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Crystallization and preliminary X-ray crystallographic analysis of the laminarinase endo-β-1,3-glucanase from *Pyrococcus furiosus*

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Laminarinase endo-β-1,3 glucanase (LamA) from *Pyrococcus furiosus* is an enzyme which displays its main hydrolytic activity on the β-1,3-glucose polymer laminarin. This laminarinase is remarkably resistant to denaturation: its secondary structure is unchanged in 8 *M* guanidinium chloride. This protein belongs to the family 16 glycosyl hydrolases, which are enzymes that are widely distributed among bacteria, fungi and higher plants. Single crystals of *P. furiosus* LamA have been obtained by the hanging-drop vapour-diffusion method using 2-methyl-2,4-pentanediol as a precipitant agent. A complete data set has been collected under cryocooling at a synchrotron source. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 44.36$, $b = 84.76$, $c = 69.23$ Å, $\alpha = 90$, $\beta = 104.97$, $\gamma = 90^\circ$, and diffract to 2.15 Å resolution.

1. Introduction

Laminarinase endo-β-1,3-glucanase from the extremophilic archaeon *Pyrococcus furiosus* (LamA) displays high conformational stability: its tertiary structure is partially preserved and its secondary structure is unchanged in 8 *M* guanidinium chloride (GdmCl; Chiaraluce *et al.*, 2002). This enzyme displays its highest hydrolytic activity on the β-1,3-glucose polymer laminarin and has some hydrolytic activity on the β-1,3-1,4-glucose polymers lichenan and barley β-glucan (Gueguen *et al.*, 1997). On the basis of substrate specificity, this protein belongs to the laminarinase subfamily of the family 16 glycosyl hydrolases (Allouch *et al.*, 2003), which are enzymes that are widely distributed among bacteria, fungi and higher plants (Coutinho & Henrissat, 1999). The β-1,3-glucanases can play various physiological roles. In plants, they have been implicated in cell differentiation and defence against fungal pathogens (Grenier *et al.*, 1993; Coutinho *et al.*, 2003). In fungi, β-1,3-glucanases are important in morphogenetic processes, β-glucan mobilization and fungal pathogen–plant interactions (Bachman & McClay, 1996). In bacteria, β-1,3-glucanases hydrolyze 1,3-β-glucosyl linkages, but they usually require a region of unsubstituted contiguous 1,3-β-linked glucosyl residues (Gueguen *et al.*, 1997).

A considerable number of primary sequences of family 16 are available; however, detailed structural information on the laminarinase subfamily is not available among the few crystal structures solved (Allouch *et al.*, 2003). A structural comparison between the *P. furiosus* LamA and the other subfamily members of the family 16 glycosyl hydrolases may help in identifying the factors responsible for substrate specificity.

The presence of at least one metal-binding site is one of the common features in the crystal structures of family 16 glycosyl hydrolases (Coutinho & Henrissat, 1999; Allouch *et al.*, 2003) and calcium has been reported to protect *P. furiosus* LamA from heat-induced inactivation (Gueguen *et al.*, 1997). Interestingly, in 8 *M* GdmCl *P. furiosus* LamA can interact with calcium to give a significant regaining of tertiary structure (Chiaraluce *et al.*, 2004). The presence of residual tertiary interactions under these particular solvent conditions, which would induce complete unfolding for most polypeptide chains, is a peculiar property of this protein. The X-ray structure of *P. furiosus* LamA will provide us with information about the possible structural determinants of its unusual stability. Moreover, knowledge of the three-dimensional structure of the active site will provide a deeper understanding of the mechanism of the hydrolytic activity of *P. furiosus* LamA on the β-1,3-glucose polymer laminarin.

2. Materials and methods

The *P. furiosus* endo-β-1,3-glucanase was produced in *Escherichia coli* BL21(DE3) with pLUW530 and purified according to Gueguen *et al.* (1997) with an additional size-exclusion chromatography step on Superdex 75 (Amersham Pharmacia). The protein concentration was determined at 280 nm using ε_{280} = 83 070 M^{-1} cm^{-1} calculated according to Gill & von Hippel (1989). Enzyme activity was determined as described by Gueguen *et al.*

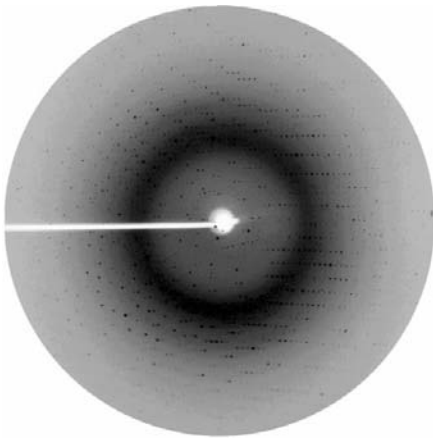

Figure 1
Diffraction pattern of a *P. furiosus* LamA crystal. The first frame of the data collection is shown. The data set was collected on the XRD1 beamline at ELETTRA (Basovizza, TS). The wavelength was set at 1.0 Å.

(1997). Calcium-depleted protein was obtained by extensive dialysis with 250 µ*M* EDTA or EGTA in 10 m*M* Tris–HCl pH 7.5.

P. furiosus LamA was concentrated to about 20 mg ml^{-1}. Crystallization was achieved at 293 K by the sitting-drop vapour-diffusion technique (McPherson, 1990). In the final conditions, the reservoir solution contained 0.1 *M* HEPES [*N*-(2-hydroxylethyl)piperazine-*N*′-2-ethanesulfonic acid] in the pH range 7.5–8.0 and 60–70% MPD (2-methyl-2,4-pentanediol). 2 µl of the protein sample was mixed with an equal amount of the reservoir solution and allowed to equilibrate. Crystals grew in 2–4 d.

The best X-ray data set was collected as 1.0° oscillation frames on the XRD1 beamline at ELETTRA (Basovizza, TS). The wavelength was set at 1.0 Å. The data were collected at 100 K from a frozen crystal. The data were indexed with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

The protein has been crystallized using MPD as a precipitant agent. The crystals grew in one week and have an elongated form. The identity of the crystallized protein was assessed by sequencing the N-terminus of the protein from a solution obtained by dissolving several crystals in water. The results of this experiment showed that the crystallized *P. furiosus* LamA lacks the first 33 residues, as reported by Gueguen *et al.* (1997).

Table 1
Data-collection statistics.

Data in parentheses are for the last resolution shell.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	44.36
b (Å)	84.76
c (Å)	69.23
α (°)	90
β (°)	104.97
γ (°)	90
Resolution range (Å)	50–2.15 (2.23–2.15)
$R_{\rm merge}$ (%)	8.5 (21.4)
χ^2	0.86
Completeness (%)	99.8 (98.8)
$I/\sigma(I)$	13.4
Observed reflections	452496
Indexed reflections	20792

Data were collected at the ELETTRA synchrotron source to a resolution of 2.15 Å (Fig. 1). Data indexing performed with *DENZO* indicated that the crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 44.36, b = 84.76, c = 69.23 Å, α = 90, β = 104.97, γ = 90°.

The data scaling gave an $R_{\rm merge}$ value of 8.5% for 20 755 indexed reflections (Table 1).

A value of $V_{\rm M}$ = 2.2 Å^3 Da^{-1} has been calculated according to Matthews (1968), assuming the presence of two asymmetric units in the unit cell and that each asymmetric unit contains two molecules. From this calculation, the crystal solvent content is about 43%.

Enzymes that belong to the different subfamilies of the family 16 glycosyl hydrolases show a similar fold and varying substrate specificity. The sequence identity between the members of different subfamilies is 10–25% and the sequence similarity is restricted to the invariant residues involved in catalysis (Allouch *et al.*, 2003). Similarly, the molecular architecture of *P. furiosus* LamA may be envisaged as resembling that of κ-carrageenase from *Pseudoalteromonas carrageenovora* (PDB code 1dyp; Michel *et al.*, 2001; Chiaraluce *et al.*, 2004) and that of other family 16 glycosyl hydrolases whose three-dimensional structures have been solved (Coutinho & Henrissat, 1999).

We are trying to solve the structure by molecular replacement using as search models polyalanine-truncated models derived from the coordinates of the family 16 glycosyl hydrolases reported so far in the Protein Data Bank. A derivative search with Hg^{II} binding to the Cys residue of the protein has been attempted and appears promising.

P. furiosus LamA is the only known member of the family 16 glycosyl hydrolases which conserves its tertiary structure in the presence of 8 *M* GdmCl. Knowledge of the three-dimensional structure of *P. furiosus* LamA will reveal the structural features responsible for its high stability with respect to the other members of the family 16 glycosyl hydrolases.

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