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Crystallization and preliminary X-ray analysis of human leukocyte cell-derived chemotaxin 2 (LECT2)

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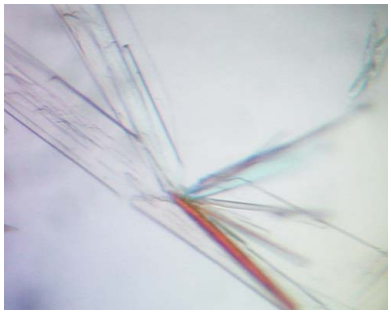

Human leukocyte cell-derived chemotaxin 2 (LECT2) is a chemotactic factor for neutrophils that plays multifunctional roles in liver regeneration, regulation of neuritic development and proliferation of chondrocytes and osteoblasts. In addition, the C-terminal region of LECT2 belongs to the zinc metalloendopeptidase M23 (PF01551) family. Purified LECT2 was crystallized using the sitting-drop vapour-diffusion method at 293 K. Crystals of selenomethionine-substituted LECT2 that diffracted X-rays to 1.94 Å resolution were obtained using a reservoir solution consisting of 0.2 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5, 25%(*w*/*v*) PEG 8000. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 59.4, b = 63.5, c = 64.0 Å. The calculated Matthews coefficient (V_M = 2.10 Å^3 Da^{-1}, solvent content 40%) indicates that the crystal consists of two molecules per asymmetric unit.

1. Introduction

Leukocyte cell-derived chemotaxin 2 (LECT2) was originally purified from the culture supernatants of phytohaemagglutinin-activated (PHA) human T-cell leukaemia SKW-3 cells as a chemotactic factor for human neutrophils (Yamagoe *et al.*, 1996) and was subsequently found to be identical to chondromodulin-II (chM-II), a growth-stimulating factor for chondrocytes and osteoblasts (Hiraki *et al.*, 1996; Shukunami *et al.*, 1999). In addition, homologous genes have been identified in many vertebrates (Yamagoe, Watanabe *et al.*, 1998; Fujiki *et al.*, 2000; Kokkinos *et al.*, 2005; Chayka *et al.*, 2005; Lin *et al.*, 2007; Li *et al.*, 2008).

Human LECT2 is preferentially expressed in the livers and hepatoma cell lines and is secreted into the bloodstream (Yamagoe, Mizuno *et al.*, 1998; Segawa *et al.*, 2001). Accumulating evidence suggests that LECT2 plays multifunctional roles in several tissues. For instance, LECT2 participates in liver regeneration (Sato *et al.*, 2004*a*,*b*), potentially plays a key role in the development of human hepatocellular carcinoma (HCC) by the repression of the growth of HCC cells (Ong *et al.*, 2011) and may be involved in the pathogenesis of hepatitis in humans through the modulation of the homeostasis of hepatic NKT cells (Saito *et al.*, 2004). Furthermore, LECT2 was found to have a prominent role in the regulation of neuritic development through a unique mechanism that differs from those of other related cytokines (Koshimizu & Ohtomi, 2010). LECT2 was also identified as a novel renal amyloid protein (Benson *et al.*, 2008; Larsen *et al.*, 2010). In addition, the polymorphism of human LECT2 (V58I substitution) was demonstrated to be associated with the incidence and severity of rheumatoid arthritis in the Japanese population (Kameoka *et al.*, 2000). A LECT2-deficient (LECT2$^{-/-}$) mouse model of inflammatory arthritis demonstrated that LECT2 directly suppresses the development of collagen antibody-induced arthritis (CAIA), probably by suppressing the production of certain crucial arthritis-related cytokines and chemokines (Okumura *et al.*, 2008). In spite of its biological significance, however, the molecular basis underlying the function of LECT2 remains unclear.

Human LECT2 is a 16 kDa secreted protein consisting of 133 amino acids and three intramolecular disulfide bonds (Okumura *et al.*, 2009). Consistent with the extracellular location of the protein, the gene for LECT2 encodes a secretory signal at the N-terminus. The SignalP 3.0 server (Bendtsen *et al.*, 2004) estimated that the signal

sequence is comprised of the 18 N-terminal amino-acid residues. Search results in the Pfam database also indicated that the C-terminal region of human LECT2 belongs to the zinc metalloendopeptidase M23 (PF01551) family (Okumura *et al.*, 2009). Members of this family share the HX_nD and H*X*H motifs for binding a zinc ion, and the motifs are conserved in the LECT2 sequence. This family of enzymes possesses a catalytic activity that leads to the bacteriolysis of Gram-positive bacteria cells through the cleavage of pentaglycine interpeptides that cross-link adjacent peptidoglycan chains (Odintsov *et al.*, 2004; Firczuk *et al.*, 2005; Spencer *et al.*, 2010). However, the overall sequence identity of LECT2 with the M23 metalloendopeptidases is low (22% identity) and there is no evidence that demonstrates that human LECT2 also has the M23 metalloendopeptidase activity.

The diverse biological functions of LECT2 should be relevant to its structure. Previously, high hydrostatic pressure (HHP) was successfully applied to refold LECT2 from inclusion bodies (IBs) and the refolded LECT2 showed chemotactic activity (Zheng *et al.*, 2013). In this study we report the crystallization and preliminary X-ray analysis of the refolded LECT2, which allow the structural elucidation of the biochemical properties and multifunctional roles of LECT2.

2. Materials and methods

2.1. Protein preparation

LECT2 was prepared according to the previous report with some modifications for the SeMet derivative (Zheng *et al.*, 2013). Briefly, the mature LECT2 gene (encoding 19–133 amino acids) was inserted into the pET-48b(+) vector (Novagen) between the *Sma*I and *Bam*HI sites. LECT2 with an N-terminal tag sequence containing thioredoxin (Trx), hexahistidine (His_6) and a HRV3C cleavage site (Trx sequence GSGSGHTSGGGGSNNNPPTPTPSSGSG-His_6-SAALEVLFQGP) was overexpressed in *Escherichia coli* Rosetta-gami 2(DE3) cells (Novagen) grown in Lysogeny-Broth (LB) medium containing 20 mg l^{-1} kanamycin, 17 mg l^{-1} chloramphenicol, 6 mg l^{-1} tetracycline and 0.01%(*v*/*v*) antifoaming agent at 310 K. When the OD_{600} value reached ∼0.5, the cells were transferred into M9 medium supplemented with amino acids (100 mg l^{-1} L-lysine, L-phenylalanine and L-threonine; 50 mg l^{-1} L-isoleucine, L-leucine, L-valine and L-selenomethionine) and grown for an additional 30 min at 298 K. The expression of SeMet-substituted LECT2 (LECT2^{SeMet}) was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 m*M* and the culture was then further incubated at 298 K overnight. The harvested cells were resuspended in the lysis buffer (20 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 10 m*M* imidazole) with the addition of 0.1% protease inhibitor cocktail (Nacalai Tesque) and 0.1 m*M* 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (ABSF) and were disrupted by sonication. The cell lysate was centrifuged at 40 000*g* at 277 K for 30 min to separate IBs from the soluble fraction. The suspension of LECT2^{SeMet} IBs was prepared to a protein concentration of 0.5 mg ml^{-1} with the refolding buffer (50 m*M* Tris–HCl pH 8.0, 500 m*M* L-arginine) and subjected to HHP (200 MPa) for 16 h at room temperature. The refolded LECT2^{SeMet} solution was dialysed against 20 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl to remove L-arginine and then centrifuged for 30 min at 40 000*g* at 277 K. The supernatant was loaded onto a Ni–NTA superflow (Qiagen) column equilibrated with lysis buffer. After washing with the lysis buffer, the fusion protein bound to the resin was treated with HRV3C protease overnight to remove the N-terminal tag sequence. The cleaved protein, which has two additional amino acids (GP) at the N-terminus of the mature LECT2-coding sequence, was further purified by cation-exchange chromatography using a Resource S (GE Healthcare) column equilibrated with 20 m*M* sodium phosphate buffer pH 6.0 and was eluted with a linear gradient of 0–1 *M* NaCl in 20 m*M* sodium phosphate buffer pH 6.0. The purified LECT2^{SeMet} was concentrated to 10 mg ml^{-1} in the buffer consisting of 10 m*M* Tris–HCl pH 7.0, 1 m*M* iminodiacetic acid (IDA) and 50 µ*M* $ZnCl_2$ using Vivaspin-20 (5 kDa cutoff, treated with Tween 20, hydrozart membrane; Vivascience) prior to crystallization trials. The protein concentration was determined by the absorbance at 280 nm with the molar extinction coefficient of 16 305 M^{-1} cm^{-1} (Pace *et al.*, 1995) and the molecular weight of 14 572.

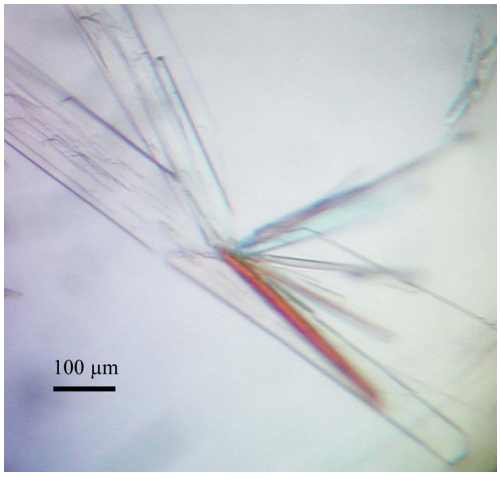

Figure 1
Crystal aggregate of LECT2^{SeMet} grown in 0.2 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5 and 25%(*w*/*v*) PEG 8000 at 293 K.

2.2. Crystallization

All crystallization experiments were performed at 293 K using the sitting-drop vapour-diffusion method. The initial crystallization screening was performed using the commercially available kit Crystal Screen HT (Hampton Research) in the 96-well Intelli Plate (Art Robbins). Each sitting drop was prepared by mixing 0.6 µl of protein solution with 0.6 µl of reservoir solution and was equilibrated against 30 µl of reservoir solution. Crystallization conditions were optimized to obtain crystals of better quality by varying the pH and precipitant concentrations using 24-well Cryschem Plates (Hampton Research). In the optimization step, each sitting drop was prepared by mixing 1.0 µl of protein solution with 1.0 µl of reservoir solution and was equilibrated against 0.5 ml of reservoir solution at 293 K.

2.3. X-ray data collection and processing

The crystals were mounted on cryoloops (Hampton Research) and flash-cooled in a stream of nitrogen at 95 K using a mixture of 30% ethylene glycol and 70% reservoir solution as the cryoprotectant. The X-ray diffraction data were collected on beamline BL-5A at the Photon Factory (Tsukuba, Japan) using an ADSC Quantum 210r CCD detector. A crystal of LECT2^{SeMet} was used to collect a single-wavelength anomalous diffraction (SAD) data set at the selenium peak wavelength of 0.9792 Å, with a 360° sweep in 0.5° oscillation steps and an exposure time of 1 s per image. The crystal-to-detector distance was 194.7 mm. The data set was indexed, integrated and scaled by the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The Matthews coefficient and solvent content were calculated from the space group, the unit-cell parameters and the molecular weight of

Table 1
Crystal parameters and data-collection statistics of LECT2^{SeMet}.

Values in parentheses are for the highest-resolution shell.

Beamline	Photon Factory BL-5A
Wavelength (Å)	0.9792
Crystal-to-detector distance (mm)	194.7
Total rotation range (°)	360
Oscillation range (°)	0.5
Exposure time (s)	1
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 59.4, b = 63.5, c = 64.0
Resolution (Å)	50.0–1.94 (2.01–1.94)
Unique reflections	18525 (1764)
Multiplicity	14.1 (11.5)
Completeness (%)	99.7 (97.2)
R_{merge}†	0.072 (0.224)
$\langle I/\sigma(I)\rangle$	51.9 (12.8)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average.

LECT2^{SeMet} using the *MATTHEWS_COEF* program (Matthews, 1968) from the *CCP*4 (Winn *et al.*, 2011) program suite.

3. Results and discussion

LECT2^{SeMet} was expressed as IBs in *E. coli* Rosetta-gami 2(DE3) cells (Novagen). The LECT2^{SeMet} IBs were refolded by HHP treatment with 200 MPa and the refolded LECT2^{SeMet} was purified by Ni-affinity resin and cation-exchange chromatography. During the initial crystallization screening, the most promising crystals were obtained in the solution containing 0.2 *M* ammonium sulfate, 0.1 *M* sodium cacodylate trihydrate pH 6.5 and 30%(*v*/*v*) polyethylene glycol (PEG) 8000. After two-dimensional grid optimization of crystallization conditions (pH *versus* the concentration of PEG 8000), the best crystal of LECT2^{SeMet} was obtained using the reservoir solution consisting of 0.2 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5 and 25%(*w*/*v*) PEG 8000. Fig. 1 shows typical crystals of LECT2^{SeMet}. The crystals appear as rod clusters, with approximate dimensions of 0.56 × 0.05 × 0.01 mm for a typical crystal from the cluster. In X-ray diffraction experiments, the rod cluster was broken and one crystal was used to collect a SAD data set. The crystal diffracted X-rays to 1.94 Å resolution (Fig. 2). The space group of the crystal was $P2_12_12_1$ with unit-cell parameters of a = 59.4, b = 63.5 and c = 64.0 Å. According to the collected diffraction data, the value for completeness became worse at the higher resolution than at 1.9 Å. For this reason, the diffraction data set was processed with a resolution range of 50.0–1.94 Å with 99.7% completeness and an R_{sym} of 7.2%. The data-collection statistics are summarized in Table 1. The $\langle I/\sigma(I)\rangle$ value is high enough at 1.94 Å resolution (12.8); therefore, the crystals could diffract X-rays to a higher resolution. The Matthews coefficient analysis indicated that the crystals contained two molecules per asymmetric unit, with a V_M (Matthews, 1968) of 2.10 Å^3 Da^{-1} and a solvent content of 40%. The determination of the three-dimensional structure will be performed by the SAD method using the data set for the LECT2^{SeMet} crystal. The crystal structure of LECT2 will be helpful in understanding the structural basis for the biological functions of LECT2.

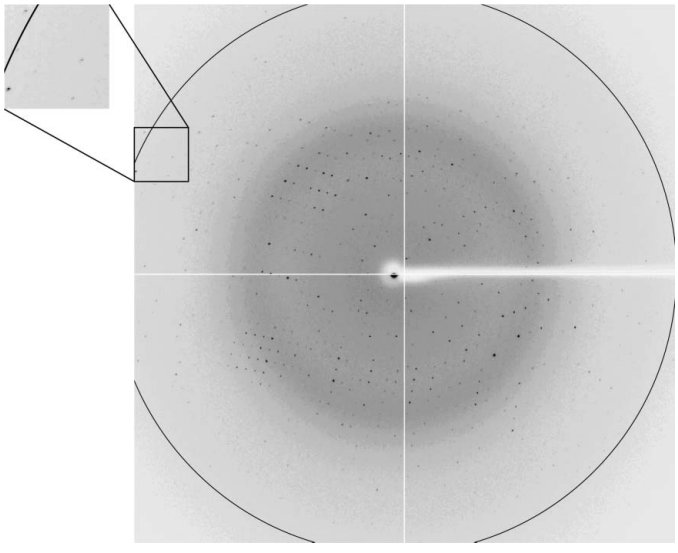

Figure 2
X-ray diffraction image (0.5° oscillation) from a LECT2^{SeMet} crystal. The data set was analysed at 1.94 Å resolution (indicated by the circle).

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