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Crystallization and preliminary X-ray analysis of heparinase II from *Pedobacter heparinus*

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Heparinase II from *Pedobacter heparinus* (formerly *Flavobacterium heparinum*), which acts on both heparin and heparan sulfate, is one of several glycosaminoglycan-degrading enzymes produced by this organism. This enzyme, with a molecular weight of 84 kDa, utilizes a lytic mechanism to cleave the α(1–4) glycosidic bond between hexosamine (D-glucosamine) and L-iduronic or D-glucuronic acid, resulting in a product with an unsaturated sugar ring at the non-reducing end. The enzyme was crystallized by the hanging-drop vapour-diffusion method. The crystals belong to orthorhombic space group $P2_12_12_1$ and diffract to 2 Å resolution. There are two molecules in the asymmetric unit, consistent with the finding that recombinant heparinase II functions as a dimer in solution.

1. Introduction

Glycosaminoglycans (GAGs) are negatively charged linear polysaccharides usually composed of repeating units of uronic acid and sulfated *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) (Ernst *et al.*, 1995). The GAG chains are generally attached at the reducing end to a protein core, forming proteoglycans (PGs). PGs are important components of the vertebrate extracellular matrix and play critical roles in the formation of the skeleton (Iozzo, 1998). The presence of GAGs is usually associated with higher animals, but they are not unique to eukaryotes. Several specialized microorganisms also produce unsulfated forms of these polymers (DeAngelis, 2002). In addition to their structural role, GAGs act as critical modulators of a number of biochemical signalling events and play a role in cell growth and differentiation, adhesion and tissue morphogenesis (Lander & Selleck, 2000; Kawashima *et al.*, 2002; Esko & Selleck, 2002). Heparan sulfate (HS) glycosaminoglycan (Stringer & Gallagher, 1997) has emerged as key player in many different biological processes ranging from cancer (Linhardt *et al.*, 1986; Sugahara & Kitagawa, 2002), angiogenesis (Linhardt *et al.*, 1992), anticoagulation (Petitou *et al.*, 1999), viral and microbial pathogenesis (Shukla *et al.*, 1999; Menozzi *et al.*, 2002) to multiple aspects of development (Perrimon & Bernfield, 2000).

Two different mechanisms are utilized by enzymes that degrade HS. Heparinases are polysaccharide lyases that employ an eliminative mechanism (Linhardt *et al.*, 1986), while glucuronidases act *via* a hydrolytic mechanism (Myette *et al.*, 2002). The soil bacterium *Pedobacter heparinus* (formerly *Flavobacterium heparinum*; Steyn *et al.*, 1998) produces three different heparinases. They differ in size, charge properties and substrate specificities (Lohse & Linhardt, 1992; Nader *et al.*, 1990) and display sequence similarities only to proteins from several other microorganisms (http://afmb.cnrs-mrs.fr/CAZY/PL.html). In the CAZY database, heparinase I has been assigned to the PL13 family and heparinase III to the PL12 family, while heparinase II is an as yet unclassified enzyme.

Heparinase II is unique in its ability to cleave both heparin and heparan sulfate-like regions of HS (Nader *et al.*, 1990). It is a basic protein consisting of 772 residues. An N-terminal signal sequence of 25 residues is cleaved upon export to the periplasmic space to yield the mature protein with a MW of 84 545 Da (Su *et al.*, 1996). This enzyme degrades heparin and heparan sulfate through β-elimination of the α(1–4) glycosidic bond between glucosamine and uronic acid (either glucoronic or iduronic acid) sugars, in an endolytic non-random manner (Rhomberg *et al.*, 1998).

Although heparinase II exhibits a stronger affinity towards heparin, its turnover rate of heparan sulfate is higher (Lohse & Linhardt, 1992) and it prefers long polymer substrates to shorter oligosaccharide substrates. Sequence analysis showed that heparinase II posses a Cardin–Weintraub heparin-binding consensus which reads ^{444}FFKRTIAH^{451} (Godavarti & Sasisekharan, 1996). Extensive biochemical and site-directed mutagenesis revealed that His451 as well as Cys348 are essential for heparin degradation (Shriver, Hu, Pojasek *et al.*, 1998; Shriver, Hu & Sasisekharan, 1998). It was also proposed that within the single substrate-binding pocket of heparinase II,

there are two proximate 'active sites': one site contains residues positioned for the catalytic cleavage of heparin, while the other contains residues that are positioned for the cleavage of heparan sulfate (Rhomberg *et al.*, 1998). Here, we report the crystallization of this enzyme.

2. Methods and results

2.1. Protein expression and purification

Recombinant heparinase II (residues 26–772) was purified from *P. heparinus* cells (ATCC No. 13125) overexpressing the cloned gene product under the control of a heparin-sensitive promoter (Blain *et al.*, 2002). The bacteria were cultivated at 296 K with 225 rev min^{-1} shaking for aeration, in FH medium (Su *et al.*, 2001) supplemented with 10% crude-grade heparin (Dongying Hi-tech Chemical Industry Co., Dongying City, Haochun West Road, Shandong Province, People's Republic of China) and 0.1 mg ml^{-1} trimethoprim (Sigma–Aldrich Chemie, Steinheim, Germany). The cells were harvested when the optical density of the culture reached an A_{600} of 2.5 and were lysed using a French Press. 30 ml of cleared lysate (centrifugation for 30 min at 45 000 rev min^{-1}, 277 K) was loaded on a water-cooled (277 K) 25 × 800 mm SP Sepharose Fast Flow column (Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with buffer *A* (10 m*M* sodium phosphate pH 7.5). The column was washed with 50 volumes of buffer *A* and the protein was eluted in a linear gradient of 0–1 *M* NaCl in buffer *A* over ten column volumes. The heparinase II-containing fractions were loaded onto a hydroxyapatite column (Bio-Gel HTP, BioRad Laboratories, Richmond, Canada) pre-equilibrated with buffer *B* (10 m*M* sodium phosphate pH 6.0). The column was washed with ten column volumes of buffer *B* with the addition of 100 m*M* NaCl. The protein was eluted in three column volumes with a salt gradient of 400–700 m*M* NaCl in 25 m*M* sodium phosphate pH 6.0. Purified heparinase II was concentrated by ultrafiltration using a Centriprep-50 and the buffer was exchanged to 10 m*M* sodium phosphate pH 7.5, 100 m*M* NaCl, 20 m*M* sodium formate, 5 m*M* dithiothreitol (DTT) using a 5 ml HiTrap desalting column (Amersham Biosciences AB, Uppsala, Sweden) to yield a protein with a concentration of 6.5 mg ml^{-1}. The protein showed a single band on a 12.5%(*w*/*v*) SDS–PAGE gel. Analysis of purified heparinase II by dynamic light scattering (DynaPro MSPRII, Protein Corporation, Piscataway, NJ, USA) showed that the protein was monodisperse with a molecular weight of 150 kDa, indicating the presence of a dimer in solution. This was confirmed by size-exclusion chromatography (Superdex 200 10/300Gl, Amersham Biosciences AB, Uppsala, Sweden), which yielded an apparent molecular weight of 162 kDa on a calibrated column. Enzyme activity was measured spectrophotometrically by monitoring at 232 nm the formation of unsaturated oligosaccharide products released from heparin (Fluka Chemie Neu-Ulm, Switzerland). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

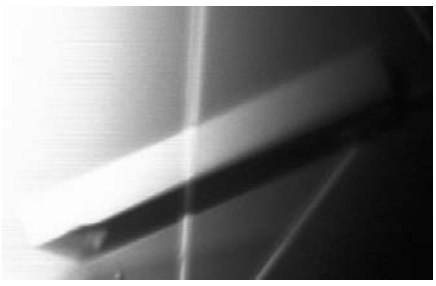

Figure 1
Crystal of heparinase II grown by the hanging-drop vapour-diffusion method.

2.2. Crystallization

Crystallization conditions for heparinase II were initially found using the PEG/Ion screen (Hampton Research Laguna Niguel, CA, USA) with the hanging-drop vapour-diffusion method at 292 K. Small cubic shaped crystals appeared from drops containing 0.5 µl heparinase II (6.5 mg ml^{-1} in 10 m*M* sodium phosphate pH 7.5, 100 m*M* NaCl, 20 m*M* sodium formate) and 0.8 µl reservoir solution [20%(*w*/*v*) polyethylene glycol (PEG) 3350, 200 m*M* sodium dihydrogen phosphate monohydrate pH 4.5] equilibrated over 60 µl reservoir solution. Crystals appeared within 3–7 d. They showed anisotropic diffraction extending to 3 Å resolution in the best direction. Screening around the initial conditions led to crystals [obtained from 17%(*w*/*v*) PEG 3350, 200 m*M* sodium phosphate pH 5] that had a different morphology (rectangular plates) but the same unit-cell parameters. Well diffracting crystals were obtained by microseeding into drops consisting of 1 µl protein solution and 1 µl reservoir solution consisting of 16–17.5%(*w*/*v*) PEG 3350 and 200 m*M* sodium phosphate pH 5. Rectangular prism-shaped crystals measuring approximately 0.05 × 0.05 × 0.75 mm grew overnight at 292 K (Fig. 1). These crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 70.0, b = 119.3, c = 200.7 Å, and contain two molecules in the asymmetric unit. The Matthews coefficient V_M is 2.49 Å^3 Da^{-1} (Matthews, 1968) and the estimated solvent content is 51%. Occasionally, we found in the same drops crystals that could be indexed with a three times longer a axis, a = 210 Å. We have not investigated these crystals in detail at this time.

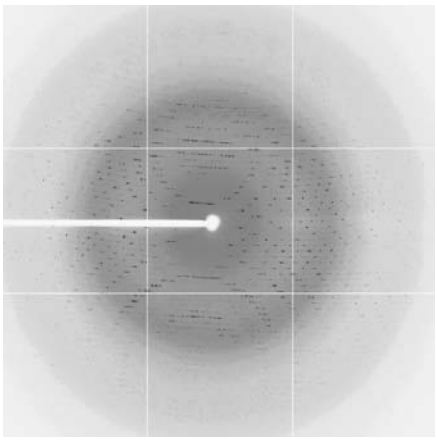

Figure 2
Oscillation frame obtained at beamline X25, NSLS, BNL, showing the extent of the diffraction pattern. The oscillation range is 1° and the exposure time was 7 s.

Table 1
Statistics of diffraction data collected from a single heparinase II crystal.

Values in parentheses refer to the last resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 70.0, b = 119.3, c = 200.7, α = 90.0, β = 90.0, γ = 90.0
Crystal mosaicity (°)	0.25
Resolution range (Å)	50–2.00 (2.07–2.00)
Total No. reflections measured	817975
No. unique reflections	112668
Average redundancy	7.3 (7.1)
Average $I/\sigma(I)$	10.6 (3.3)
R_{sym}†	0.075 (0.393)
Completeness (%)	98.6 (98.0)

† $R_{sym} = \sum |I_{obs} - I_{avg}| / \sum I_{avg}$.

2.3. Data collection and processing

Diffraction data were collected at beamline X25, NSLS, Brookhaven National Laboratory on an ADSC Q315 area detector. Crystals were transferred briefly from mother liquor to a cryoprotectant solution [22.5%(*w*/*v*) PEG 3350, 0.07 *M* sodium phosphate pH 5, 2.17 *M* sodium formate], mounted in a nylon loop (Hampton Research, Laguna Niguel, CA, USA) and placed directly in a cold nitrogen stream at 100 K. The crystal-to-detector distance was set to 280 mm. 1° oscillation

images were recorded with an exposure time of 7 s (Fig. 2). A native data set was collected at a wavelength of 0.97940 Å to 2.00 Å resolution (Table 1). Images were processed with the *HKL*2000 program package (Otwinowski & Minor, 1997) with statistics as shown in Table 1. Structure determination is in progress.

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