Crystal Structure of Chitosanase from Bacillus circulans MH-K1 at 1.6-Å Resolution and Its Substrate Recognition Mechanism*

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Chitosanase from Bacillus circulans MH-K1 is a 29-kDa extracellular protein composed of 259 amino acids. The crystal structure of chitosanase from B. circulans MH-K1 has been determined by multiwavelength anomalous diffraction method and refined to crystallographic R = 19.2% (Rproj = 23.5%) for the diffraction data at 1.6-Å resolution collected by synchrotron radiation. The enzyme has two globular upper and lower domains, which generate the active site cleft for the substrate binding. The overall molecular folding is similar to chitosanase from Streptomyces sp. N174, although there is only 20% identity at the amino acid sequence level between both chitosanases. However, there are three regions in which the topology is remarkably different. In addition, the disulfide bridge between Cys50 and Cys124 joins the secondary structure of the MH-K1 chitosanase, which is hydrolyzed by chitinase (EC 3.2.1.14). Chitinase is one of the key enzymes in plant defense systems against fungal infection (1, 2). It is widely distributed in microorganisms and plants, and the primary structures of these molecules have been reported for many sources. They are classified on the basis of the amino acid sequence similarities into either family 18 or 19 among the 72 glycosyl hydrolase families (3–5). The three-dimensional structures of these chitosanases is available only for the crystal structure of N174 chitosanase (28).

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¶ The abbreviations used are: MH-K1 chitosanase, chitosanase from B. circulans MH-K1; N174 chitosanase, chitosanase from Streptomyces sp. N174; N106 chitosanase, chitosanase from Nocardioiodes sp. N106; MAD, multiwavelength anomalous diffraction.

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and will discuss here the reaction mechanism and specificity of substrate recognition of this enzyme.

**EXPERIMENTAL PROCEDURES**

**Crystallization**—The recombinant MH-K1 chitosanase was purified and crystallized as previously reported (32). Crystallization was performed at 20 °C by the sitting drop vapor diffusion method using ammonium sulfate as the precipitant. Crystals belong to the orthorhombic space group P2₁2₁2₁ with unit-cell dimensions of a = 43.3 Å, b = 129.0 Å, and c = 57.7 Å.

**Data Collection and Processing**—Intensity data on native crystals were collected using synchrotron radiation with a wavelength of 1.00 Å at BL-6A of Photon Factory (KEK, Tsukuba, Japan). Four full sets were measured around two different rotation axes in which two sets were measured mainly for weak reflections at higher resolution with longer exposure time (20 s/degree), and the other two were measured for strong reflections at lower resolution with shorter exposure time (4 s/degree) to avoid saturation of these reflection intensities. A screenless Weissenberg camera for macromolecular crystals was used with a 0.1-mm aperture collimator and a cylindrical cassette of radius 286.5 mm for high resolution data (1.6 Å) and 429.7 mm for low resolution data (2.5 Å)/(35). For the multilambda anomalous diffraction (MAD) method, intensity data of the K₃PtCl₄ derivative were collected using one crystal at BL-18B of Photon Factory at four wavelengths (1.000 Å for remote 1, 1.0721 Å for edge, 1.0722 Å for peak, and 1.0728 Å for remote 2) determined from a fluorescence scan. All data were collected at room temperature. Diffraction intensities were recorded on 200 × 400-mm imaging plates (Fuji Photo Film, Co., Ltd.) and read on a Fuji BAS 2000 scanner (34). The intensity data were processed using the program DENCH and merged using the program SCALEPACK (Table II).

**Phase Determination**—Many heavy atom compounds were extensively screened, but only the K₃PtCl₄ derivative was found to be effective. This derivative was prepared by soaking crystals with 0.1 mM K₃PtCl₄ in 81% (w/v) saturated ammonium sulfate in Tris-HCl buffer for 24 h. The refinement of heavy atom parameters and calculation of MAD phases were performed with the program MLPHARE in the CCP4 package (Table II). MAD phases were initially calculated at 4.0 Å resolution, and the obtained electron density map was improved by solvent flattening and histogram mapping using the program DM in the CCP4 package (37, 38).

**Model Building and Refinement**—The C coherent X-ray crystallographic map of the MAD phased electron density map using the program TURBO-FRODO was phase extended to 3.0 Å resolution. Phases calculated from the partially constructed model were combined with the MAD phases using the programs SFALL and SIGMAA in the CCP4 package (38, 40). The map was again improved by solvent flattening, and phases were extended to 3.0-Å resolution. The molecular envelope was re-estimated from the partial backbone model with the aid of the molecular model of N174 chitosanase (Protein Data Bank code 1GHK) for comparison of the whole structure using the program MAMA in the CCP4 package (41). After 80% of the total polyanamine model had been constructed, this model was refined against the merged native data at 5.0–1.8-Å resolution. All refinements were performed using the X-PLOR program (42). This refined model was subjected to molecular dynamics and simulated annealing refinement with slow cooling from 3000 to 300 K at 5.0–1.6-Å resolution. After repeated manual rebuilding and fitting the model into the Fₐ — F₀ and F₀ — F₀ maps, positional and individual atomic B factors were carried out. The stereochemistry of the final model was analyzed using the program PROCHECK (43).

### Table I

| Subclass | Subclass II (Serratia sp. No. 7-M) | Subclass III (B. circulans MH-K1) |
|----------|----------------------------------|----------------------------------|
| Degradation | GlcN-GlcN | GlcN-GlcN | GlcN-GlcN |
| points | GlcNAc-GlcN | GlcNAc-GlcN | GlcNAc-GlcN |

### Table II

| Data collection | Native 1 | Native 2 | Native 3 | Native 4 | Merged native | Remote 1 | Edge | Peak | Remote 2 |
|-----------------|----------|----------|----------|----------|---------------|----------|------|------|----------|
| Rotation axis | c* | a* | c* | a* | c* | a* | c* | a* | c* | a* |
| Wavelength (Å) | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 |
| Resolution (Å) | 1.60 | 1.60 | 2.50 | 2.50 | 1.60 | 2.50 | 1.60 | 2.50 | 3.00 | 3.00 |
| Outer shell (Å) | 1.63–1.60 | 1.63–1.60 | 2.54–2.50 | 2.54–2.50 | 1.63–1.60 | 2.59–2.50 | 3.11–3.00 | 3.11–3.00 | 3.11–3.00 | 3.11–3.00 |
| No. of observed reflections | 137,671 | 141,857 | 66,529 | 67,615 | 462,029 | 41,369 | 29,289 | 24,330 | 29,289 | 24,330 |
| No. of unique reflections | 29,340 | 30,230 | 8,880 | 8,913 | 40,769 | 34,299 | 10,196 | 10,267 | 10,231 |
| Rmerge | 0.044 | 0.044 | 0.078 | 0.047 | 0.085 | 0.041 | 0.039 | 0.039 | 0.038 |
| Outer shell | 0.598 | 0.728 | 0.277 | 0.163 | 0.656 | 0.198 | 0.112 | 0.090 | 0.092 |
| Completeness (%) | 68.4 | 74.0 | 76.8 | 76.8 | 95.1 | 76.3 | 80.6 | 81.2 | 80.9 |
| Phase determination | 50.5 | 52.4 | 52.4 | 52.4 | 83.7 | 55.7 | 59.7 | 61.2 | 59.8 |
| Rmerge (50–4.0 Å) | 0.88 | 0.84 | 0.84 |
| Phasing power (50–4.0 Å) | 29 | 30 | 30 |
| Acentric | 0.87 | 1.02 | 1.01 |
| Centric | 0.71 | 0.79 | 0.77 |

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**a** Rmerge = Σ[I(h) − ⟨I(h)⟩]/Σ[I(h)], where ⟨I(h)⟩ is the mean value of the reflection h for all measurements of I(h).

**b** Rmerge = Σ[|F(measured) − F(calculated)|]/Σ|F(measured)|, where F(measured) is the structure factor of the data at the wavelength, F(calculated) is the structure factor of the data collected at 1.000 Å, and Fcalculated is the calculated contribution of anomalous scatter.

**c** Phasing power Fobs/⟨F⟩, where Fobs is the mean of calculated heavy atom structure factor amplitude and E is the root mean square lack of closure error.
RESULTS AND DISCUSSION

Structure Determination—The crystal structure of MH-K1 chitosanase was solved by MAD phasing of the K\textsubscript{2}PtCl\textsubscript{4} derivative. Although the space group could not be unambiguously determined between P\textsubscript{2} \textsubscript{1} \textsubscript{2} \textsubscript{1} \textsubscript{2} \textsubscript{1} and P\textsubscript{2} \textsubscript{2} \textsubscript{2} \textsubscript{2} \textsubscript{1} from the results of the preliminary diffraction experiments (32), the MAD phasing gave a reasonable solution only when the former space group was employed. The electron density with initial MAD phases at 4.0-Å resolution enabled us to incorporate six α helices (α\textsubscript{3}, α\textsubscript{7}, α\textsubscript{8}, α\textsubscript{9}, α\textsubscript{10}, and α\textsubscript{12}) corresponding to 67 of the total of 259 amino acid residues. Subsequently, phases calculated from these portions were combined with the MAD phases, and then phases were improved and extended to 3.0-Å resolution by solvent flattening. Despite repeated combination and improvement of the phases, residues 89–120, corresponding to the platinum binding site and a few loop regions between α helices, were not well fitted to the electron density map. Finally, the polyalanine model with 80% of the total structure was constructed, which was used for refinement against the merged native data at 5.0–1.8-Å resolution. At this stage, side chains of the α helices could be easily identified in the 2\(F_o\) – \(F_c\) map, and α helix and β sheet were identified in the region of residues 89–120 in \(F_o\) – \(F_c\) maps. During the course of refinement, the free R factor (44) dropped from 47.7 to 23.5% for 5% of the total reflections. The crystallographic R factor for the final model, including 150 water molecules and an SO\textsubscript{4}\textsuperscript{2-} ion lying in the crystallographic 2-fold axis, was 19.2% at 1.6-Å resolution (Table III). Fig. 1 shows the final 2\(F_o\) – \(F_c\) map in the highly hydrophobic core region. The stereochemistry of the final model was reasonable, with no residues lying at unfavorable regions in the Ramachandran plot. The root mean square deviations from standard values were 0.009 Å for bond lengths and 1.375° for angles (Table III).

Overall Structure—The overall molecular structure of MH-K1 chitosanase is shown in Fig. 2, which shows 14 α helices and 5 β strands. The overall folding of MH-K1 chitosanase is similar to that of N174 chitosanase (28) except for three regions described below, the longest dimensions of the both molecules being 57 and 55 Å, respectively (Fig. 3a). The amino acid sequences were aligned between MH-K1 and N174 chitosanases on the basis of their conformational comparison of the secondary structure (Fig. 4). Despite the folding similarity,
there was only 20% identity in the amino acid sequences of both chitosanases (18). While there was a significantly conserved segment in the N-terminal region (residues 19–69), its sequence alignment from the secondary structure was difficult in the region of residues 90–119. Three marked differences in the molecular structures were observed between MH-K1 and N174 chitosanases. First, there were two additional helices (a1 and a2) in the N-terminal region of MH-K1 chitosanase, which were 16 residues longer than that of N174 chitosanase. Second, there were two β strands (β4 and β5) following to the α6 helix in the top region of the upper domain, whereas there was only an α5 helix in N174 chitosanase. They were located in the unaligned region described above (residues 90–119 in MH-K1 chitosanase and residues 70–93 in N174 chitosanase). Third, the secondary structures were completely different at the C-terminal regions of both chitosanases, a helical structure (α14) in MH-K1 chitosanase and two β sheets (β4 and β5) in N174 chitosanase. In addition, the disulfide bridge between Cys50 and Cys124 joined the β1 strand and the α7 helix only in MH-K1 chitosanase, which is not conserved among other chitosanases.

Domain Structure—The molecule was shown to be composed of two globular domains, the upper and lower domains. Two
backbone helices shown in yellow in Fig. 3a, the α8 and α9 helices in MH-K1 chitosanase (α7 and α8 helices in N174 chitosanase), connect the upper and lower domains. Val147 and Tyr148 (Val121 and Tyr122 in N174 chitosanase) localized between the two helices are conserved between MH-K1 and N174 chitosanases. Although the lengths of the two helices in both chitosanases were almost the same, the angles formed by the two helices, Asp133–Val147 and Val147–Gly160 in MH-K1 chitosanase, Asp107–Val121 and Val121–Gly134 in N174 chitosanase, were different, being approximately 110 and 130°, respectively. In other words, the cleft formed by the upper and lower domains of MH-K1 chitosanase is more open than that of N174 chitosanase. The relative orientations of the upper and lower domains were slightly different. This difference in relative orientation of the upper and lower domains affects the size and shape of cleft. In superposition of the overall structures between MH-K1 and N174 chitosanases (45), the root mean square deviation for the corresponding 129 Cα-atoms was 2.03 Å (MH-K1: residues 23–40, 47–68, 77–88, 120–130, 134–147, 149–159, 164–178, 199–216, and 232–239; N174: 8–25, 32–53, 57–68, 94–104, 108–121, 123–133, 137–151, 179–196, and 210–217). Nevertheless, the domain structures of MH-K1 and N174 chitosanases were very similar in each domain, and individual root mean square deviations for the upper and lower domains were 0.98 Å (Fig. 3b) and 1.35 Å (Fig. 3c), respectively. Although the sequence identity between MH-K1 and N174 chitosanases was only 20%, the superimposed Cα-atoms of each domain showed a high degree of similarity in both secondary and tertiary structures.

Substrate Recognition—A highly conserved sequence segment was found in the N-terminal region of the procaryotic MH-K1, N174, and N106 chitosanases (18, 46). A site-directed

![Fig. 4. Amino acid sequence alignment of MH-K1 (GenBank™ accession number D10624) and N174 (GenBank™ accession number L40408) chitosanases based on their secondary structures.](image_url)

**TABLE III**

| Parameter                  | Value              |
|----------------------------|--------------------|
| No. of reflections (I ≥ 2σ(I)) | 38,353             |
| Resolution (Å)             | 5.0–1.6            |
| No. of nonhydrogen protein atoms | 2037               |
| No. of ions (SO₄²⁻)        | 1 on 2-fold axis   |
| No. of water molecules     | 150                |
| R factor (%)               | 19.2               |
| Rfree (%)                  | 23.5               |
| Root mean square deviation from ideal geometry | 0.009             |
| Bond angles (degrees)      | 1.375              |

a R factor = Σ|Fobs| - |Fcalc| / Σ|Fobs|, where Fobs and Fcalc are the observed and calculated structure factor amplitudes.

The Rfree value was calculated from a set of reflections (5%) randomly, which were omitted from structure refinement.

Although the sequence identity between MH-K1 and N174 chitosanases was only 20%, the superimposed Cα-atoms of each domain showed a high degree of similarity in both secondary and tertiary structures.

Substrate Recognition—A highly conserved sequence segment was found in the N-terminal region of the procaryotic MH-K1, N174, and N106 chitosanases (18, 46). A site-directed
mutagenesis study revealed that both Glu22 and Asp40 localized within the conservative N-terminal region in N174 chitosanase are essential for catalytic activity (47). These two residues are conserved in MH-K1 chitosanase (Glu37 and Asp55) as shown in Fig. 4, which suggests that these two are also catalytic residues. This was also supported by mutagenesis of Asp55 of this chitosanase.2

To understand their substrate specificities, the surface electrostatic potentials of MH-K1 and N174 chitosanases were calculated using the program GRASP (48). There were no marked differences in electrostatic distribution at the potential substrate binding cleft, where the electrostatic potentials were negative in both chitosanases. However, there was a significant difference in the shape of the substrate binding cleft (Fig. 5). There was an insertion region (residues 70–74) after β3 strand in the upper domain of MH-K1 chitosanase, which formed a protrusion at the roof of the cleft (shown in pink in Fig. 5a). On the other hand, the shape of the cleft created by the lower domain was also different. The C-terminal region of MH-K1 chitosanase was composed of α14 helix, whereas there were two β strands (β4 and β5) in N174 chitosanase. The α14 helix extended toward the cleft, which covered a hollow observed in N174 chitosanase and provided the flat base of the cleft in MH-K1 chitosanase. Consequently, the cleft in MH-K1 chitosanase was a little smaller than that in N174 chitosanase. In addition, the relative orientation of the upper and lower domains was slightly different in these two chitosanases as discussed above. These structural and orientational differences affecting the substrate binding cleft should account for the differences in substrate recognition between MH-K1 and N174 chitosanases. To examine the substrate recognition mechanism, a substrate analogue (chitosan hexamer; hexa-β-(1,4)-D-glucosamine, GlcN6) was adjusted into the cleft of MH-K1 chitosanase (Fig. 6). This binding model was constructed on the basis of the structure of human lysozyme complexed with tetrasaccharide (49) and the speculative chitosan hexamer model of N174 chitosanase (28). The interaction between the cleft and the substrate analogue was specified only at three subsites, C, D, and E, among the six sugar binding sites, whereas the positions of A, B, and F sugars were more speculative due to the loose interaction between the cleft and sugars. In subsites A–F, the F sugar was assigned as the reducing end of the sugar. Panel a was drawn using the program GRASP (48). b, the substrate cleavage positions for the partially acetylated chitosan in MH-K1 and N174 chitosanases.

**Fig. 5.** Comparison of the molecular surfaces of the substrate binding cleft of MH-K1 (a and c) and N174 chitosanases (b and d). The viewpoint of a and b is the same as that of Fig. 3a. The viewpoint of c and d, which directly shows the cleft opening space, is rotated 90° around a vertical line from that of a and b. The catalytic residues in the cleft are indicated in yellowish green. This figure was drawn by GRASP (48).

**Fig. 6.** Binding of substrate in MH-K1 chitosanase. a, a binding model with a substrate analogue (chitosan hexamer; hexa-β-(1,4)-D-glucosamine, GlcN6) in the active site. The subsites in the binding site of the cleft (six sugar binding sites) A–F are labeled, where F is the reducing end of the sugar. Panel a was drawn using the program GRASP (48). b, the substrate cleavage positions for the partially acetylated chitosan in MH-K1 and N174 chitosanases.
model with chitosan hexamer, these two catalytic residues have to move to make close contact with the linkage between D and E sugars. Glu\(^{37}\) fixed on the long central helix may act as a general acid, while Asp\(^{55}\) located on the \(\beta\) sheet loop between \(\beta1\) and \(\beta2\) may act as a general base to polarize the attacking water molecule. The temperature factors of this \(\beta\) sheet loop and the insertion loop between \(\beta3\) and \(\alpha5\) were significantly higher than those of the other regions (Fig. 2). These loops with high temperature factors may be structurally flexible so that they can make suitable contacts and for efficient recognition with the substrates.

MH-K1 chitosanase (subclass III) can split the GlcN-GlcNAc linkage (corresponding to the D-E sugar linkage) but cannot split GlcNAc-GlcN but can cleave GlcN-GlcNAc. The environments around the acetyl group of GlcNAc in the substrate were investigated on the basis of the binding model of MH-K1 chitosanase with chitosan hexamer. In Fig. 7, an artificial substrate model (GlcN-GlcNAc-GlcNAc, corresponding to C-D-E sugars), where the acetyl groups are located on both D and E sugars of the chitosan hexamer, is accommodated in the cleft of MH-K1 chitosanase. In this model, two catalytic residues (Glu\(^{37}\) and Asp\(^{55}\)) are located near the cleavage bond of the substrate. The acetyl group on the C-2 atom of the D sugar (pink) is too close to the atoms at the base of the cleft, causing a significant steric hindrance. However, the acetyl group on the C-2 atom of the E sugar (cyan) could be located at a suitable depth in the cleft without any steric hindrance. Based on this substrate binding model, we concluded that MH-K1 chitosanase can accommodate only the GlcN-GlcNAc motif at the D-E subsite of its substrate binding cleft; i.e. it cannot bind the GlcNAc-GlcN motif. This may be the reason why MH-K1 chitosanase cannot cleave the GlcN-GlcNAc linkage but the GlcNAc-GlcN linkage. This model shows that the size and shape of the cleft are such that the substrate sugar with the acetyl groups at positions suitable for the specific cleavage reaction can be accommodated in the active site, which affords reaction specificity for substrate recognition of this chitosanase.

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