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Crystallization and preliminary X-ray crystallographic studies of the thermoactive pullulanase type I, hydrolyzing α-1,6 glycosidic linkages, from *Fervidobacterium pennivorans* Ven5

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Crystals of the thermoactive recombinant *F. pennivorans* type I pullulanase, purified from the supernatant of a *Bacillus subtilis* culture, have been obtained by the vapour-diffusion method in the presence of the inhibitor β-cyclodextrin (2 m*M*) by mixing protein (15 mg ml^{-1}) with an equal volume of crystallization solution containing 0.1 *M* bis–tris propane pH 6.5, 50 m*M* $MgCl_2$ and 15% polyethylene glycol 3350. Crystals diffracted to 3.0 Å using conventional Cu $K\alpha$ radiation and belong to space group $P2_12_12_1$, with unit-cell parameters a = 76.8, b = 96.2, c = 98.5 Å. The asymmetric unit contains one monomer. A preliminary 26% complete data set has been collected at 2.2 Å resolution using synchrotron radiation.

1. Introduction

Hyperthermophiles are microorganisms that have their optimal temperature for growth above 353 K. Knowledge of the molecular adaptations of their macromolecules, in particular enzymes, that allow these organisms to survive and function optimally under these harsh conditions will enhance our fundamental understanding of macromolecule stabilization and operation under extreme conditions. Furthermore, because of the outstanding thermal and chemical stability of their biocatalysts, hyperthermophiles are of great interest for industrial and biotechnological applications (Ladenstein & Antranikian, 1998). This is illustrated by the wide application of thermostable DNA polymerases in the polymerase chain reaction, as well as the anticipated role for polymer-degrading enzymes of extremophilic origin, such as amylases, pullulanases, xylanases and proteases, in industrial applications (Aguilar, 1996).

Pullulanases are debranching extracellular enzymes that are specifically able to hydrolyze α-1,6 glycosidic linkages in polysaccharides. They are the only enzymes known to be able to hydrolyze the α-1,6 bonds of the linear α-glucan pullulan, producing maltotriose. They furthermore hydrolyze α-1,6 linkages in branched oligosaccharides such as amylopectin, the main component of starch, forming linear α-1,4 linked oligomers. Pullulan-degrading enzymes can be classified into four groups (Spreinat & Antranikian, 1990) as follows. Pullulanase type I specifically hydrolyses α-1,6 glycosidic linkages in pullulan and branched oligosaccharides. Pullulanase type II (amylopullulanase) attacks α-1,6 linkages in pullulan and branched substrates as well as α-1,4 glycosidic linkages in substrates other than pullulan. Furthermore, two additional pullulan-degrading enzymes have been described, pullulan hydrolase type I (neopullulanase) and II (isopullulanase), that hydrolyze the α-1,4 glycosidic linkages in pullulan to form panose and isopanose, respectively (Kuriki *et al.*, 1989; Sakano *et al.*, 1972). No three-dimensional structure of a pullulanase is currently available. To our knowledge, crystals have been reported only for the type II pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* (Knapp *et al.*, 1995). All type I pullulanases contain the four conserved regions typical of the amylolytic enzyme family (glycosyl hydrolase family 13; Bertoldo *et al.*, 2000). However, the pullulanase type II from *P. furiosus*, which is very closely related to the enzyme from *P. woesei*, shows only low homology with α-amylases and the four conserved regions could not be identified in the sequence (Rüdiger *et al.*, 1995; Dong *et al.*, 1997). Several α-amylase three-dimensional structures are available and reveal that the four conserved regions are located in a domain that adopts the classical $(\alpha\beta)_8$- or TIM-barrel fold (Kadziola *et al.*, 1994; Machius *et al.*, 1995). The recent elucidation of the crystal structure of the α-amylase II from *Thermoactinomyces vulgaris*, which is able to hydrolyze the α-1,4 linkages in pullulan in addition to those in amylose (neopullulanase activity), and that of the isoamylase from *Pseudomonas amyloderamosa* (hydrolyzing α-1,6 linkages in amylopectin and glycogen) revealed a widening of the active site in comparison with other α-amylases, which could allow these enzymes to accommodate substrates containing α-1,6 glycosidic bonds (Kamitori *et al.*, 1999; Katsuya *et al.*, 1998).

The extreme thermophilic anaerobic bacterium *F. pennivorans* Ven5 produces a thermostable type I pullulanase (Koch *et al.*, 1997; Bertoldo, Duffner, Jorgensen *et al.*, 1999). This pullulanase is a dimer consisting of two identical 93 kDa subunits, is optimally active at 353 K and has a half-life for thermal inactivation of 2 h at this temperature. The *F. pennivorans* Ven5 type I pullulanase gene (*pul*A) was overexpressed in *Escherichia coli* (Bertoldo, Duffner, Jorgensen *et al.*, 1999). Furthermore, in order to obtain large amounts of the pullulanase for crystallization trials, the *pul*A gene was cloned and expressed in the industrial strain *B. subtilis* (Bertoldo, Duffner, Groudieva *et al.,* 1999). Unlike the *E. coli* intracellular expression, the pullulanase in *B. subtilis* is secreted in the supernatant in very high yield. Possibly as a consequence of *in vivo* proteolytic activity, the resulting gene product is 83 kDa. Based on size-exclusion chromatography this protein is monomeric, suggesting that the N-terminal 118 amino acids play a role in subunit association in the intact enzyme. However, the truncated pullulanase displays similar specific activity to the native enzyme (38 *versus* 75 U mg^{-1}; these values are not considered to differ significantly because both enzymes have been isolated from different sources using different purification protocols). Furthermore, the temperature and pH optimum, thermostability, substrate specificity and inhibition pattern by cyclodextrins are identical (Bertoldo, Duffner, Groudieva *et al.*, 1999). Therefore, in spite of the difference in oligomerization state, the enzyme produced by *B. subtilis* represents the full-length enzyme very well. In this article, we present for the first time crystallization conditions for a pullulanase type I from a thermophilic bacterium.

2. Materials and methods

The expression of the *F. pennivorans* Ven5 pullulanase gene in *B. subtilis* and the purification of the protein secreted in the culture supernatant have been described elsewhere (Bertoldo, Duffner, Groudieva *et al.*, 1999).

All chemicals used for crystallization experiments were of highest purity available. The enzyme was dialysed against 25 m*M* sodium acetate pH 5.6 and concentrated to a protein concentration of 15 mg ml^{-1}. The inhibitor β-cyclodextrin was added (2 m*M* final concentration) and the protein was allowed to stand for at least 1 h. Using the sitting-drop vapour-diffusion method, a random screening protocol was used to search for crystallization conditions (Jancarik & Kim, 1991). A combination of available screens and screens developed in-house were used. Promising conditions were further optimized by a finer grid search. Suitable cryo-solutions were screened by addition of varying concentrations of polyethylene glycol 400 or glycerol to the stabilizing solution (final crystallization condition with increased precipitant concentration, see below).

Initial X-ray diffraction data was collected using Cu $K\alpha$ radiation produced by a MacScience/Siemens rotating-anode generator operated at 45 kV/100 mA and 105 K. 180 frames (1° rotation) were collected on an 18 cm MAR image plate. The crystal-to-detector distance was 120 mm and the exposure time was 240 s. Data were indexed, scaled and merged using *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997). Preliminary synchrotron-radiation data at 100 K were collected at beamline X11 at the EMBL Outstation, DESY, Hamburg using a wavelength of 0.9092 Å, a crystal-to-detector distance of 150 mm, 120 s exposure, 1° rotation per frame and a MAR CCD detector.

3. Results and discussion

The initial screening condition, containing 0.1 *M* 1,3-bis[tris(hydroxymethyl)-methylamino] propane (bis-tris propane) pH 6.5, 200 m*M* $MgCl_2$, 20% polyethyleneglycol 8000 and 2 m*M* β-cyclodextrin, resulted in rapid formation of small needle-like crystals. The final protein concentration in the drops was 7.5 mg ml^{-1}. This condition was further optimized to 0.1 *M* bis–tris propane pH 6.5, 50 m*M* $MgCl_2$ and 15% polyethyleneglycol 3350 and resulted in crystals growing up to 1 mm in the longest direction and 0.2 mm in the directions perpendicular to the needle axis (Fig. 1). Crystals were soaked for 1 min in 0.1 *M* bis–tris propane pH 6.5, 50 m*M* $MgCl_2$, 17.5% polyethyleneglycol 3350, 20% polyethylene glycol 400 and were flash-frozen in a nitrogen-gas stream at 105 K (Oxford Cryosystems Cryostream), immediately followed by data collection.

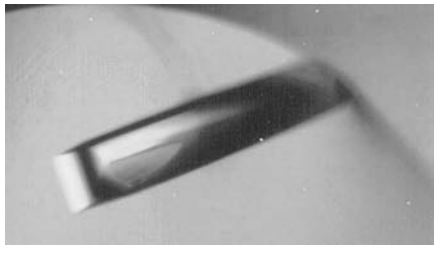

Figure 1
F. pennivorans pullulanase crystal grown in 0.1 *M* bis–tris propane pH 6.5, 50 m*M* $MgCl_2$ and 15% polyethylene glycol 3350. The crystals diffract to at least 2.2 Å at the EMBL beamline X11 at the synchrotron in Hamburg.

From the symmetry of the reciprocal lattice (*mmm*) and the systematic extinctions $h = 2n + 1$, $k = 2n + 1$, $l = 2n + 1$, the space group of the crystals was assigned as $P2_12_12_1$, with unit-cell parameters $a = 76.8$, $b = 96.2$, $c = 98.5$ Å. The data set is 99.7% complete to 3.0 Å, with 97.9% in the last shell from 3.11 to 3.0 Å and 73% of the data with $I > 3\sigma$ in this shell. The symmetry R factor of the individual frames ranged between 9 and 14% and for the whole data set is 11.7% (24.7% in the last shell). The asymmetric unit contains one monomer of 83 kDa. This results in a typical packing density for protein crystals of 2.1 $Å^3$ Da^{-1} and a solvent content of 64% (Matthews, 1968).

A preliminary, 26% complete data set was collected at the synchrotron at the EMBL outstation at DESY, Hamburg. This data shows considerable higher maximum resolution of 2.2 Å with better statistics. The R factor ranges from 3 to 6% per batch and the overall R factor is 4.1% (24.3% in the last shell from 2.28 to 2.20 Å).

In addition to solving the structure of this type I pullulanase, our laboratories are currently working on the structure of the *P. woesei* type II pullulanase. With the structures of these two pullulanases and other available structures in the α-amylase family, we aim to achieve further insight into the degree of structural conservation between the different amylolytic enzymes and into the adaptations that allow discrimination between α-1,4 and α-1,6 linkages in linear and branched oligosaccharides. The structures might furthermore add to the knowledge of the factors responsible for achieving more robust proteins and biocatalyst thermostability.

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