyzes chitin and is composed of an N-terminal catalytic domain, two fibronectin type III-like domains, and a C-terminal chitin-binding domain (45 residues, Ala655–Gln699), which binds specifically to insoluble chitin. ChBD_{ChiA1} has a compact and globular structure with the topology of a twisted \( \beta \)-sandwich. This domain contains two antiparallel \( \beta \)-sheets, one composed of three strands and the other of two strands. The core region formed by the hydrophobic and aromatic residues makes the overall structure rigid and compact. The overall topology of ChBD_{ChiA1} is similar to that of the cellulose-binding domain (CBD) of Eucaryia chrysanthemi endoglucanase Z (CBD_{EC}). However, ChBD_{ChiA1} lacks the three aromatic residues aligned linearly and exposed to the solvent, which probably interact with cellulose in CBDs. Therefore, the binding mechanism of a group of ChBDs including ChBD_{ChiA1} may be different from that proposed for CBDs.

Chitinase (EC 3.2.1.14) is a glycosyl hydrolase that catalyzes the hydrolytic degradation of chitin, a fibrous insoluble polysaccharide made of \( \beta \)-1,4-N-acetyl-D-glucosamine residues. Chitinases are found in a wide variety of organisms that possess chitin as a constituent (fungi, insects, and crustaceans) and that do not possess chitin as well (bacteria, plants, and vertebrates). The roles of chitinases in these organisms are diverse (1). Invertebrates require chitinases for partial degradation of old exoskeletons. Fungi produce chitinases to modify chitin, which is used as an important cell wall component. Bacteria produce chitinases to digest chitin and utilize it as carbon and energy sources. It is suggested that the production of chitinases by higher plants is a part of defense mechanisms against fungal pathogens (2).

Bacillus circulans WL-12 is a Gram-positive bacterium identified as being lytic for yeast and fungal cell walls (3). The bacterium has been reported to secrete multiple chitinases into culture medium containing chitin as an inducer (4, 5). Among these chitinases, A1 encoded by the chiA gene is thought to be the key enzyme in the chitinase system of this bacterium, because chitinase A1 (ChiA1) (4) is produced most abundantly and exhibits the highest activity as to the hydrolysis of colloidal chitin (4) and a high affinity to insoluble chitin. ChiA1 (M\(_{\text{r}}\) = \( \sim \)74,000) contains three discrete functional domains: an N-terminal catalytic domain (CatD) (417 amino acid residues), a tandem repeat of fibronectin type III-like (FnIII) domains (duplicate 95 residues), and a C-terminal chitin-binding domain (45 residues) (6). Such a structural form of discrete catalytic and substrate-binding domains (7) as in ChiA1 has also been observed for some other polysaccharide-degrading enzymes, including cellulas (8, 9), xylanases (10), amylases, and a \( \beta \)-1,3-glucanase (11).

The C-terminal chitin-binding domain (ChBD_{ChiA1}) is required for ChiA1 to bind specifically to insoluble chitin and to hydrolyze it efficiently (4, 6). We have found that ChBD_{ChiA1} does not bind to chito-oligosaccharides or soluble derivatives of chitin but does bind to insoluble or crystalline chitin. So far, two roles of the cellulose-binding domains (CBDs) in their hydrolysis activities have been suggested: (i) CBDs may enhance the cellulase activities by concentrating the cellulases on a cellulose surface (13, 14), and (ii) CBDs may disrupt noncovalent interactions including hydrogen bonds between adjacent glucose units (15). The chitin-binding domains (ChBDs) are also considered to have similar roles to those of CBDs, but the roles of ChBDs remain unclear. Although a large number of studies have been performed on the structures of the CBDs of cellulases (8, 16–21), little is known about the tertiary structures of the ChBDs of chitinases.

The structures of at least three chitinases have already been determined. They are (i) endochitinase from barley seeds, Hordeum vulgare (22), (ii) hevamine with combined chitinase and lysozyme activities from a plant, Hveeza brasiliensis (23), and (iii) chitinase A from a Gram-negative soil bacterium, Serratia marcescens (24). However, the two plant chitinases do not contain ChBDs that are separated from their catalytic domains. It is suggested that the remaining chitinase, S. marcescens chitinase A, contains a putative ChBD in its N-terminal region (ChiN) (residues Ala\(^24\)–His\(^{137}\)) (1), but no sequence similarity exists between ChiN and ChBD_{ChiA1} (see “FnIII Domains” below).
mains”). Therefore, the three-dimensional structure of a ChBD whose function has clearly been identified is necessary for elucidation of its binding mechanism with chitin and its role in the subsequent catalytic activity. Further, some CBDs studied so far have the ability to bind to soluble chito-oligosaccharides (17) and chitin (25) as well as crystalline cellulose. On the other hand, ChBD\textsubscript{CHI1} only binds to insoluble chitin; specifically, it does not bind to soluble chito-oligosaccharides, soluble chitin derivatives, or cellulose. Thus, it is important to approach the mechanism by which this rather small domain consisting of 45 amino acids exhibits this binding specificity to insoluble chitin from the viewpoint of its tertiary structure.

Here we present the three-dimensional structure of the chitin-binding domain of Chitinase A1 (B. circulans) determined by NMR using a uniformly $^{15}$N-labeled domain. To the best of our knowledge, this is the first reported structure of a ChBD that binds specifically to chitin. Analysis involving structures and sequential alignments allowed us to suggest that the chitin-binding mechanism of ChBD\textsubscript{CHI1} is different from the currently proposed cellulose-binding mechanism of the CBDs of cellulases, which are supposed to interact with cellulose via three aromatic rings arranged linearly on the surfaces of CBDs.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Cells of Escherichia coli BL21(DE3) harboring the expression pET3a plasmid (Novagen, Madison, WI) with the ChBD\textsubscript{CHI1} gene were grown in M9 minimal medium containing 100 μg/ml ampicillin, 0.1% glycerol, 4.0 g/liter d-glucose, and 0.5 g/liter $^{15}$NH\textsubscript{4}Cl as a sole nitrogen source. The cells were incubated at 30 °C with shaking, and expression of the protein was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside for 24 h. The cells were collected by centrifugation and disrupted by sonication in 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. The soluble fraction was collected by ammonium sulfate precipitation (60% saturation) and then dialyzed against 5 mM potassium phosphate buffer (pH 6.0). The obtained protein has a T7 tag consisting of 14 residues (H\textsubscript{2}N-Met-Ala-...-Ser-Glu-Thr) at its N terminus. The protein was stable and did not lose activity even after 22 h at 30 °C. The samples for most of the NMR measurements were obtained from 1 liter of an M9 minimal culture. The obtained protein has a T7 tag consisting of 14 residues (H\textsubscript{2}N-Met-Ala-...-Ser-Glu-Thr) at its N terminus. The protein was stable and did not lose activity even after 22 h at 30 °C. The samples for most of the NMR measurements were obtained from 1 liter of an M9 minimal culture. The obtained protein has a T7 tag consisting of 14 residues (H\textsubscript{2}N-Met-Ala-...-Ser-Glu-Thr) at its N terminus. The protein was stable and did not lose activity even after 22 h at 30 °C. The samples for most of the NMR measurements were obtained from 1 liter of an M9 minimal culture.

**NMR Spectroscopy**—All NMR experiments were performed with a Bruker DRX500 or DRX800 spectrometer equipped with a triple resonance ($^{1}H$, $^{15}N$, and $^{13}C$) probe with a self-shielded triple axis gradient coil. Most spectra were recorded at 310 K. For $^{1}H$ and $^{15}N$ resonance assignments (26), two-dimensional $^{15}N$-HSQC, two-dimensional TOCSY (with a mixing time of 50 ms), three-dimensional $^{15}N$-edited TOCSY (with a mixing time of 70 ms), and two-dimensional H\textsubscript{NN} TOCSY and three-dimensional H\textsubscript{NNH} TOCSY spectra (with mixing times of 308 ms) (27), spectra were acquired. The numbers of complex points and spectral widths in the three-dimensional (H\textsubscript{NNH} TOCSY experiment with a Bruker DRX500) were 20,620 Hz ($^{15}N$, $^{1}H$, and $^{13}C$) and 681,928 Hz ($^{1}H$, $^{15}N$). For dihedral angle constraints, a two-dimensional HMOC-J spectrum (26) was acquired with the DRX800. The $^{15}N$ dimension was recorded with an acquisition time of 403 ms (400 complex data sets). The digital resolution was 0.5 Hz after zero-filling and subsequent Fourier transformation. For interproton distance constraints (26), two-dimensional HMQC-J TOCSY (with a mixing time of 50 ms), three-dimensional $^{15}N$-edited TOCSY, and three-dimensional (H)NNH TOCSY experiments were included. The program WANGEINGATE and Water-flip-back techniques (26). The three-dimensional $^{15}N$-edited TOCSY experiment included the sensitivity enhancement and gradient echo methods for the indirect $^{15}N$ dimension (26). All other indirect dimensions were recorded in the States-TPPI manner (26). For 1H and 15N resonance assignments (26), two-dimensional $^{15}N$-edited TOCSY, two-dimensional H\textsubscript{NNH} TOCSY, three-dimensional (H)NNH TOCSY experiments included. The program WANGEINGATE and Water-flip-back techniques (26). The three-dimensional $^{15}N$-edited TOCSY experiment included the sensitivity enhancement and gradient echo methods for the indirect $^{15}N$ dimension (26). All other indirect dimensions were recorded in the States-TPPI manner (26). The NMR data were processed and analyzed using the nmrPipe (28) and Pipp (29) software packages, respectively. Stereospecific assignments of the methyl groups of the leucine and valine residues were achieved with 15% fractionally $^{13}$C-labeled ChBD\textsubscript{CHI1} dissolved in 99.8% $^{2}H_{2}O$ as described (30).

**Results**

**Resonance Assignments**—The amide $^{1}H$ and $^{15}N$ resonances were assigned at first using two-dimensional H\textsubscript{NN}/H TOCSY and three-dimensional (H)NNH TOCSY spectra (27), magnetization being transferred from an amide $^{15}N$ spin to those of both preceding and following residues through a $^{15}N$ homonuclear Hartmann-Hahn mixing (TOCSY) scheme. Thus, the two-dimensional and three-dimensional spectra provided observable resonances at the frequencies of (ω\textsubscript{NN}, ω\textsubscript{N}) and (ω\textsubscript{NN}, ω\textsubscript{N}, ω\textsubscript{NN}), respectively, where $i$ equals $j−1$, $j$, or $j+1$. Figs. 1, A and B, show examples of the sequential connectivities of the amide groups of residues (Tyr\textsubscript{462}–Tyr\textsubscript{470}) in the two-dimensional H\textsubscript{NNH} TOCSY and three-dimensional (H)NNH TOCSY spectra, respectively. The combination of the two-dimensional and three-dimensional spectra provided secure and three-dimensional sequential connectivities, which account for 63% of the expected connectivities. Next, additional information regarding $^{1}H$-$^{15}N$ distances and amino acid types was obtained from the three-dimensional $^{15}N$-edited NOE and three-dimensional $^{15}N$-edited TOCSY spectra, respectively. This information compensated for the ambiguity of the amide sequential connectivities that were not resolved in the above $^{1}$NN-based experi-
ments. Finally, we accomplished the assignment of all the amide resonances except for those of Asn$^671$, Gly$^672$, the T7 tag region, and three proline residues. The region containing Asn$^671$ and Gly$^672$ may undergo a conformational exchange, because these residues form the turn connecting the $\beta_x$- and $\beta_z$-strands (see “Structure Description”). Fig. 1C shows a two-dimensional $^{15}$N-$^1$H HMQC spectrum with the amide resonance assignments. The well dispersed resonance peaks in both the $^1$H and $^{15}$N dimensions reflect the stable structure of ChBD-Chia1. The proton resonances of the $\alpha$ sites and side chains were assigned using the two-dimensional TOCSY and three-dimensional $^{15}$N-edited TOCSY spectra. The methyl groups of the four leucine and two valine residues were assigned stereospecifically using 15% fractionally $^{13}$C-labeled ChBD-Chia1. The methyl groups of the other two residues, Leu$^{678}$ and Val$^{658}$, could not be assigned stereospecifically owing to their overlapping $^1$H chemical shifts. Overall, we assigned 95% of the expected $^1$H and $^{15}$N resonances of the main chain and side chains.

Constrains for the Structure Calculation—The distance constraints based on NOE were extracted from two-dimensional NOESY and three-dimensional $^{15}$N-edited NOESY spectra with mixing times of 150 ms. By comparing the intensities of well resolved cross-peaks in a series of preliminary two-dimensional NOESY spectra obtained with different mixing times, i.e. 50, 100, 150, 200, and 250 ms (data not shown), we judged that the mixing time of 150 ms did not cause severe spin diffusion during the NOE transfer of magnetization. Fig. 2A summarizes the sequential and medium-range NOE connectivities along with the secondary structures, $^1$H-$^1$H chemical shift indices (38), amide hydrogen exchange rates, and $^3J_{\text{HNHA}}$, coupling constants observed for ChBD-Chia1. The $\beta$-strand regions are characterized by the strong intensities of the NOE cross-peaks between $\alpha$ protons and the amide protons of the subsequent residues ($d_{N(i), i+1}$). The locations of the $\beta$-strands are also indicated by positive deviations of the $^1$H chemical shift values from those observed in random coils, which are represented by the $^1$H chemical shift index of $+$1 (38), slow rates of exchange of the amide protons with the solvent, and $^3J_{\text{HNHA}}$ coupling constants larger than 8.5 Hz. Interstrand NOE connectivities were observed among $^1$H and $^1$H atoms ($^1$H-$^1$H, $^1$H-$^1$H, and $^1$H-$^1$H), as shown by the arrows in Fig. 2B.

Overall, 493 NOE-based distance, 20 hydrogen bond, and 33 dihedral angle constraints were collected and used for the structure calculations (Table I).

Structure Determination—Solution structures were calculated through the standard simulated annealing protocol in the program X-PLOR 3.851 (35). Out of 100 calculated structures, 30 final structures, which showed the lowest energy values, no distance constraint violation of $>0.3$ Å, and no dihedral angle constraint violation of $>5^\circ$, were selected for further analyses. Superpositioning of these 30 structures is shown in Fig. 3A, and a summary of the constraints and structural statistics is given in Table I. The backbones converged well, as indicated by the r.m.s. deviation of 0.326 Å from the mean structure for the backbone $C^\alpha, C^\beta, N$ atoms of all the residues except for the N-terminal Ala$^{655}$ and C-terminal Glu$^{699}$ residues. The r.m.s. deviation for all heavy atoms in the same regions is 0.700 Å. The Asn$^671$ and Gly$^672$ residues, whose amide signals could not be observed in $^1$H-$^{15}$N HSQC spectra, are in the loop region connecting the $\beta_x$- and $\beta_z$-strands, and the calculated coordinates of the loop diverged due to the few constraints in the loop.

Almost the same structures were obtained upon calculation with the program DYANA 1.5 (34) with the same constraints as those used in the X-PLOR calculation. The average DYANA target function value of the best 30 structures selected out of the 100 calculated ones was $0.32 \pm 0.10$ (one S.D.), and the r.m.s. deviations of all 60 structures that were calculated with X-PLOR and DYANA were 0.354 Å for the backbone atoms and 0.713 Å for all of the heavy atoms in the same regions as
**TABLE I**  
**Structural statistics for ChBD ChlA1**

These statistics comprise the ensemble of the final 30 simulated annealing structures from Ala655 to Gln699 calculated with X-PLOR 3.851 (35). All variances are quoted 6 S.D.

| Statistical Category | Value |
|----------------------|-------|
| Total number of distance constraints | 513 |
| Intraresidual | 149 |
| Sequential (| 1 | ) | 128 |
| Medium range (| 1 | 4) | 51 |
| Long range (| 1 | > 4) | 165 |
| Hydrogen bonds | 20 |
| Number of dihedral angle constraints | 33 |
| Maximum violation of distance constraints | 0.11 Å |
| Maximum violation of dihedral angle constraints | 1.3° |
| Maximum violation of van der Waals distances | 0.0 Å |
| r.m.s. deviations from experimental constraints |
| Distance | 0.0068 ± 0.0011 Å |
| Angle | 0.171 ± 0.056° |
| r.m.s. deviations from idealized covalent geometry |
| Bonds | 0.0019 ± 6.94 X 10^-5 Å |
| Angles | 0.550 ± 0.008° |
| Impropers | 0.339 ± 0.004° |
| X-PLOR potential energy (E_{total}) | 74.6 ± 2.2 |
| PROCHECK Ramachandran plot statistics (residues Trp656-Try670, Lys673-Leu698) |
| Residues in most favored regions | 70.0% |
| Residues in additional allowed regions | 30.0% |
| Residues in generously allowed regions | 0.0% |
| Residues in disallowed regions | 0.0% |
| r.m.s. deviations from the mean coordinate positions (residues Trp656-Leu698) |
| Backbone heavy atoms | 0.326 Å |
| All heavy atoms | 0.700 Å |
Structure Description—ChBDChiA1 has a compact and globular structure, as shown in Fig. 3B. It contains two antiparallel β-sheets (Fig. 2C). One sheet is composed of three strands designated as β2 (Gln666–Tyr670), β3 (Lys673–Cys677), and β5 (Trp696–Leu698), while the other is composed of two strands designated as β1 (Thr660–Tyr662) and β4 (His681–Ser683). No region characteristic of an α-helix exists. β5 consists of three residues and forms an antiparallel β-sheet with the three C-terminal residues of β3. Fig. 2B shows the hydrogen bond networks and NOE connectivities among these five β-strands that were used for the structure calculation. The two antiparallel β-sheets formed by these hydrogen bond networks fold into the topology of a twisted β-sandwich with an angle of about 180° rotation about the vertical axis. The molecular orientation in the left-hand image is almost the same as in A and B. The figures were generated with the program GRASP (52).

Fig. 3. Tertiary structures of ChBDChiA1. A, a stereoview of the best-fit superpositioning of the final 30 structures, which were calculated by means of the simulated annealing procedure of X-PLOR 3.85 (35). The backbone atoms (N, Cα, and C) in the regions colored purple (Trp666–Tyr670 and Lys673–Leu698) are superimposed. The side chains of the residues forming the hydrophobic core are also shown in magenta. B, schematic ribbon drawing of the representative structure. The secondary structure elements and both end residues of each β-strand are shown. The figure was drawn with the programs MOLSCRIPT (50) and RASTER3D (51). The direction of the molecule is the same as in A, C, mapping of the electrostatic potential on the solvent-accessible surface of ChBDChiA1. Blue, a positive potential; red, a negative potential. The right-hand image was generated from the left one by 180° rotation about the vertical axis. The molecular orientation in the left-hand image is almost the same as in A and B. The figures were drawn with the programs MOLMOL (36).

Fig. 4. Structural comparison of CBD_{EGZ} and ChBD_{ChiA1}. A, schematic ribbon drawing of the structure of the 62-amino acid C-terminal cellulose-binding domain of endoglucanase Z (Cel5) (CBD_{EGZ}) secreted by E. chrysanthemi determined by NMR by Brun et al. (8); B, drawing of ChBD_{ChiA1}. The aromatic and hydrophobic residues conserved well among various chitinases, which are indicated by the gray backgrounds in Fig. 5, are also shown. The corresponding residues between CBD_{EGZ} and ChBD_{ChiA1} are drawn in the same colors. The two cysteine residues of CBD_{EGZ} forming the disulfide bridge (Cys5–Cys61) are also shown in orange in A. The three residues of CBD_{EGZ}, which are involved in the cellulose-binding (Trp18, Trp43, and Tyr44), are colored green in A. Both images were drawn with the program MOLMOL (36).
Structure of the Chitin-binding Domain of Chitinase A1

Comparison of ChBD_{ChA1} and CBDs—Chitin differs chemically from cellulose only in that each C2 hydroxyl (-OH) group in cellulose is replaced by an acetamide (-NHCOCH_{3}) group in chitin. Thus, the mechanism by which ChBD_{ChA1} binds to chitin was expected to be similar to the mechanism by which CBDs bind to cellulose. The most accepted model for the binding of CBDs to cellulose is that aromatic rings arranged in the flat face of a CBD are stacked on every other pyranose ring of polysaccharides through hydrophobic interactions (20). The involvement of aromatic residues in the interactions has been observed using NMR (41), site-directed mutagenesis (16, 25), and chemical modification (42). In CBD_{EGZ}, for example, three aromatic residues, Trp^{18}, Trp^{32}, and Tyr^{34}, are involved in the interaction with cellulose. Moreover, the other two aromatic residues, which correspond to Trp^{43} and Tyr^{44} in CBDEGZ, are also missing or replaced by another aromatic side chain in CBD_{EGZ}, as shown in Fig. 4A. Recently, Simpson et al. confirmed the involvement of these three aromatic residues in the interaction with cellulose on site-directed mutagenesis of the intact endoglucanase Z (also referred to as Cel5) (25).

Despite the similarity between the overall topologies of CBD_{EGZ} and ChBD_{ChA1}, ChBD_{ChA1} lacks a region that corresponds to the loop ranging from Val^{15} to Gln^{22} of CBD_{EGZ} (colored purple in Fig. 4A). This loop region of CBDEGZ contains only three charged residues (+Lys^{62}, +Lys^{67}, and −Glu^{688}), and the surface of the molecule is dominated by noncharged residues, as shown in Fig. 3C.

The similarity of the tertiary structure of ChBD_{ChA1} to known structures was examined with the DALI server version 2.0 (39), pairs with Z scores of more than 2.0 being assumed to be similar. The results showed that the structure of ChBD_{ChA1} is similar to that of the CBD of endoglucanase Z (CBD_{EGZ}) (Protein Data Bank code 1aiw) secreted by a Gram-negative bacterium, Eruinia chrysanthemi (8), which is similar to that of the CBD of endoglucanase Z (CBD_{EGZ}) (Protein Data Bank code 1aiw) secreted by a Gram-negative bacterium, Eruinia chrysanthemi (8), as indicated by the Z score of 2.8 and r.m.s. deviation (C^\alpha atoms) of 2.4 Å (Fig. 4). No other similar structure (characterized by a Z score > 2.0) was found in the DALI data base. The sequence of CBD_{EGZ} is also similar to that of ChBD_{ChA1} (Fig. 5) (CLUSTAL W (40) alignment score, 19%) (see “Discussion”).

FIG. 5. Amino acid sequence alignment of ChBD_{ChA1} with the domains of other chitinases and CBD_{EGZ}. After alignment with the program CLUSTAL W (40), all of the sequences were further aligned on the basis of the three-dimensional structures of ChBD_{ChA1} and CBD_{EGZ}. Amino acid sequences well conserved are indicated by gray backgrounds. The amino acid sequences shown are for B. circulans WL-12 chitinase D (53) (ii), S. marcescens 2170 chitinase C (54) (iii), Aeromonas sp. strain 108-24 chitinase II (55) (iv, vii), Janthinobacterium lividum iv, (viii) chitinase (56), Aeromonas sp. strain 108-24 chitinase (57) (vi), Aeromonas casei extracellular chitinase A (58) (ix), Alteromonas sp. strain O-7 chitinase 85 (12) (x), and the C-terminal CBD of E. chrysanthemi endoglucanase Z (EGZ or Cel5) (8) (xi). The sequences are classified into two groups, i.e., the ChBD_{ChA1} group displayed in the upper part (lines i-v) and the CBD_{EGZ} group displayed in the lower part (lines vi-xi). The numbers at the left and right of each sequence represent the first and last residue positions in the sequence, respectively. The numbers at the top and bottom represent the sequence numbers of ChBD_{ChA1} and CBD_{EGZ}, respectively. The boxes at the top and bottom represent the ß strand regions of ChBD_{ChA1} and CBD_{EGZ}, respectively. The three residues of CBD_{EGZ} (Trp^{18}, Trp^{43}, and Tyr^{44}), which are involved in the cellulose-binding, are indicated by diamonds at the top. The numbers in brackets indicates the aligned scores estimated with the program CLUSTAL W (40) between ChBD_{ChA1} and the respective sequences. The score corresponds to 100 for identical sequences.

FIG. 6. The residues that may interact with chitin. The side chain atoms of these residues are shown in a space-filling model on a ribbon representation of ChBD_{ChA1} in stereo.

The similarity of the tertiary structure of ChBD_{ChA1} to known structures was examined with the DALI server version 2.0 (39), pairs with Z scores of more than 2.0 being assumed to be similar. The results showed that the structure of ChBD_{ChA1} is similar to that of the CBD of endoglucanase Z (CBD_{EGZ}) (Protein Data Bank code 1aiw) secreted by a Gram-negative bacterium, Eruinia chrysanthemi (8), which is similar to that of the CBD of endoglucanase Z (CBD_{EGZ}) (Protein Data Bank code 1aiw) secreted by a Gram-negative bacterium, Eruinia chrysanthemi (8), as indicated by the Z score of 2.8 and r.m.s. deviation (C^\alpha atoms) of 2.4 Å (Fig. 4). No other similar structure (characterized by a Z score > 2.0) was found in the DALI data base. The sequence of CBD_{EGZ} is also similar to that of ChBD_{ChA1} (Fig. 5) (CLUSTAL W (40) alignment score, 19%) (see “Discussion”).
ChBD-ChiA1 probably recognizes the solid surface conformation of crystallin chitin but not that of cellulose or the flexible conformations of soluble substrates. On the other hand, the binding specificities of the CBDs of some cellulases are rather broad. CBD-Chb1, CBD-Chb1 (41), CBD-Exl (17), and CBD-Xyla (10) bind to both crystalline cellulose and soluble cello-oligosaccharides, and CBD-Exz (CBD-CalA) (25), CBD-Cha (43), CBD-Chb1 (44), CBD-Cex (18), CBD-Csea (45), and Cip-Cbd (20) bind to both cellulose and chitin. Thus, we suggest that the binding mechanism of ChBD-ChiA1 is different from that of CBDs.

Two Groups of ChBDs—Brun et al. (8) pointed out that the stWWst motif, which corresponds to Ala41, Asn42, Trp43, Tyr44, Thr45, and Ala66 in CBD-Exz, is not conserved in CBD-Chb1. Fig. 5 shows the sequence alignment of ChBD-ChiA1 with the domains of other chitinases and CBD-Exz. In CBD-Exz and the five chitinases listed on the lower lines (Fig. 5, vi–xi), the Trp-Trp, Tyr-Trp, or Trp-Tyr (Arom-Arom) sequence is conserved at the site where CBD-Exz holds the Trp43-Tyr44 sequence. On the contrary, ChBD-ChiA1 and the four chitinases listed on the upper lines (i–v) have no such sequence at the corresponding site. The lack of the Arom-Arom sequence in ChBD-ChiA1 is more apparent in the tertiary structure shown in Fig. 4B. Therefore, the chitinases in the group including ChBD-ChiA1 (the ChBD-ChiA1 group) probably have different binding surfaces for substrates from those of the chitinases and cellulases in the other group including CBD-Exz (the CBD-Exz group).

We searched for the residues of ChBD-ChiA1 that may be involved in the binding to chitin. The criteria for the search were that (i) such residues are well conserved at least in the ChBD-ChiA1 group (lines i–v in Fig. 5); (ii) they exist on the surface of the molecule; and (iii) they are hydrophobic or aromatic residues contributing to the hydrophobic interaction with chitin. The last criterion is based on the observation that more ChBD-ChiA1 bound to chitin at a pH nearer to its isoelectric point (about 9.0) or in the presence of more NaCl in the solution. The residues that meet these criteria are His681 (27.1), Thr682 (34.4), Trp687 (22.5), Pro689 (38.6), and Pro693 (45.5), the values in parentheses indicating the solvent accessibility. These residues are localized on one face of the molecule, which is different from the interaction surface of CBD-Exz, as shown in Fig. 6. Further, Thr682 and Pro693 are specific residues to the ChBD-ChiA1 group, i.e. they are not conserved in the CBD-Exz group (lines vi–xi in Fig. 5). In CBD-Exz, the three aromatic residues involved in the interaction with cellulose, Trp18 (49.2), Trp43 (36.0), and Tyr44 (45.9%), are exposed much on the surface of the molecule, but in ChBD-ChiA1, no aromatic residue with such high solvent accessibility was found. His681 (27.1) and Thr682 (22.5%) are the two aromatic residues that are most exposed to the solvent. Instead, Thr682 (34.4) and Pro689 (38.6%) in ChBD-ChiA1 have much higher solvent accessibility values than the corresponding residues of CBD-Exz, Thr45 (5.0) and Pro49 (0.9%), respectively. Thus, in the ChBD-ChiA1 group, residues such as threonine and proline as well as aromatic residues may also be involved in the interaction with chitin. Since ChBD-ChiA1 binds only to the crystalline form of chitin, the domain is expected to recognize the characteristic conformation of crystallin chitin, such as extended polysaccharide chains aligned in parallel to each other. Thus, the binding specificity of ChBD-ChiA1 to solid chitin is probably due to the different binding mechanism from that of CBD-Exz despite their similar tertiary structures.

We anticipate that the hydrophobic effect is the major contribution to the interaction between ChBD-ChiA1 and chitin, as mentioned above. However, since ChBD-ChiA1 is eluted from a chitin affinity column at pH 3.0, the negatively charged unique residue, Glu688, may also be involved in the interaction (Fig. 3C). The involvement of polar residues (Gln, Asn) in the interaction with cellulose through hydrogen bonding to oxygen atoms or hydroxyl groups of glucose moieties has been observed in the CBDs (20, 46).

FnIII Domains—The N-terminal domain of chitinase A from a soil bacterium, S. marcescens, is likely to interact with chitin because the intact S. marcescens chitinase A exhibits significant chitin-binding activity (47) and comprises this N-terminal domain (ChiN) (residues Ala24–His137) and a C-terminal catalytic domain (residues Val159–Ala442 and Asp317–Val563), which is significantly homologous to the catalytic domain of B. circulans chitinase A (ChiA1). However, no similarity was found in the sequence (CLUSTAL W (32) alignment score, 10%), overall topology, or arrangements of the aromatic rings between ChiN and ChBD-ChiA1. ChiN (114 residues, 11 β-strands) is more than twice the size of ChBD-ChiA1 (45 residues, 5 β-strands), and the tertiary structure of ChiN rather resembles those of the FnIII modules found in various animal proteins despite no sequence similarity between ChiN and FnIII domains (48). Although ChiA1 also contains two consecutive FnIII domains between the catalytic (CatD) and binding (ChBD) domains (49), these FnIII domains, interestingly, do not bind to chitin (6).

Since ChiN has nine aromatic residues, some of them may be involved in the interaction with chitin in the similar way to how the three consecutive aromatic rings of CBDs interact with cellulose.

A Disulfide S–S Bond—CBD-Exz has a disulfide bridge (Cys4–Cys81) between the two extremities of the domain, whereas ChBD-ChiA1 has no disulfide bridge at the corresponding site (Fig. 4). To be precise, Cys61 of CBD-Exz corresponds to the outside of the C-terminal end of ChiA1, and no cysteine residue exists around the region in ChiA1 corresponding to the region containing Cys4 in CBD-Exz. As a protein engineering approach, the introduction of a disulfide bridge into ChiA1 would increase the stability of the protein against changes in heat, pH, ionic strength, etc. more and would make a mutated protein more appropriate for various industrial uses.

REFERENCES

1. Jolles, P., and Muzzarelli, R. A. A. (1999) Chitin and Chitinases, Birkhauser Verlag, Basel.
2. Boller, T. (1985) in Cellular and Molecular Biology of Plant Stress (Key, J. L., and Kouse, T., eds) pp. 247–262, Alan R. Liss Inc., New York.
3. Tanaka, H., and Phaff, H. J. (1976) J. Bacteriol. 126, 176–181.
4. Watanabe, T., Oyanagi, W., Suzuki, K., and Tanaka, H. (1999) J. Bacteriol. 172, 4017–4022.
5. Alam, M. M., Mizutani, T., Isho, M., Nikiadou, N., and Watanabe, T. (1996) J. Ferment. Bioeng. 82, 28–38.
6. Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S., and Tanaka, H. (1994) J. Bacteriol. 176, 4465–4472.
7. Cohen-Kupiec, R., and Chet, J. (1998) Curr. Opin. Biotechnol. 9, 270–277.
8. Brun, E., Moriaud, F., Gans, P., Blackledge, M. J., Barras, F., and Marion, D. (1997) Biochemistry 36, 16074–16086.
9. Reinikainen, T., Russhonen, L., Nevanen, T., Laakosen, L., Kraulis, P., Jones, T. A., Knowles, J. K., and Teeri, T. T. (1999) Proteins 42, 475–482.
10. Nagy, T., Simpson, P., Williamson, M. P., Hazlewood, G. P., Gilbert, H. J., and Orosee, L. (1998) FEBS Lett. 429, 312–316.
11. Watanabe, T., Kasahara, N., Aida, K., and Tanaka, H. (1999) J. Bacteriol. 174, 156–160.
12. Tsujibo, H., Orikoshi, H., Tanno, H., Fujimoto, K., Miyamoto, K., Imada, C., Okamoto, Y., and Inamoto, T. (1999) J. Bacteriol. 181, 575–581.
13. Gilkes, N. R., Warren, R. A., Miller, R. C. J., and Kilburn, D. G. (1988) J. Biol. Chem. 263, 10401–10407.
14. Tonnme, P., Van Tilbeurgh, H., Petersson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., and Claeseney, M. (1998) Eur. J. Biochem. 270, 575–581.
15. Din, N., Gilkes, N. R., Tekant, B., Miller, R. C., Warren, R. A. J., and Kilburn, D. G. (1991) Bio/Technology 9, 1096–1099.
16. Matti, L., Kontturi, M., Kerovu, J., Linder, M., Annila, A., Lindeberg, G., Reinikainen, T., and Drakenberg, T. (1997) Protein Sci. 6, 294–303.
17. Matti, L., Kontturi, M., Linder, M., Drakenberg, T., and Annila, A. (1998) Eur. J. Biochem. 256, 279–286.
18. Xu, G. Y., Ong, E., Gilkes, N. R., Kilburn, D. G., Muhandiram, D. R., Harris-Brandt, M., Carver, J. P., Kay, L. E., and Harvey, T. S. (1995) Biochemistry 34, 6993–7009.
19. Johnson, P. E., Tomme, P., Joshi, M. D., and McIntosh, L. P. (1996) Biochemistry 35, 13935–13966.
20. Tornoto, J., Lamed, R., Chirino, A. J., Morag, E., Bayer, E. A., Shoham, Y., and...
