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Crystallization of Recombinant Chitobiase from *Serratia marcescens*

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We are currently investigating the biochemical and structural properties of both chitin degrading enzymes chitinase and chitobiase from *Serratia marcescens*. Previously we have reported the first crystallization and characterization of chitinase crystals (Vorgias *et al.*, 1992). In this communication we present the first crystallization of chitobiase. The protein was synthesized in *Escherichia coli* and purified to homogeneity using cation exchange chromatography and fast protein liquid chromatography. The crystals have the shape of small prisms and the space group is $P2_1$ with $\beta = 101{\cdot}0°$ and unit cell dimensions $a = 63{\cdot}2$ Å, $b = 133{\cdot}2$ Å, $c = 55{\cdot}1$ Å. They diffract X-rays to about 2·5 Å resolution and are suitable for three-dimensional structural analysis.

Keywords: recombinant chitobiase; crystallization; *Serratia marcescens*

Chitin, a cellulose-like biopolymer is widely distributed in the marine and terrestrial biotopes. The enzymatic degradation of chitin by many microorganisms takes place in two successive steps. Chitinase first digests chitin releasing most *N*,*N*′-diacetylchitobiose (chitobiose) oligomers of *N*-acetylglucosamine and a small quantity of *N*-acetylglucosamine. Chitobiase then hydrolyses chitobiose to *N*-acetyl-D-glucosamine and can also hydrolyse chitotriose and chitotetraose at lower rates (Muzzarelli, 1977).

The gene encoding the chitobiase protein was isolated from a *Serratia marcescens* genomic library and inserted in pEMBL18 resulting in the plasmid pCBI as described by Kless *et al.* (1989). In contrast to *Serratia marcescens*, where chitobiase is synthesized after induction with chitin, *Escherichia coli* cells carrying the pCBI plasmid express the chitobiase gene constitutively. Chitobiase activity is mainly localized in the periplasm of the *E. coli* cells and the amount of chitobiase produced was high enough to establish and scale up a proper purification protocol in order to prepare a reasonable amount of enzyme for crystallization experiments.

E. coli cells carrying the cloned chitobiase gene were grown overnight in L-Broth medium supplemented with 125 μg/ml of ampicillin. Next day the cells were diluted 50-fold and grown for several hours at 30 °C. The cells were harvested by low speed centrifugation and washed once with 50 mM-Tris·HCl, pH 7·0, 100 mM-NaCl. The cells were fractionated to periplasmic and cytoplasmic fractions, essentially as described by Jannatipour *et al.* (1987), and slightly modified. The cells were suspended in 3 ml/g of buffer A (10 mM-Na-phosphate, pH 8·0, 0·5 mM-EDTA, 0·1 mM-PMSF‡ and 0·5 M-sucrose), 100 μl of 2 mg/ml lysozyme solution per ml of suspension was added and incubated on ice for 30 minutes. An equal volume of ice-cold water was added and the mixture kept on ice for the next 30 minutes. Finally the extract was adjusted to 18 mM-$MgCl_2$ and centrifuged at 10,000 ***g*** for 20 minutes. The supernatant was the periplasmic fraction of the *E. coli* cells where chitobiase was one of the major proteins. Chitobiase extract was further fractionated by precipitation with 50 to 75% saturated ammonium sulphate. The protein was dissolved in buffer B (10 mM-Na-phos-

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‡ Abbreviations used: PMSF, phenylmethylsulphoryl fluoride.

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phate, pH 8·0, 1 mM-EDTA, 0·1 mM PMSF, 1 mM dithiothreitol) in large volume to keep the total ionic strength low. The enriched chitobiase fraction was directly applied to an S-Sepharose CL-6B, equilibrated in buffer B, and bound protein was eluted with a 15 to 20 column volume of 0 to 0·5 M-NaCl gradient in buffer B, at a flow rate of 40 ml/hour. The chitobiase activity assay was used to select and combine the proper fractions which were also checked by 0·1% SDS/10% PAGE (Laemmli, 1970). The chitobiase fractions were combined and diluted three times with buffer C (i.e. buffer B adjusted to pH 7·0) and applied on a Mono-S column on a fast protein liquid chromatography system. The Mono-S column was run at a flow rate of 2 ml/min and the bound chitobiase was eluted with a very shallow gradient of 100 to 300 mM-NaCl at 1 ml/min. The enzyme was concentrated on the same column to 10 mg/ml and immediately used for crystallization experiments. In a routine preparation 20 mg of highly purified chitobiase was recovered from 30 g of *E. coli* cells.

The crystallization of chitobiase was screened using "50 standard crystallization conditions" at 4 °C and 16 °C (Jancarik & Kim, 1991). The protein was at a concentration of 10 mg/ml in buffer C. Using hanging-drop vapour diffusion, 2·5 to 5 μl of protein solution were mixed with an equal volume of precipitating solution and equilibrated against 1·0 ml of precipitating solution (Davies & Segal, 1971). Useful crystals were obtained in the pH range 5·4 to 5·8 with 15 to 17% polyethylene glycol 6000 in the presence of 7·5% to 10% isopropanol. The crystals appeared within 24 to 48 hours and have dimensions 0·1 mm × 0·2 mm × 0·2 mm.

The crystals were mounted in glass capillaries and diffraction data measured using the EMBL X31 beam line in HASYLAB/DESY on the DORIS storage ring. Data were collected using an imaging plate scanner with a crystal to plate distance of 200 to 400 mm and radiation wavelength 1·009 Å (1 Å = 0·1 nm).

The space group $P2_1$ and unit cell dimensions, $a = 63{\cdot}2$ Å, $b = 133{\cdot}2$ Å, $c = 55{\cdot}1$ Å, $\beta = 101{\cdot}0°$ were determined from the oscillation images. The molecular mass of recombinant chitobiase is 95,000 Da and the unit cell volume $0{\cdot}455 \times 10^6$ Å^3 gives $V_m = 2{\cdot}4$ Å^3/Da, assuming that there are two protein molecules in the unit cell. Although the crystals were quite small, the diffraction extended to about 2·5 Å. We are currently working on improving the crystallization procedure and searching for heavy atoms derivatives.

In summary, the overexpression of recombinant chitobiase in *E. coli* and the simple and fast purification scheme have made crystallization trials possible. In a relatively short time we obtained crystals which diffract to medium resolution. Preliminary X-ray diffraction data are reported for the chitobiase crystals and we will start to use the diffraction data for the structural determination of the protein.

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