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Crystallization and preliminary crystallographic analysis of recombinant hyaluronate lyase from *Streptococcus suis*

Hyaluronate lyase is an important surface enzyme of many streptococcal species. The enzyme degrades several biologically important connective tissue components, which facilitates the spreading of the bacteria throughout the host tissues and presumably provides energy and a carbon source for bacterial cells. Recombinant hyaluronate lyase was expressed in *Escherichia coli* and was crystallized using the hanging-drop vapour-diffusion method. The crystals belonged to space group $P222_1$, with unit-cell parameters $a = 58.08$, $b = 101.32$, $c = 103.47$ Å and one molecule in the asymmetric unit. Diffraction data were collected to 2.50 Å resolution.

1. Introduction

Hyaluronic acid is present in many mammalian tissues, including synovial fluid, cartilage, skin and brain, and forms a major part of the extracellular matrix (Hynes & Walton, 2000). Hyaluronate lyase enzyme is present in a wide variety of bacteria, including several pathogens, and catalyses the degradation of hyaluronic acid. Pathogenic bacteria cause degradation of the hyaluronic acid at their entry points, such as skin and mucosal surfaces, in order to spread the infection. This degradation results in decreased viscosity of the extracellular matrix and aids in the penetration of bacteria and associated products, including secreted toxins, into the host's tissues. Hyaluronic acid, chondroitin sulfates and to a smaller extent dermatan sulfates of the host connective tissue are the main targets of hyaluronidases for degradation. The degradation of these essential components leads to spreading of the bacterial infection and presumably provides energy and a carbon source for the bacterial cells. Hyaluronate lyase may also provide the disaccharide degradation product as a carbon source for further catabolism, growth and enhanced colonization at the site of infection (Homer *et al.*, 1997; Seddon *et al.*, 1990).

Hyaluronate lyases from various species show different specificities towards polysaccharide substrates (Mishra *et al.*, 2006). The enzyme is found in two forms, the first closely associated with bacterial cells and the second a cell-free form that is presumably released from bacterial cells by the action of sortase enzymes (Pallen *et al.*, 2001; Ton-That *et al.*, 2000). The cell-attached hyaluronidase is anchored to the surface of the bacterial cell wall through a covalent linkage to the cross-bridges of the peptidoglycan of the wall (Schneewind *et al.*, 1995; Rigden & Jedrzejewski, 2003). Streptococci from Lancefield groups A, B, C and G have been shown to possess hyaluronate lyase activity (Günther *et al.*, 1996). Hyaluronate lyase requirement has been shown for the full virulence of *Streptococcus pneumoniae* in a mouse model (Berry & Paton, 2000). However, group B *S. agalactiae* containing a mutated hyaluronate lyase gene have been associated with endocarditis (Granlund *et al.*, 1998; Rolland *et al.*, 1999) suggesting that the presence of active hyaluronate lyase is not always essential in causing invasive disease. Hyaluronidases are produced by a variety of organisms, including mammals, insects, leeches and bacteria. Besides these well known sources, phage-encoded hyaluronidases from *S. pyogenes* and *S. equi* have also been identified (Meyer, 1971; Kreil, 1995).

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Table 1

Summary of data-collection statistics.

Values in parentheses are for the last resolution shell.

Space group	$P222_1$
Unit-cell parameters (Å)	$a = 58.08, b = 101.32, c = 103.47$
Molecules in asymmetric unit	1
Resolution range (Å)	38.63–2.50 (2.59–2.50)
Total no. of reflections	228902
No. of unique reflections	21600
Completeness (%)	99.1 (98.0)
$R_{\text{merge}}^{\dagger}$	0.185 (0.373)
Reduced χ^2	0.94 (1.01)
Average $I/\sigma(I)$	9.8 (6.2)

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

2. Materials and methods

2.1. Cloning, expression and purification

The gene encoding hyaluronate lyase (Hyl; 05SSU1214) was amplified and cloned into the vector pET-30a (Novagen). Positive cloning was confirmed by double-enzyme digestion and was verified by direct DNA sequencing. Competent *Escherichia coli* strain BL21(DE3) cells were transformed with positive recombinant plasmid and cultured in LB broth with 50 mg l⁻¹ kanamycin. Cells were grown at 310 K to an OD₆₀₀ of 0.6 and were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was incubated for a further 4 h for protein expression. The following procedure was performed at 277 K. The cells were harvested by centrifugation at 5000g, resuspended in affinity buffer A (50 mM sodium phosphate buffer pH 8.0 containing 0.25 M NaCl, 10 mM imidazole) and lysed by sonication on ice. To collect the soluble supernatant, the sonicated cells were centrifuged at 18 000 rev min⁻¹ for 30 min. Post-centrifugation, the clear supernatant was loaded onto a column containing Ni-NTA His-binding resin (Novagen) previously equilibrated with buffer A as used for resuspending the cells. To wash the nonspecifically bound protein from the column, an increased concentration of imidazole was used in buffer B (50 mM sodium phosphate buffer pH 8.0 containing 0.25 M NaCl, 20 mM imidazole). For the elution of the target protein, buffer C (50 mM sodium phosphate buffer pH 8.0 containing 0.25 M NaCl, 250 mM

imidazole) was used. To further purify the protein by FPLC, the eluted protein was first dialysed against buffer consisting of 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM mercaptoethanol, 1 mM EDTA and concentrated to the desired concentration and volume using a 10 kDa molecular-weight cutoff Centricon (Amicon). The protein sample was loaded onto a Superdex 200 column with an ÄKTA purifier system (GE Healthcare). The purified protein was examined using SDS-PAGE (Laemmli, 1970) and was found to be nearly homogeneous (Fig. 1).

2.2. Protein crystallization and X-ray diffraction analysis

Adopting the hanging-drop vapour-diffusion method and using Crystal Screen, Crystal Screen 2, PEG/Ion, Index and SaltRx (Hampton Research), initial crystallization screening was carried out at 291 K. By mixing 1 µl each from the crystallization solutions and protein solutions (at a concentration of 5, 10 or 15 mg ml⁻¹) drops were set up against 250 µl reservoir solution. Crystals were normally obtained in a few days to a few weeks. Successful conditions with crystals were further optimized by varying the protein concentrations, pH and ionic strengths of the contributing components, performing crystallization at different temperatures [277, 291 K and room temperature (RT, 300 K)], and utilizing Additive Screen. The crystals from the optimized conditions were used for X-ray diffraction analysis (Figs. 2a and 2b).

Using the crystals from these drops, X-ray diffraction data were collected using an in-house X-ray source (Rigaku MicroMax-007 HF desktop rotating-anode X-ray generator with a Cu target operated at

Figure 1

SDS-PAGE analysis of hyaluronate lyase protein. Lane M, protein marker (labelled in kDa). Lanes 1 and 2, purified hyaluronate lyase fractions after size-exclusion chromatography.

(a)

(b)

Figure 2

(a) Single crystal of hyaluronate lyase grown by the hanging-drop method. (b) Diffraction pattern obtained from the hyaluronate lyase crystal.

40 kV and 20 mA) and an R-Axis IV⁺⁺ imaging-plate detector at a wavelength of 1.5418 Å. The crystals were first cryoprotected by soaking them for a short period of time in a solution consisting of 50% each of the crystal-growth reservoir solution and glycerol. The crystal was flash-cooled in a cold nitrogen-gas stream at 100 K and data were collected. The data were analysed using *DENZO* and *SCALEPACK* from the *HKL-2000* suite (Otwinowski & Minor, 1997) and had a completeness of 100% to 2.6 Å resolution.

Data-collection statistics are given in Table 1. The relatively poor merging statistics may be related to the poor mount of the crystal on the magnet, as was later noticed.

Molecular-replacement methods of phase determination were attempted, but the identity and similarity of the searched model to known structures was very low; therefore, as an attempt to solve the structure of this important enzyme selenomethionine-labelled protein will be expressed and crystallized.

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