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Crystallization and preliminary X-ray crystallographic analysis of chitosanase from *Bacillus circulans* MH-K1.

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Abstract

Chitosanase, an enzyme which hydrolyzes chitosan, isolated from *Bacillus circulans* MH-K1, was crystallized by a vapor-diffusion procedure at 293 K using ammonium sulfate as a precipitant. Rod-shaped colorless crystals, which grew to $0.05 \times 0.15 \times 1.2$ mm within a week, belong to the orthorhombic system and the space group $P222_1$ or $P2_12_12$ with unit-cell dimensions of $a = 43.3$, $b = 57.7$, and $c = 128.0$ Å. The asymmetric unit is thought to contain one chitosanase molecule (29 024 Da). The crystals diffract X-rays to at least 2.3 Å resolution and are suitable for high-resolution X-ray structure analysis.

Chitin is a homopolymer of β-(1,4)-linked *N*-acetylglucosamine. Chitinase (E.C. 3.2.1.14) is an enzyme which hydrolyzes chitin specifically. Chitinase is one of the key enzymes which participate in the plant defense system against fungal infection (Verburg & Huynh, 1991; Huynh *et al.*, 1992). Usually chitosan is produced by partially or fully deacetylation of chitin at alkaline pH. Chitosan is hydroylzed by chitosanase (E.C. 3.2.1.132). It is also thought that plant chitosanase is responsible for plant defence system (El Ouakfaui & Asselin, 1992). Chitosanase is not clearly distinguished from chitinase at the molecular level. The major reason for this problem comes from the structural similarity of the substrates in these two enzymes; their only difference is the degree of deacetylation. But the amino-acid sequences of chitosanase have shown no homology with those of various chitinases.

Bacillus circulans MH-K1 was isolated from soil as a chitosanase producer (Yabuki, Hirano, Ando, Fujii & Amemiya, 1987). Biochemical properties of the purified chitosanase from *B. circulans* MH-K1 have been characterized (Yabuki, Uchiyama, Suzuki, Ando & Fujii, 1988), and the chitosanase gene has been cloned (Ando *et al.*, 1992). The protein coded by this cloned gene was composed of 301 amino acids containing a signal peptide with 42 amino acids. The calculated molecular weight of mature chitosanase is 29 024 Da for 259 amino acids.

In order to understand the catalytic mechanisms of chitosanase and chitinase, determination of their three-dimensional structures is essential. Crystals of a few chitinases have been obtained (Rozeboom, Budiani, Beintema & Dijkstra, 1990; Vorgias, Kingswell & Dauter, 1992; Hart, Ready & Robertus, 1992; Song, Hwang, Kim & Suh, 1993), and the crystal structures of chitinases from *Hordeum vulgare* (Hart, Monzingo, Ready, Ernst & Robertus, 1993), from *Hevea brasiliensis* (Terwisscha van Scheltinga, Kalk, Beintema & Dijkstra, 1994) and from *Serratia marcescens* (Perrakis *et al.*, 1994) have been reported. On the other hand, the crystallization of chitosanase has been hitherto reported only for that from *Streptomyces* N174 (Marcotte, Hart, Boucher, Brzezinski & Robertus, 1993).

We report here the crystallization and a preliminary X-ray diffraction study of chitosanase from *B. circulans* MH-K1 as the first step toward structure determination. *B. circulans* MH-K1 chitosanase shares 40–45% primary sequence similarity with *Streptomyces* N174 chitosanase (Masson, Denis & Brzezinski, 1994). The crystals obtained in these two chitosanases have different crystallization conditions and crystallographic data (Marcotte *et al.*, 1993).

For the construction of hyper expression, the chitosanase gene of *B. circulans* MH-K1 was inserted into pNU210 (named pNUE). *B. brevis* 47-5Q was transformed by this recombinant plasmid (unpublished results). Using this expression system, chitosanase is obtainable in gram order from 1 l of cultivation medium (unpublished results). Chitosanase produced by *B. brevis* 47-5Q/pNUE was purified as a usual manner (Yabuki *et al.*, 1988).

The purified chitosanase solution was concentrated to about 30 mg ml^{-1} by using a Centriprep-10 (Amicon), and then dialyzed against 20 m*M* Tris–maleate buffer (pH 6.5). The protein solution (5 µl) was mixed with 13 µl of ammonium sulfate solution [90%(w/v) saturated ammonium sulfate in Tris–HCl buffer, final pH 6.7–7.6]. Crystallization was performed by the sitting-drop vapor-diffusion method at 293 K. The final concentration of ammonium sulfate in the protein solution was about 65%(*w*/*v*). The protein solution was then equilibrated against the reservoir solution with 75%(*w*/*v*)

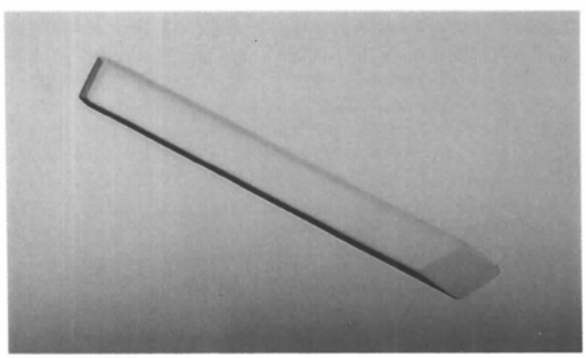

Fig. 1. A crystal of chitosanase from *Bacillus circulans* MH-K1. The size of the crystal is about $0.05 \times 0.15 \times 1.2$ mm.

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ammonium sulfate. Rod-shaped colorless crystals appeared after 24 h and grew to 0.05 × 0.15 × 1.2 mm on average within a week (Fig. 1).

A crystal was mounted in a glass capillary with the mother liquor. For crystallographic characterization, precession photographs were taken using a Huber precession/rotation camera with Cu $K\alpha$ radiation generated by an M18X X-ray generator (MAC Science Co. Ltd, Tokyo), running at 50 kV and 90 mA (Fig. 2). X-ray diffraction pattern symmetry indicated that the crystals belong to the orthorhombic system. The unit-cell parameters are $a = 128.3$, $b = 57.7$ and $c = 128.0$ Å, which were determined by a least-squares fit of diffraction spots recorded on the imaging-plate with a DIP100S rotation camera system (MAC Science Co. Ltd, Tokyo). The space group is determined by systematic absences of reflections as either $P222_1$ (No. 17) or $P2_122_1$ ($P2_12_12$, No. 18). There are two possibilities because of the fact that all the reflections on the a* axis are too week to determine without ambiguity whether the screw axis exists (Fig. 2). The space group will be determined uniquely at the stage of structure analysis. Assuming that the asymmetric unit contains one molecule of chitosanase, the V_m value is calculated to be 2.8 Å^3 Da^{-1}, which is consistent with those obtained for water-soluble proteins (Matthews, 1968). The crystals are of good quality and stable toward exposure to X-rays. The crystals diffract X-rays to at least 2.3 Å resolution and are suitable for high-resolution X-ray structure analysis. Data collection on the native crystals and a search for heavy-atom derivatives are in progress.

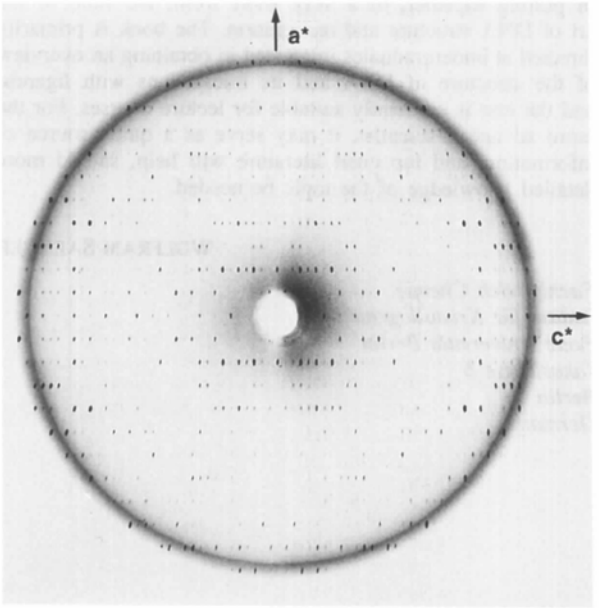

Fig. 2. Precession photograph ($h0l$ zone) of a chitosanase crystal. The precession angle is 11.4° and the crystal-to-film distance is 100 mm. Exposure time is 87 h.

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