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Crystallization and preliminary X-ray diffraction study of thermostable RNase HIII from *Bacillus stearothermophilus*

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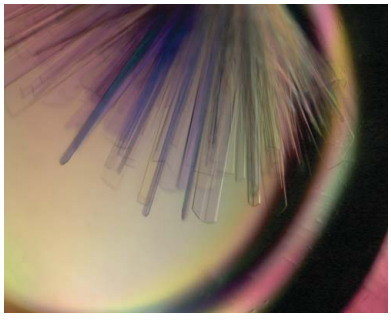

A thermostable ribonuclease HIII from *Bacillus stearothermophilus* (Bst RNase HIII) was crystallized and preliminary crystallographic studies were performed. Plate-like overlapping polycrystals were grown by the sitting-drop vapour-diffusion method at 283 K. Native X-ray diffraction data were collected to 2.8 Å resolution using synchrotron radiation from station BL44XU at SPring-8. The crystals belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 66.73, b = 108.62, c = 48.29 Å. Assuming one molecule per asymmetric unit, the V_M value was 2.59 Å^3 Da^{-1} and the solvent content was 52.2%.

1. Introduction

Ribonuclease H (RNase H) endonucleolytically hydrolyzes the RNA of RNA/DNA hybrids in the presence of divalent cations (Crouch & Dirksen, 1982). The enzyme is widely present in various organisms. The RNase H enzymes are classified into two major families, type 1 and type 2, based on amino-acid sequence differences (Ohtani *et al.*, 1999*a*). These enzymes are thought to be involved in DNA replication, repair and/or transcription (Kogoma & Foster, 1998; Murante *et al.*, 1998; Qiu *et al.*, 1999; Rydberg & Game, 2002).

In prokaryotes, three different RNases H (RNases HI, HII and HIII) have so far been identified (Ohtani *et al.*, 1999*a*,*b*; Itaya *et al.*, 1999). RNases HI are members of the type 1 RNase H family, while RNases HII and HIII are members of the type 2 RNase H family. Crystal structures are available for RNases HI (Katayanagi *et al.*, 1990, 1992; Yang *et al.*, 1990; Ishikawa *et al.*, 1993) and HII (Lai *et al.*, 2000; Muroya *et al.*, 2001; Chapados *et al.*, 2001). Comparison of these structures indicates that RNases HI and HII share a main-chain fold containing four acidic active-site residues, despite the lack of any significant amino-acid sequence similarity. For *Escherichia coli* RNase HI, a catalytic mechanism and a substrate-recognition mechanism have been proposed (Kanaya, 1998; Goedken & Marqusee, 2001; Tsunaka *et al.*, 2005). However, compared with RNases HI and HII, much less is known about the structure and function of RNase HIII as no structural information is available.

RNase HIII shows poor amino-acid sequence identity to RNase HII (Ohtani *et al.*, 1999*a*,*b*). For example, *Bacillus subtilis* RNase HIII shows 18% amino-acid sequence identity to *B. subtilis* RNase HII. Nevertheless, they are classified into the same family because several sequence motifs are conserved in their sequences. Single bacterial cells usually contain two different RNases H (Ohtani *et al.*, 1999*a*,*b*). For example, *E. coli* cells contain RNases HI and HII, while *B. subtilis* cells contain RNases HII and HIII. Biochemical characterization of *B. subtilis* RNase HIII indicated that this enzyme is more closely related to RNase HI than to RNase HII in enzymatic properties, suggesting that RNase HIII functions as a substitute for RNase HI in *B. subtilis* (Ohtani *et al.*, 1999*b*).

We have recently cloned the gene encoding RNase HIII from the thermophilic bacterium *B. stearothermophilus* (Bst RNase HIII) and biochemically characterized the recombinant protein (Chon *et al.*, 2004). Bst RNase HIII is composed of 310 amino-acid residues and shows an amino-acid sequence identity of 47.1% to *B. subtilis* RNase HIII. Bst RNase HIII closely resembles *B. subtilis* RNase HIII in enzymatic properties, such as its requirement for divalent cations, its

pH optimum and the cleavage mode of the substrate. However, Bst RNase HIII is much more stable than *B. subtilis* RNase HIII, suggesting that Bst RNase HIII is more suitable for crystallographic studies than *B. subtilis* RNase HIII. Crystallization and preliminary X-ray crystallographic analysis of *B. subtilis* RNase HIII have been reported (Kwak *et al.*, 2001); however, this crystal structure has not yet been solved.

Here, we report the crystallization and preliminary crystallographic studies of Bst RNase HIII.

2. Experimental procedures

2.1. Overproduction and purification

Bst RNase HIII was overproduced in the *rnh* mutant strain of *E. coli* MIC2067(DE3) as described previously (Chon *et al.*, 2004). The cells were collected by centrifugation, resuspended in 10 m*M* Tris–HCl pH 7.5 containing 1 m*M* EDTA (TE buffer) and disrupted by sonication on ice. The resultant cell lysate was centrifuged at 30 000*g* for 30 min at 277 K to remove insoluble materials. Ammonium sulfate was added to the supernatant to a concentration of 70% and the resultant precipitate was collected by centrifugation at 15 000*g* for 30 min at 277 K. The precipitate was then dissolved in TE buffer and dialyzed against TE buffer. The protein was purified by the following three column-chromatographic steps. The protein solution was first loaded onto a HiTrap Heparin HP column (Amersham Biosciences) equilibrated with TE buffer. The protein was eluted from the column with a linear gradient of 0–0.5 *M* NaCl. The fractions containing Bst RNase HIII, which eluted from the column at approximately 0.3 *M* NaCl, were collected and dialyzed against TE buffer containing 0.1 *M* NaCl. The protein solution was then loaded onto a HiTrap SP HP column (Amersham Biosciences) equilibrated with TE buffer containing 0.1 *M* NaCl. The protein was eluted from the column with a linear gradient of 0.1–0.5 *M* NaCl and the fractions containing Bst RNase HIII, which eluted from the column at approximately 0.3 *M* NaCl, were collected. The protein solution was finally loaded onto a HiLoad 16/60 Superdex 200pg column (Amersham Biosciences) equilibrated with 20 m*M* sodium acetate pH 4.8 containing 0.1 *M* NaCl, which was used as the running buffer. Purified Bst RNase HIII was concentrated using a Vivapore 10/20 ml concentrator (Vivascience) to about 16 mg ml^{-1}, filtrated using Ultrafree-MC (Millipore) and stored in 20 m*M* sodium acetate pH 4.8 containing 0.1 *M* NaCl at 253 K. This procedure yielded roughly 10 mg of highly purified Bst RNase HIII from 1 l culture. The protein concentration was determined from UV absorption using a cell with an optical path length of 1 cm and an A_{280} value of 0.73 for 0.1% solution. The purity of the protein was confirmed by SDS–PAGE (Laemmli, 1970) followed by staining with Coomassie Brilliant Blue.

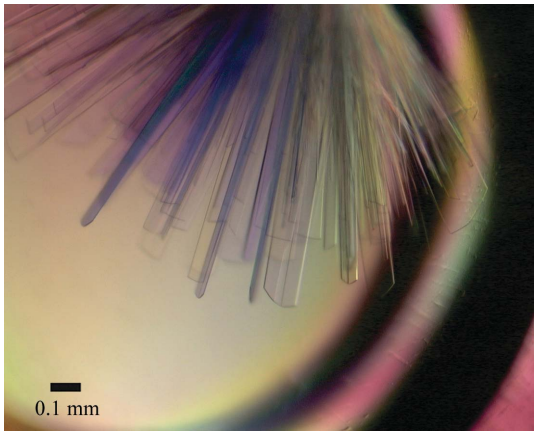

Figure 1
Plate-like polycrystals of Bst RNase HIII.

Table 1
Statistics of data collection.

Values in parentheses refer to the highest resolution shell (2.95–2.80 Å).

X-ray wavelength (Å)	0.9
Temperature (K)	100
Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 66.73, b = 108.62, c = 48.29
Resolution range (Å)	29.0–2.80
No. of measured reflections	34825
No. of unique reflections	8930
$R_{\rm merge}$† (%)	11.4 (28.2)
Data completeness (%)	98.4 (98.4)
Average $I/\sigma(I)$	5.3 (2.4)

† $R_{\rm merge} = \sum|I_{hkl} - \langle I_{hkl}\rangle|/\sum I_{hkl}$, where I_{hkl} is the intensity measurement for the reflection with indices hkl and $\langle I_{hkl}\rangle$ is the mean intensity for multiply recorded reflections.

2.2. Crystallization

The crystallization condition was initially screened using crystallization kits from Hampton Research (Crystal Screens I, II and Cryo). The conditions were surveyed using the sitting-drop vapour-diffusion method at 293 K. Drops were prepared by mixing 1 µl each of the protein solution and the reservoir solution and were vapour-equilibrated against 100 µl reservoir solution. Needle-like polycrystals appeared after a few days using Crystal Screen Cryo solution No. 17 (0.085 *M* Tris–HCl pH 8.5, 0.17 *M* lithium sulfate, 25.5% PEG 4000, 15% glycerol). The crystallization condition was further optimized and plate-like polycrystals suitable for X-ray diffraction analysis appeared when the drop was prepared by mixing 2 µl protein solution and 3 µl reservoir solution and vapour-equilibrated against 100 µl reservoir solution at 283 K. The crystal usually grew such that single crystals overlapped cohesively. The single crystals were separated from each other using Micro Tools (Hampton Research).

2.3. Data collection

A crystal of Bst RNase HIII was mounted on a CryoLoop (Hampton Research) without adding cryoprotectant and then flash-frozen in a nitrogen-gas stream at 100 K. X-ray diffraction data were collected at 100 K on the BL44XU station at SPring-8, Japan using a DIP6040 multiple imaging-plate diffractometer. A total of 180 images were recorded with an exposure time of 10 s per image and an oscillation angle of 1.0°. The data were processed using the programs *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results

The crystals appeared after a few days and grew to maximum dimensions of 0.6 × 0.1 × 0.05 mm after about 10 d (Fig. 1). The crystals diffracted to 2.8 Å. A total of 34 825 measured reflections were merged into 8930 unique reflections with an $R_{\rm merge}$ of 11.4%. The crystals belong to the primitive orthorhombic space group $P2_12_12$, with unit-cell parameters a = 66.73, b = 108.62, c = 48.29 Å. Table 1 summarizes the data-collection statistics. Based on the molecular weight and the space group, the crystal was assumed to contain one protein molecule per asymmetric unit, giving a $V_{\rm M}$ value of 2.59 Å^3 Da^{-1} and a solvent content of 52.2%. These values are within the ranges frequently observed for protein crystals (Matthews,

1968), suggesting that this crystal is suitable for structural determination. Two heavy-atom derivatives have also been found and determination of the crystal structure by multiple isomorphous replacement is in progress.

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