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Crystallization and preliminary X-ray studies of β-1,4-galactanase from *Aspergillus aculeatus*

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Recombinant β-1,4-galactanase from *Aspergillus aculeatus* has been crystallized and characterized by X-ray diffraction. Crystals were obtained in hanging drops by vapour-diffusion under the conditions 30% PEG 400, 0.2 *M* $CaCl_2$ and 0.1 *M* Na HEPES buffered to pH 7.5. The crystals diffract to 2.3 Å resolution and belong to one of the orthorhombic space groups $I222$ or $I2_12_12_1$. The unit-cell dimensions are a = 60.42, b = 88.94 and c = 129.08 Å. With one molecule in the asymmetric unit, the corresponding solvent content is ~48%.

1. Introduction

A major component of the plant cell wall is pectin, which is a complex network of polysaccharides. Pectin is comprised of smooth regions of homogalacturonan and hairy regions of rhamnogalacturonan. Rhamnogalacturonan consists of alternating α1–2 linked rhamnoses and α1–4 linked galacturonic acids. The C4 position of rhamnose can serve as an attachment site for carbohydrate side chains such as arabinan, galactan and arabinogalactan. These side chains make this region highly ramified, which is the reason why it is referred to as the 'hairy region' of pectin. β-1,4-Galactanase from *Aspergillus aculeatus* is one of the plant cell-wall degrading enzymes which act on rhamnogalacturonan. It hydrolyses unsubstituted galactan to galactose and galactobiose. From a sequence analysis, Henrissat & Bairoch (1996) classified this enzyme as belonging to family 53 of the glycosyl hydrolases, a family of the clan GH-A, for which no three-dimensional structures are available. The cDNA encoding β-1,4-galactanase has been cloned and overexpressed in *A. oryzae* to produce recombinant enzyme in large quantities (Christgau *et al.*, 1995). Its primary sequence comprises 334 amino-acid residues, giving a theoretical molecular weight of 36758 Da. The enzyme has an unusual distribution of amino acids with charged side chains: it contains 45 Asp and Glu residues but only 14 Arg and Lys residues. This explains its very low isoelectric point, which has been measured to be 2.85 (Christgau *et al.*, 1995). The pH optimum for the enzyme is between 4.0 and 4.5 and it must therefore carry a significant negative charge in its functional state. The β-1,4-galactanase displays a fairly high temperature optimum around 313–318 K, in line with some of the rhamnogalacturonan-degrading enzymes isolated from *A. aculeatus*, rhamnogalacturonan acetylesterase and rhamnogalacturonase A (Kofod *et al.*, 1994; Kauppinen *et al.*, 1995).

2. Methods and results

2.1. Crystallization

β-1,4-Galactanase from *A. aculeatus* was overexpressed in a *A. oryzae* host system and the recombinant β-1,4-galactanase was purified as described previously (Christgau *et al.*, 1995). Crystallization conditions were screened by the sparse-matrix method (Jancarik & Kim, 1991). The hanging drops were equilibrated at room temperature by vapour diffusion. A protein solution of concentration corresponding to an OD_{280} of 33 was used for the initial experiments. A droplet composed of 2 µl protein solution and 2 µl reservoir solution was mixed and equilibrated against 0.5 ml of a reservoir solution. Crystals grew in three of the drops in less than 20 h under the following conditions: (i) 18%(*w*/*v*) PEG 8000, 0.2 *M* CaAc and 0.1 *M* sodium cacodylate (pH 6.5), (ii) 28%(*v*/*v*) PEG 400, 0.2 *M* $CaCl_2$ and 0.1 *M* Na HEPES (pH 7.5) and (iii) 30%(*w*/*v*) PEG 4000, 0.2 *M* $MgCl_2{\cdot}6H_2O$ and 0.1 *M* Tris–HCl (pH 8.5), showing that crystallization was favoured by the presence of a PEG compound and a small divalent cation. The types and concentrations of PEG compounds, salts and buffers were varied and the optimized crystallization conditions were 30%(*v*/*v*) PEG 400, 0.2 *M* $CaCl_2$ and 0.1 M Na HEPES (pH 7.5) used in hanging drops containing 8 µl protein and 4 µl solution.

2.2. Molecular mass determination

SDS–PAGE showed a molar mass of 44 kDa for the purified recombinant enzyme, slightly higher than the 43 kDa estimated from the wild-type enzyme (Christgau *et al.*, 1995). As

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Table 1
Data-collection statistics.

The complete and outermost resolutions are the resolution ranges for the complete data set and highest resolution shell, respectively. The completenesses are the percentages of obtained reflections compared to the theoretically obtainable measurements. Overall and outermost $I/\sigma(I) > 2$ values are the percentage of intensities higher than two standard deviations. $R_{\rm merge}$ is the internal R factor, $R_{\rm merge} = \sum_{hkl}\sum_i |I(hkl)_i - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I(hkl)_i$. Multiplicity is the number of redundant measurements.

Number of measured reflections	70510
Number of unique reflections	15382
Complete resolution range (Å)	30.0–2.3
Outermost resolution shell (Å)	2.42–2.30
Overall completeness (%)	97.5
Completeness in outermost resolution shell (%)	95.6
Overall $I/\sigma(I) > 2$ (%)	82.3
$I/\sigma(I) > 2$ in outermost resolution shell (%)	61.4
Overall $R_{\rm merge}$ (%)	9.6
$R_{\rm merge}$ in outermost resolution shell (%)	39.2
Overall multiplicity	4.6
Multiplicity in outermost resolution shell	4.4

the molar mass from the primary sequence was 36.7 kDa, this suggests that the β-1,4-galactanase, like the rhamnogalacturonan acetylesterase and rhamnogalacturonase A from *A. aculeatus*, is extensively glycosylated (Mølgaard *et al.*, 1998; Petersen *et al.*, 1997). Examination of the preliminary sequence shows that it contains the consensus sequence for *N*-glycosylation at Asn51 and Asn112. To obtain a realistic measure of the molecular mass, a MALDI (matrix-assisted laser desorption/ionization) spectrum was recorded in the linear mode using a Biflex instrument (Bruker, Bremen, Germany) with the Multiprobe ion source. Dihydroxy acetophenone was used as the matrix. The resulting spectrum shows seven peaks in the range 37973–38949 Da, with intervals of approximately 160 Da between the peaks. Based on the calculated theoretical molecular weight of the enzyme, this corresponds to one glycosylation site with two GlcNAc and between five and eleven hexose units. The highest peak at 38312 Da corresponds to two GlcNAc and seven hexose units.

2.3. Data collection and processing

Data collection was performed with an in-house Rigaku R-AXIS IIC image-plate system equipped with an RU-200 rotating-anode generator operated at 50 kV and 180 mA. A 0.5 mm collimator was employed and a graphite monochromator was used to select monochromatic Cu $K\alpha$ radiation. A native data set was collected at 291 K using a 2° oscillation per frame and a crystal-to-detector distance of 100 mm. Each of the 59 frames were exposed for 60 min. The diffraction pattern extends to a resolution of 2.3 Å (Fig. 1). Integrated intensities were obtained using the *HKL* package (Gewirth, 1994) and further data processing with the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). The diffraction symmetry and the systematically absent reflections, $h + k + l = 2n + 1$, were consistent with the two chiral space groups $I2_12_12_1$ and $I222$. The unit-cell parameters were determined to be $a = 60.4$, $b = 88.9$ and $c = 129.1$ Å. The data-collection statistics are shown in Table 1. The solvent content was estimated using the Matthews formula (Matthews, 1968) assuming the molar mass of the unglycosylated enzyme, to give a volume-to-mass ratio V_m of 2.36 and a solvent content of 48%. Wukowitz & Yeates (1995) have shown that all known proteins which form crystals with the same space-group ambiguity and one molecule per asymmetric unit actually crystallize in the space group $I222$. We will, therefore, initiate the subsequent structure determination in this space group.

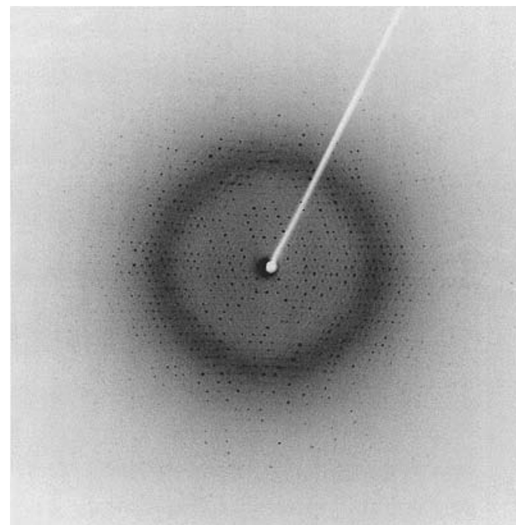

Figure 1
The diffraction pattern obtained from a crystal of β-1,4-galactanase after 30 min exposure with a 2° oscillation.

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