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## Crystallization and preliminary crystallographic study of oligomers of the haemolytic lectin CEL-III from the sea cucumber *Cucumaria echinata*

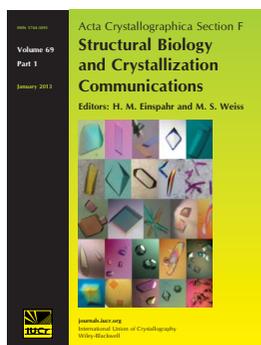
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## Crystallization and preliminary crystallographic study of oligomers of the haemolytic lectin CEL-III from the sea cucumber *Cucumaria echinata*

CEL-III is a  $\text{Ca}^{2+}$ -dependent haemolytic lectin isolated from the marine invertebrate *Cucumaria echinata*. This lectin binds to Gal/GalNAc-containing carbohydrate chains on the cell surface and, after conformational changes, oligomerizes to form ion-permeable pores in cell membranes. CEL-III also forms soluble oligomers similar to those formed in cell membranes upon binding of specific carbohydrates in high-pH and high-salt solutions. These soluble and membrane CEL-III oligomers were crystallized and X-ray diffraction data were collected. Crystals of soluble oligomers and membrane oligomers diffracted X-rays to 3.3 and 4.2 Å resolution, respectively, using synchrotron radiation and the former was found to belong to space group C2. Self-rotation functional analysis of the soluble oligomer crystal suggested that it might be composed of heptameric CEL-III.

### 1. Introduction

The sea cucumber *Cucumaria echinata* contains four  $\text{Ca}^{2+}$ -dependent, Gal/GalNAc-specific lectins (CEL-I–IV) (Hatakeyama *et al.*, 1994). While CEL-I and CEL-IV are C-type lectins, composed of two and four C-type carbohydrate-recognition domains, respectively (Hatakeyama, Ohuchi *et al.*, 1995; Hatakeyama *et al.*, 2002), CEL-III has been found to belong to the ricin (R)-type lectins, which show sequence similarities with the B-chain of ricin, a toxic plant lectin (Villafranca & Robertus, 1981; Araki & Funatsu, 1987). CEL-III shows strong haemolytic activity towards erythrocytes as well as cytotoxicity towards some cultured cell lines (Oda *et al.*, 1997; Hatakeyama, Nagatomo *et al.*, 1995). These activities manifest in target cell membranes through the formation of ion-permeable pores, composed of CEL-III oligomers inserted in the membrane (Hatakeyama *et al.*, 1996; Kouzuma *et al.*, 2003; Kouriki-Nagatomo *et al.*, 1999). The crystal structure of CEL-III monomer has been solved (Uchida *et al.*, 2004; PDB entry 1vcl) and it was found to be composed of three domains, in which domains 1 and 2 have  $\beta$ -trefoil folds containing the QXW (Gln–X–Trp) motif (Hazes, 1996) in their carbohydrate-binding sites (Hatakeyama *et al.*, 2007; PDB entries 2z48 and 2z49), resembling the B-chain of ricin. Domain 3 possesses a unique fold, composed of long  $\beta$ -strands and two  $\alpha$ -helices. These  $\alpha$ -helices are accommodated in a cleft formed between domains 1 and 2, stabilizing the association between these domains and domain 3. Previous studies have suggested that binding of CEL-III to the cell surface carbohydrate chains *via* its domains 1 and 2 triggers an extensive conformational change in domain 3, leading to its oligomerization (Kouzuma *et al.*, 2003). The hydrophobic region in domain 3, including two  $\alpha$ -helices H8 and H9 (residues 320–354), is supposed to play an important role in such an oligomerization process (Hisamatsu *et al.*, 2008, 2009).

While CEL-III forms oligomers composed of hexamers or heptamers in cell membranes during haemolytic action, structures of similar size have also been found to form in solution under conditions of high pH and high salt concentration, in which the protein-bound disaccharides contain  $\beta$ -galactoside structures, such as lactose (Gal $\beta$ 1-4Glc) and lactulose (Gal $\beta$ 1-4Fru) (Hatakeyama *et al.*, 1996; Kuwahara *et al.*, 2002). This finding suggests that the oligomerization



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process is triggered by binding to  $\beta$ -galactoside-containing carbohydrate chains on the cell surface and is further promoted by a hydrophobic environment on cell membrane surfaces. It appears likely that high salt concentration and high pH conditions facilitate conformational changes of domain 3 in place of the hydrophobic environment on the cell membrane.

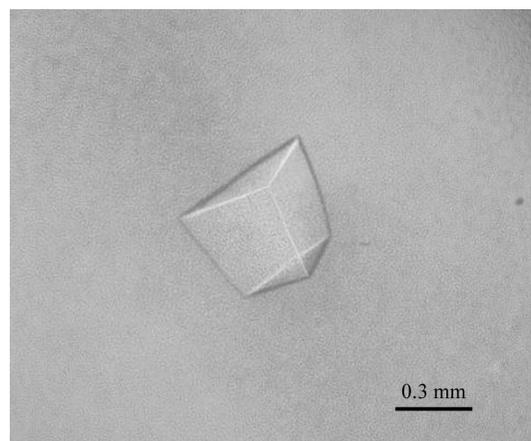
For elucidation of CEL-III's haemolytic mechanism, crystals of CEL-III oligomers, formed in erythrocyte membranes as well as in solution, were prepared and preliminary crystallographic analyses performed.

## 2. Materials and methods

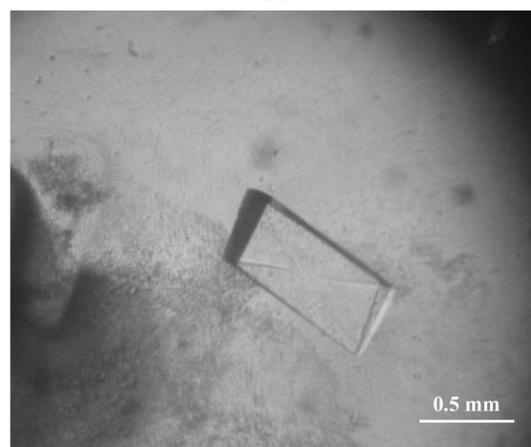
### 2.1. Purification and oligomerization of CEL-III

CEL-III was extracted from the body fluid of *C. echinata* and purified by affinity column chromatography using lactose-Cellulofine and GalNAc-Cellulofine columns, followed by a gel-filtration column, as previously reported (Hatakeyama *et al.*, 1996).

Soluble CEL-III oligomer was prepared essentially as previously described (Hatakeyama *et al.*, 1996). Briefly, CEL-III ( $2 \text{ mg ml}^{-1}$ ) was incubated in the presence of  $1 \text{ M NaCl}$ ,  $0.1 \text{ M glycine-NaOH pH } 10$ ,  $0.01 \text{ M CaCl}_2$ ,  $0.1 \text{ M lactulose}$  at  $298 \text{ K}$  for  $2 \text{ h}$ . As X-ray scattering analyses have shown that the resulting CEL-III oligomers, composed of six or seven monomers, mostly associate as core oligomers to produce larger formations of  $\sim 21$ -mer with relatively weak inter-



(a)



(b)

**Figure 1**  
Crystals of the soluble oligomer (a) and the membrane oligomer (b) of CEL-III.

**Table 1**

Data-collection statistics.

Values in parentheses are for the highest shell.

Space group	C2
Unit-cell parameters	
$a$ (Å)	218.3
$b$ (Å)	230.7
$c$ (Å)	133.6
$\beta$ (°)	126.9
Wavelength (Å)	1.000
Resolution (Å)	50.0–3.30 (3.36–3.30)
Total reflections	557027
Unique reflections	79710
$\langle I/\sigma I \rangle$	21.3 (5.1)
Redundancy	7.0 (7.0)
Completeness (%)	98.7 (100)
$R_{\text{merge}}^\dagger$ (%)	9.6 (48.5)

$^\dagger R_{\text{merge}} = 100 \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$ , where  $I_i(hkl)$  is the observed intensity and  $\langle I(hkl) \rangle$  is the average intensity of multiple observations of symmetry-related reflections.

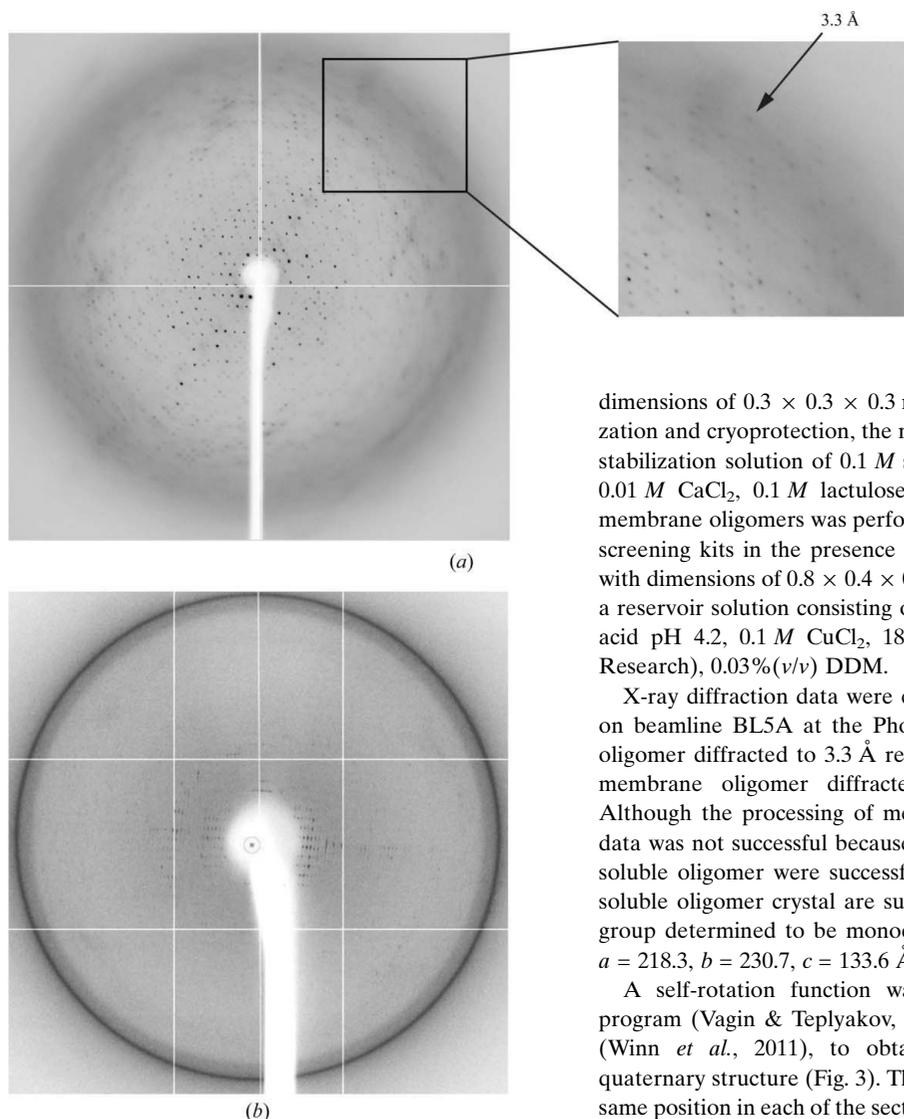
actions (Fujisawa *et al.*, 1997), they were then treated with  $0.1\%$  of *n*-dodecyl- $\beta$ -D-maltoside (DDM), to force dissociation into core oligomers, and dialysed against  $0.01 \text{ M Tris-HCl pH } 7.6$ ,  $0.15 \text{ M NaCl}$ . The CEL-III oligomers were next concentrated to  $12 \text{ mg ml}^{-1}$  using a  $100 \text{ kDa}$  cutoff ultrafiltration membrane (Vivaspin, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and stabilized by dialysis against  $0.01 \text{ M Tris-HCl pH } 7.6$ ,  $0.15 \text{ M NaCl}$ ,  $0.01 \text{ M CaCl}_2$ ,  $0.1 \text{ M lactulose}$ ,  $0.5\%$  DDM at  $293 \text{ K}$  for  $1 \text{ h}$ .

Membrane oligomers were prepared from the oligomers formed in erythrocyte membranes by treatment with CEL-III. For this, CEL-III was incubated with rabbit erythrocyte suspension [ $10\%$  (v/v)] in  $0.01 \text{ M Tris-HCl pH } 7.5$ ,  $0.15 \text{ M NaCl}$ ,  $0.01 \text{ M CaCl}_2$  at  $277 \text{ K}$  for  $1 \text{ h}$  for cell lysis. After centrifugation of the resulting suspension, the pelleted membrane fraction was washed three times with  $0.01 \text{ M Tris-HCl pH } 7.5$ ,  $0.15 \text{ M NaCl}$  by centrifugation and then solubilized with  $2\%$  (v/v) of Triton X-100 in  $0.01 \text{ M Tris-HCl pH } 7.5$ ,  $0.15 \text{ M NaCl}$  at  $277 \text{ K}$  for  $1 \text{ h}$ . After another centrifugation, solubilized oligomers were purified on a lactose-Cellulofine column in  $0.01 \text{ M Tris-HCl pH } 7.5$ ,  $0.15 \text{ M NaCl}$ ,  $0.03\%$  (v/v) DDM.

### 2.2. Crystallization of CEL-III oligomers

Purified CEL-III oligomers were crystallized using a sitting-drop vapour-diffusion method. The initial screening for crystallization of the soluble oligomer was done using the screening kits Crystal Screen, Crystal Screen 2 (Hampton Research, Aliso Viejo, California, USA) and Wizard I and II (Emerald BioSystems, Inc., Bedford, Massachusetts, USA). In the initial screening of crystallization,  $1 \mu\text{l}$  of protein solution ( $10 \text{ mg ml}^{-1}$ ) in buffer consisting of  $0.01 \text{ M Tris-HCl pH } 7.6$ ,  $0.15 \text{ M NaCl}$ ,  $0.01 \text{ M CaCl}_2$ ,  $0.1 \text{ M lactulose}$ ,  $0.5\%$  (v/v) DDM was mixed with an equal volume of reservoir solution and allowed to equilibrate against  $100 \mu\text{l}$  of the reservoir solution at  $293 \text{ K}$ . As crystals could be obtained under several variable conditions, crystallization was optimized by employing various solution volumes, precipitant concentrations and pH levels.

Initial screening for crystallization of the membrane oligomer was carried out using the above screening kits in the presence of DDM. Here,  $1 \mu\text{l}$  of the membrane oligomer solution in  $0.01 \text{ M Tris-HCl pH } 7.5$ ,  $0.15 \text{ M NaCl}$  containing  $0.01 \text{ M CaCl}_2$  and  $0.1 \text{ M lactulose}$  was mixed with an equal volume of the reservoir solution containing  $0.03\%$  (v/v) DDM and allowed to equilibrate against  $0.3 \text{ ml}$  of reservoir solution at  $293 \text{ K}$ .



**Figure 2**  
X-ray diffraction patterns of the soluble oligomer (a) and the membrane oligomer (b) crystals of CEL-III.

### 2.3. Data collection and processing

Data collection from CEL-III oligomer crystals was performed on beamline BL5A of the Photon Factory at the High Energy Acceleration Research Organization (Tsukuba, Japan), using an ADSC Quantum 210r and 315r CCD detector (Area Detector Systems Corp., Poway, California, USA). Image data were processed using the *HKL-2000* program (Otwinowski & Minor, 1997).

## 3. Results and discussion

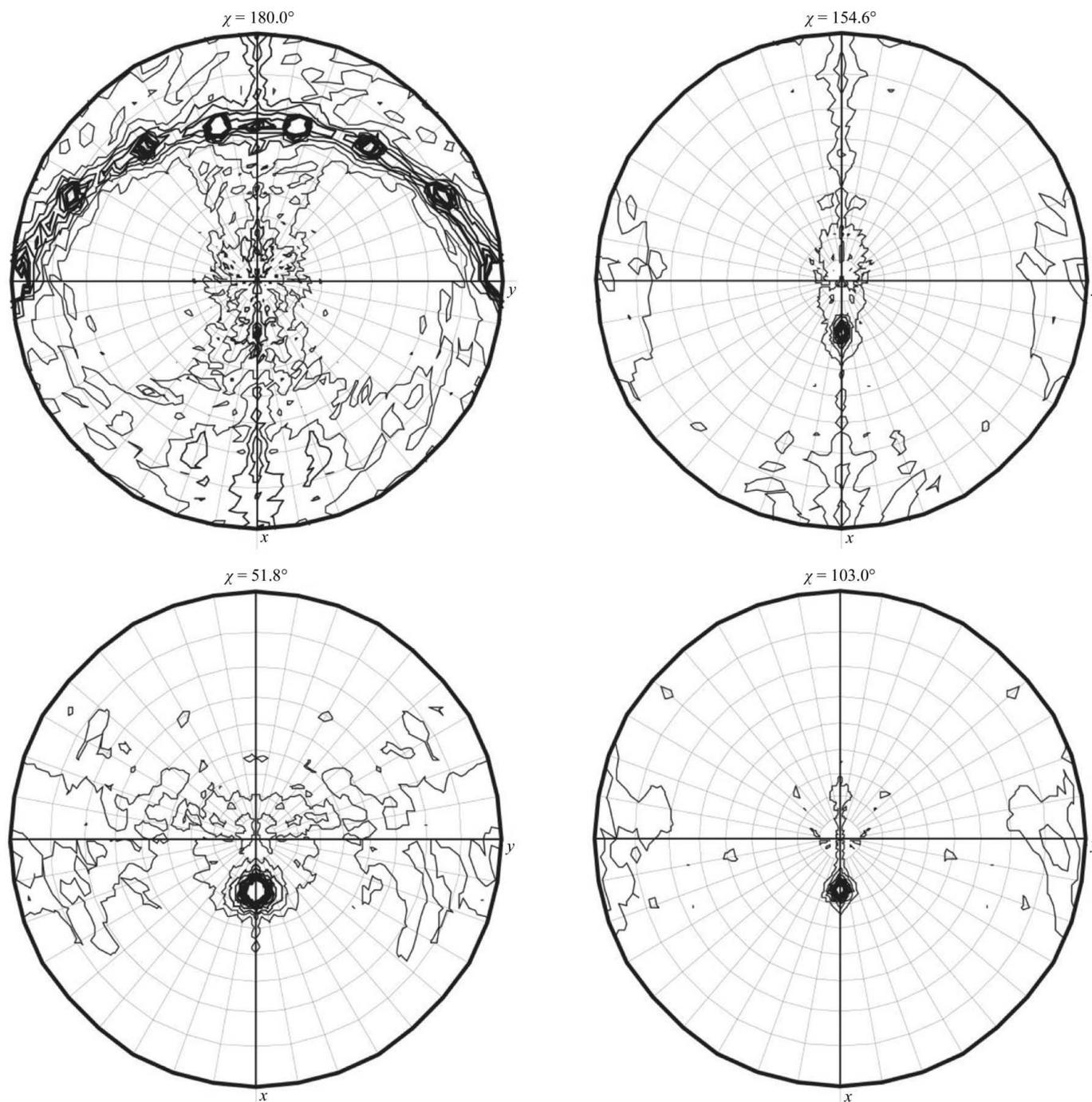
### 3.1. Crystallization of CEL-III oligomers and data collection

Rhomboid crystals of the soluble oligomer (Fig. 1a) were obtained using a reservoir solution consisting of 30% (v/v) PEG 400, 0.1 M CdCl<sub>2</sub>, 0.1 M sodium acetate pH 4.6. After condition optimization, 4–6 μl of protein solution (10 mg ml<sup>-1</sup>) in buffer consisting of 0.1 M Tris–HCl pH 7.6, 0.15 M NaCl, 0.01 M CaCl<sub>2</sub>, 0.1 M lactulose, 0.5% DDM was mixed with 2 μl of a solution consisting of 0.1 M sodium acetate pH 4.2, 0.1 M CdCl<sub>2</sub>, 30% PEG 400 and equilibrated by vapour diffusion against the same solution. Single crystals with

dimensions of 0.3 × 0.3 × 0.3 mm grew within 2 weeks. For stabilization and cryoprotection, the mother liquor was replaced by a cryo-stabilization solution of 0.1 M sodium acetate pH 4.2, 0.1 M CdCl<sub>2</sub>, 0.01 M CaCl<sub>2</sub>, 0.1 M lactulose, 35% PEG 400. Crystallization of membrane oligomers was performed using the previously mentioned screening kits in the presence of 0.03% DDM, resulting in crystals with dimensions of 0.8 × 0.4 × 0.2 mm (Fig. 1b) after 3 months, using a reservoir solution consisting of 0.1 M HEPES pH 7.5, 0.1 M acetic acid pH 4.2, 0.1 M CuCl<sub>2</sub>, 18% (v/v) Jeffamine M-600 (Hampton Research), 0.03% (v/v) DDM.

X-ray diffraction data were collected using synchrotron radiation on beamline BL5A at the Photon Factory. Crystals of the soluble oligomer diffracted to 3.3 Å resolution (Fig. 2a), while those of the membrane oligomer diffracted to 4.2 Å resolution (Fig. 2b). Although the processing of membrane oligomer crystal diffraction data was not successful because of poor-quality results, those of the soluble oligomer were successfully analysed. Data statistics for the soluble oligomer crystal are summarized in Table 1, with the space group determined to be monoclinic *C2* and unit-cell parameters of  $a = 218.3$ ,  $b = 230.7$ ,  $c = 133.6$  Å,  $\beta = 126.9^\circ$ .

A self-rotation function was computed, using the *MOLREP* program (Vagin & Teplyakov, 2010) from the *CCP4* program suite (Winn *et al.*, 2011), to obtain information on the oligomer's quaternary structure (Fig. 3). The results showed a single peak at the same position in each of the sections of  $\chi = 51.8$ , 103.0 and 154.6°, and seven peaks in the section of  $\chi = 180^\circ$ . The peaks in the sections of  $\chi = 51.8$ , 103.0 and 154.6° nearly corresponded to a sevenfold axis, and the peak in the section of  $\chi = 180^\circ$  corresponded to seven twofold axes located perpendicular to the sevenfold axis. These symmetric properties suggested that the oligomer formed a heptameric ring-like shape including one sevenfold axis and seven pseudo-twofold axes perpendicular to the sevenfold axis. Pore-forming oligomers of several bacterial toxins are known to be heptamers, such as in *Staphylococcus aureus*  $\alpha$ -haemolysin (Song *et al.*, 1996), protective antigen of anthrax toxin from *Bacillus anthracis* (Lacy *et al.*, 2004) and cytotoxin from *Vibrio cholerae* (De & Olson, 2011). The sevenfold rotation symmetry found in the present CEL-III oligomer crystal might also have been an indication of heptameric structure. Although it is still unclear whether the membrane oligomer possessed an oligomeric structure similar to the soluble oligomer, their similar sizes on SDS–PAGE (Hatakeyama *et al.*, 1996; Kuwahara *et al.*, 2002) strongly suggest that they shared almost identical oligomeric structures. The differences in the diffraction patterns of the soluble and membrane oligomers would have been due to differences in their preparation conditions. It is also possible that some membrane lipids, including glycolipids, that originated from erythrocytes may have remained bound to the membrane oligomer and thus affected the crystallization conditions and quality of the diffractions. Further analyses of CEL-III oligomer crystals are in progress.



**Figure 3**

Self-rotation function peaks calculated by *MOLREP*. Sections of  $\chi = 51.8, 103.0, 154.6$  and  $180.0^\circ$  shown.

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