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# Crystallization and preliminary X-ray analysis of chondroitinase B from *Flavobacterium heparinum*[1]

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Chondroitinase B, a glycosaminoglycan lyase from *Flavobacterium heparinum*, has been crystallized by hanging-drop vapor diffusion in space group $P2_1$ with unit-cell parameters $a = 50.6$, $b = 74.5$, $c = 58.7$ Å, $\beta = 92.9^\circ$ and one molecule in the asymmetric unit. This enzyme degrades dermatan sulfate, a glycosaminoglycan primarily made up of a disaccharide repeating unit of iduronic acid and *N*-acetylgalactosamine. A complete native data set has been collected from a single crystal to 2.2 Å resolution using a rotating-anode source.

## 1. Introduction

Glycosaminoglycans (GAGs) are a group of linear polysaccharides made up of a repeating disaccharide unit of glucosamine or galactosamine and uronic acid (reviewed in Ernst *et al.*, 1995; Jackson *et al.*, 1991). Included in this group are chondroitin sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid and heparin or heparan sulfate. GAGs possess a multitude of functions: as the carbohydrate components of proteoglycans, in cell signaling, as constituents of the basement membrane of mammalian tissues and in cell adhesion and migration (Jackson *et al.*, 1991). Dermatan sulfate, also known as chondroitin sulfate B, is a polysaccharide consisting of $\beta$-(1→4) linked disaccharide repeating units of mainly iduronic acid $\beta$-(1→3) linked to *N*-acetylgalactosamine. Iduronic acid may be substituted to varying extents by glucuronic acid, and the *N*-acetylgalactosamine moiety is usually sulfated at the C4 position.

Enzymes which degrade GAGs can be classified into two main groups, hydrolases or lyases, depending on the particular mechanism. *F. heparinum* produces two chondroitin sulfate degrading lyases: chondroitinase AC, which can cleave hyaluronic acid, chondroitin, chondroitin-4-sulfate and chondroitin-6-sulfate, and chondroitinase B, which acts exclusively on dermatan sulfate (Ernst *et al.*, 1995; Michelacci & Dietrich, 1975). Comparative analysis of the derived amino-acid sequences for chondroitinases AC and B from *F. heparinum* shows no detectable sequence homology between the two enzymes.

Chondroitinase B is a monomeric enzyme of 506 residues with an isoelectric point of 9.05 (Gu *et al.*, 1995). An N-terminal signal sequence of 25 residues is cleaved to yield the mature protein ($M_r$ = 53600 Da) upon export to the *F. heparinum* periplasmic space. Analysis of its activity on dermatan sulfate indicates that it cleaves the $\beta$-(1→4) linkages between *N*-acetylgalactosamine and iduronic acid within the polysaccharide in a random endolytic manner (Jandik *et al.*, 1994; Michelacci & Dietrich, 1975). The products resulting from the initial action of chondroitinase B on dermatan sulfate are a mixture of oligosaccharides, hexasaccharides, tetrasaccharides and an unsaturated 4-sulfated disaccharide (ΔDi-4S; Michelacci & Dietrich, 1975). Hexasaccharides or tetrasaccharides are further degraded slowly to the final ΔDi-4S product (Michelacci & Dietrich, 1975). While no detailed studies have been reported on the active-site organization or catalytic mechanism of chondroitinase B, it is expected to perform an eliminative cleavage of the 4-*O*-glycosidic bond as suggested for other polysaccharide lyases (Gacesa, 1987).

While a considerable volume of literature has accumulated on the biochemical properties of GAG-lyases (reviewed in Ernst *et al.*, 1995; Linhardt *et al.*, 1986), relatively little is known about these enzymes from a structural standpoint. Crystal structures have been reported for several pectate lyases (Lietzke *et al.*, 1994; Pickersgill *et al.*, 1994; Yoder *et al.*, 1993) and pectin lyases (Mayans *et al.*, 1997; Vitali *et al.*, 1998), two groups of enzymes related mechanistically to the GAG lyases. Two GAG lyases have been previously crystallized; chondroitinase AC from *F. heparinum* (Féthière *et al.*, 1998) and a hyaluronate lyase from *Streptococcus pneumoniae* (Jedrzejas *et al.*, 1998).

## 2. Methods and results

### 2.1. Protein expression and purification

Recombinant chondroitinase B (residues 26–506) was purified from *F. heparinum* cells

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**Table 1**
Statistics for diffraction data collected from a single chondroitinase B crystal.

Figures in parentheses refer to the resolution range 2.28–2.20 Å.

| | |
|---|---|
| Resolution range (Å) | 45–2.2 |
| Number of observations | 73516 |
| Number of unique reflections | |
| Calculated | 22184 |
| Measured | 21565 |
| Average redundancy | 3.4 |
| Average $I/\sigma(I)$ | 48.1 (30.1) |
| $R_{sym}$† (%) | 2.9 (5.2) |
| Completeness (%) | 97.2 (95.3) |

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

overexpressing the cloned gene product. Cells were grown at 296 K in shake flasks and were harvested when the culture density reached $A_{600}$ = 3.0. For purification of chondroitinase B, a procedure similar to that used to purify the wild-type enzyme was used (Gu *et al.*, 1995). However, for the recombinant enzyme purification, only three columns were employed: SP-Sepharose Big Beads (Pharmacia Biotech, Uppsala, Sweden), carboxy-sulfon (40 µm) cation exchange (J. T. Baker Inc., Phillispsburg, NJ, USA) and ceramic hydroxyapatite (20 µm; American International Chemical Inc., Natick, MA, USA). Enzyme activity was measured spectrophotometrically by monitoring the formation of unsaturated oligosaccharide products released from dermatan sulfate at 232 nm (Gu *et al.*, 1995). Chondroitinase B was apparently homogeneous following hydroxyapatite chromatography as determined by SDS–PAGE on a 12%(*w*/*v*) separating gel and staining with Coomassie Blue. Analysis of purified chondroitinase B by dynamic light scattering (DynaPro 801 Dynamic Light Scattering Instrument, Protein Solutions, Charlottesville, VA, USA) yielded a value for the ratio of polydispersity to particle radius of 22%, indicating it was monodisperse. Chondroitinase B was concentrated following purification by ultrafiltration using Centriprep-10 (750*g*, 277 K, 30 min) and Centricon-10 (4000*g*, 277 K, 1 h) concentrators to a final concentration of 6.1 mg ml$^{-1}$ in a buffer consisting of 20 m*M* Tris–HCl pH 8.0, 1 m*M* sodium phosphate pH 7.0, 3 m*M* NaCl, 0.5 m*M* phenylmethylsulfonyl fluoride, 1 µg ml$^{-1}$ aprotinin, 1 µg ml$^{-1}$ leupeptin and 1 µg ml$^{-1}$ E64. Addition of protease inhibitors following purification was found necessary in order to increase the stability of the protein while stored at 277 K. Protein concentration was determined by the method of Bradford (1977), using BSA as a standard.

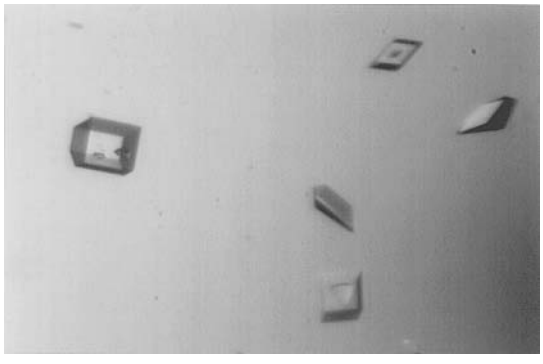

**Figure 1**
Monoclinic crystal of chondroitinase B from *F. heparinum*.

### 2.2. Crystallization

Crystals of chondroitinase B were initially found through sparse-matrix screening by hanging-drop vapor diffusion at 292 K using kits from Hampton Research (Laguna Niguel, CA, USA). Small rod-shaped crystals or small prisms appeared when 2 µl of chondroitinase B (6–8 mg ml$^{-1}$) in buffer was mixed with 4 µl of reservoir solution [19%(*w*/*v*) PEG 8000, 100 m*M* Bicine buffer pH 9.0, or 100 m*M* Tris–HCl pH 8.8, 0.15 *M* ammonium acetate, 15%(*v*/*v*) 2-methyl-2,4-pentanediol] and the drops suspended over 1 ml of reservoir solution. Crystals were found to appear overnight. Two crystal forms grew under similar conditions, although one of these (space group *C*2) often formed stacks of layered plates upon seeding and could not be grown as large single crystals in a reproducible manner. Efforts were subsequently directed at developing the second crystal form (space group $P2_1$), which showed a better and more reproducible crystal morphology. Single crystals, measuring approximately 0.3 × 0.3 × 0.3 mm, were obtained by washing and reseeding of the small prism crystals (Thaller *et al.*, 1981). Seeds were introduced into drops consisting of 2 µl protein in buffer (6.1 mg ml$^{-1}$) and 4 µl reservoir solution consisting of 16.5%(*w*/*v*) PEG 8000, 0.1 *M* Tris–HCl pH 8.8, 15%(*v*/*v*) 2-methyl-2,4-pentanediol and 0.25 *M* ammonium acetate. Crystals grew at 292 K to their full size in two to three weeks (Fig. 1).

### 2.3. Data collection and processing

Diffraction data were collected using an R-AXIS IIC imaging plate with a Rigaku RU-300 rotating-anode generator operating at 50 kV and 260 mA as the X-ray source (Molecular Structure Corporation, The Woodlands, TX, USA). Diffraction data collected from chondroitinase B crystals at room temperature were found to decay over time and the data were therefore collected under low-temperature conditions. Crystals were transferred from mother liquor to a cryoprotectant solution [22.5%(*w*/*v*) PEG 8000, 0.1 *M* Tris–HCl pH 8.7, 15%(*v*/*v*) 2-methyl-2,4-pentanediol and 0.25 *M* ammonium acetate] for 1–2 min, picked up in a mounted nylon loop (Hampton Research, Laguna Niguel, CA, USA) and placed directly in the nitrogen cold stream operating at a temperature of 100 K (Oxford Cryosystems Cryostream, Oxford, UK). Oscillation images of 1.5° were recorded with an exposure time of 25 min. Images were processed with the *HKL* program package (Otwinowski & Minor, 1997). Data were obtained for a native crystal (approximately 0.2 mm per edge) in the resolution range 45–2.2 Å, with statistics as shown in Table 1. The space group has been assigned as $P2_1$ based on the inspection of systematic absences. One chondroitinase B molecule per asymmetric unit yields a Matthews coefficient, $V_m$, of 2.06 Å$^3$ Da$^{-1}$ and an estimated solvent content of 40% (Matthews, 1968).

### 2.4. Heavy-atom derivative screening

A mercury derivative has been obtained by soaking a crystal of chondroitinase B in cryoprotectant solution containing 1 m*M* thiomersal overnight. Analysis of a difference Patterson map calculated with the isomorphous differences between 10 and 3 Å indicated two heavy-atom sites. These sites were confirmed using anomalous difference Patterson maps. The search for additional heavy-atom derivatives is under way.

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