Small-angle X-ray scattering study on CEL-III, a hemolytic lectin from Holothuroidea *Cucumaria echinata*, and its oligomer induced by the binding of specific carbohydrate

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Abstract **Hemolytic lectin CEL-III from a marine invertebrate *Cucumaria echinata* forms an oligomer upon binding of specific carbohydrate such as lactose at high pH values and in the presence of high concentrations of salt. In this study, using small-angle X-ray scattering, we characterized CEL-III and its oligomer induced by the binding of lactose. The molecular mass of the oligomer was determined as 1019 kDa from its forward scattering value, compared with 47490 Da for the monomer. This oligomer size is much larger than that estimated using SDS–polyacrylamide gel electrophoresis (SDS-PAGE, 270 kDa). The monomer has a 24.6 Å radius of gyration and can be approximated by a rod which has a 20 Å radius and a height of 75 Å, while the oligomer has a 101.4Å radius of gyration. Together with the comparison of the radii of gyration and the forward scattering of the cross-section of the monomer and oligomer, it is suggested that in aqueous solution the oligomer comprises three or four molecules of a smaller unit which was observed by SDS-PAGE (270 kDa), held by a relatively weak interaction. The scattering profile also suggests that the oligomer has a hole in its central axis which might be associated with the formation of ion-permeable pores in the erythrocyte membrane by CEL-III during the hemolytic process.**

Key words: Small-angle X-ray scattering; Lectin; Carbohydrate-binding protein; Hemolysin; Toxin

1. Introduction

CEL-III is a Ca^{2+}-dependent, Gal/GalNAc-specific lectin purified from Holothuroidea *Cucumaria echinata*, which shows hemolytic activity, especially toward human and rabbit erythrocytes [1]. We have recently found that this lectin also exhibits strong cytotoxicity toward some cultured cells such as MDCK, HeLa, and Vero cells [2]. Hemolysis is caused by the colloid osmotic rupture of the erythrocyte membrane due to the formation of ion-permeable pores by CEL-III oligomer after it has bound to carbohydrate receptors on the cell surface. The results from osmotic protection experiments using dextran with a molecular mass of over 4000 suggest that the functional radii of the pores formed by CEL-III oligomers may be smaller than 18 Å [3]. Formation of pores in the cell membrane has also been reported in the case of several bacterial toxins such as Staphylococcal α-toxin [4–9], aerolysin [10–12] and the protective antigen of the anthrax toxin [13]. Interestingly, these toxins form pores comprising toxin heptamer in the target cell membrane after their activation [14–17]. Oligomerization of these toxins can be induced not only in lipid membranes but also in solution under the appropriate conditions. For example, the Staphylococcal α-toxin oligomerizes in the presence of detergent micelles [18,19]. We found that CEL-III also forms an oligomer in solution when complexed with lactose at high pH values and in the presence of high concentrations of salt, e.g. at pH 10 and with 1 M NaCl [20]. This suggests that such non-physiological conditions may promote a conformational change triggered by the binding of the specific carbohydrate, even in aqueous solutions without a cell membrane. The resulting oligomer was similar to that formed in the erythrocyte membrane treated with CEL-III in that it had a similar size as judged by SDS-PAGE and that the oligomer only formed when complexed with specific carbohydrates such as lactose, lactulose and *N*-acetyllactosamine [20]. These findings suggest that the oligomerization of CEL-III in solution at least partly reflects the process occurring in the cell membrane, and it could be utilized as a model system for the elucidation of the hemolytic mechanism of CEL-III. Here we report the molecular parameters for CEL-III and its oligomer determined by small-angle X-ray scattering (SAXS) in order to characterize further the CEL-III oligomer induced in solution by the binding of a specific carbohydrate.

2. Materials and methods

2.1. Purification of CEL-III

The *Cucumaria echinata* samples were provided by the Fukuoka Fisheries and Marine Technology Research Center. CEL-III was purified from the body fluid of *C. echinata* using column chromatography on lactosyl-Cellulofine, GalNAc-Cellulofine, and Sephacryl S-200 as previously described [3]. Chromatography was performed at 4°C. The purified protein was dialyzed against 10 mM Tris-HCl containing 0.15 M NaCl, pH 7.5 (TBS), and stored frozen at −30°C until use.

2.2. Protein determination

The protein concentration was calculated based on an absorbance value of 1.4 at 280 nm for a 0.1% (w/v) protein solution in TBS, which had been determined with bicinchoninic acid according to the method of Smith et al. [21] using bovine serum albumin as a standard.

2.3. Preparation of the CEL-III oligomer

The CEL-III oligomer was prepared by incubating it with 10 mM

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Abbreviations: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PAGE, polyacrylamide gel electrophoresis; SAXS, small-angle X-ray scattering; TBS, Tris-buffered saline

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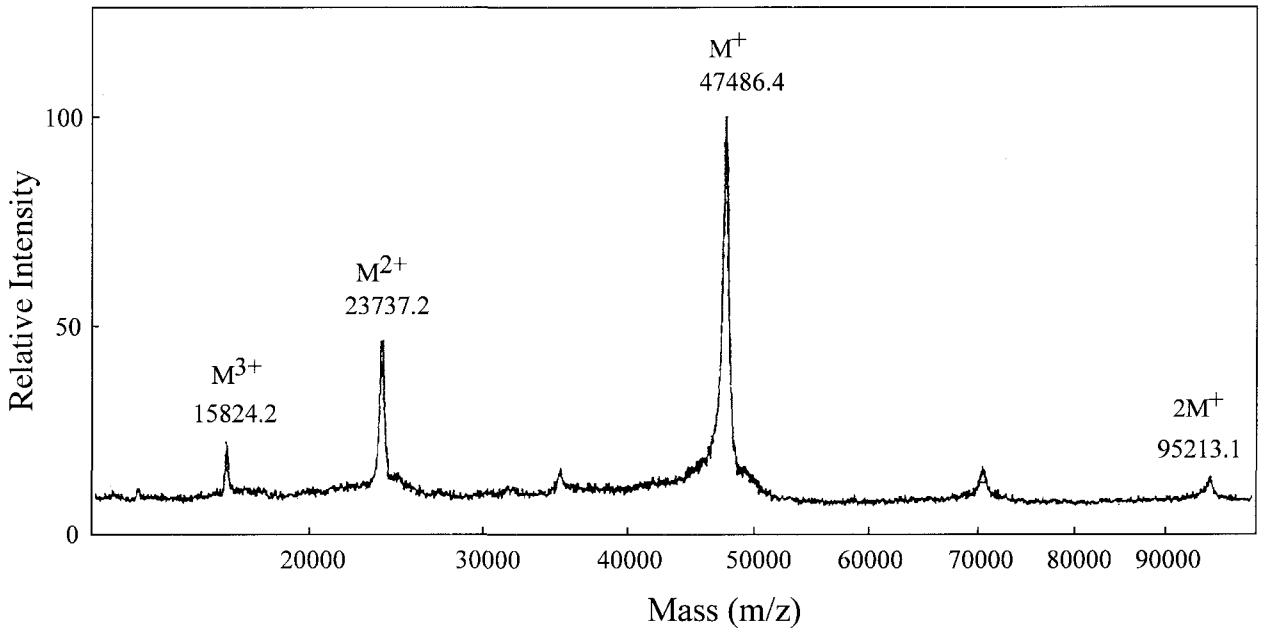

Fig. 1. MALDI-TOF mass spectrometry of CEL-III. Determined after mixing the protein with DHB as a matrix.

lactose at 20°C for 30 min in 20 mM glycine–NaOH buffer, 1 M NaCl, pH 10. The remaining small amount of monomer was removed by gel filtration on a Sephacryl S-300 column (2×40 cm).

2.4. Mass spectrometry

The mass spectrum was obtained using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Finnigan Mat Vision 2000) in linear mode at accelerating voltage of 20 kV. Bovine serum albumin was used as an external standard. Ionization was achieved with a nitrogen laser (337 nm) using 2,5-dihydroxybenzoic acid (DHB) as a matrix.

2.5. Small angle X-ray scattering

The data were collected using the small-angle scattering camera on BL-10C at the Photon Factory, Tsukuba, Japan [22], at two different camera lengths (80 and 200 cm) whose distances were calibrated by meridional reflection of dry collagen. The wavelength used was 1.488 Å. The five sample solutions (1.4–14 mg/ml) were used to avoid interparticle interference. The data were normalized to the intensity of the incident beam and the buffer was subtracted. The detailed data processing is described in another article [23].

The distance distribution function $P(r)$ was calculated using the indirect transformation program package GNOM [24]. The maximum dimension D_{max} was determined from the first zero cross-point of the $P(r)$ function. The radii of gyration were obtained using the ln $(I(S))$ vs. S^2 plot, the so-called Guinier plot [25], where $S = 2\sin\theta/\lambda$. 2θ is the scattering angle and $I(S)$ is the scattering intensity at S. The fitting ranges used for Rg determination were 20×10^{-6} Å$^{-2} < S^2 < 200\times10^{-6}$ Å^{-2} and 1×10^{-6} Å$^{-2} < S^2 < 10\times10^{-6}$ Å^{-2} for the monomer and the oligomer, respectively. The radii of gyration of the cross-sections Rc were obtained using the ln$[I(S)\cdot S]$ vs. S^2 plot, the so-called cross-section plot [26].

3. Results and discussion

Since the precise molecular mass of CEL-III monomer was required for comparison with the oligomer form, we performed MALDI-TOF mass spectrometry for the determination of the molecular mass of the CEL-III monomer. As shown in Fig. 1, the mass spectrum clearly showed three charged molecular ion peaks derived from the CEL-III monomer (M^+, M^{2+}, and M^{3+}). From the singly charged molecular ion, the molecular mass of the CEL-III monomer was found to be 47490 Da, which is a rather larger value than that previously estimated by SDS-PAGE (45 kDa) [1]. A small peak, probably corresponding to the CEL-III dimer, was also observed at m/z 95213.1.

The CEL-III oligomer was prepared by incubating the protein with 10 mM lactose in 20 mM glycine–NaOH buffer, 1 M NaCl, pH 10, under which conditions the conformational change of CEL-III was promoted, leading to oligomerization [20]. Since this preparation contained a small amount of monomer protein, the oligomer fraction was separated by gel filtration using a Sephacryl S-300 column. The structural parameters obtained from SAXS of the CEL-III monomer

Table 1
Structural parameters for CEL-III monomer and oligomer

		Monomer	S.D.	Oligomer	S.D.
Radius of gyration	Rg	24.6 Å	0.4 Å	101.4 Å	1.0 Å
Forward scattering	$I(0)/C$	1340	40	28760	460
Radius of gyration of the cross-section	Rc	15.1 A	0.1 Å	66.5 Å	0.1 Å
Forward scattering of the cross-section	$Ic(0)/C$	10.5	1.0	57.4	1.6
Maximum dimension	D_{max}	75 Å		290 Å	
Two-axis ellipsoid resolution[a]	short×long axis	22×38 Å		96×145 Å	
Cylindrical resolution[a]	radius×height	20×75 Å		88×281 Å	

[a]For model fitting $S < 0.004$ Å^{-1} and $S < 0.016$ Å^{-1} for monomer and oligomer, respectively.

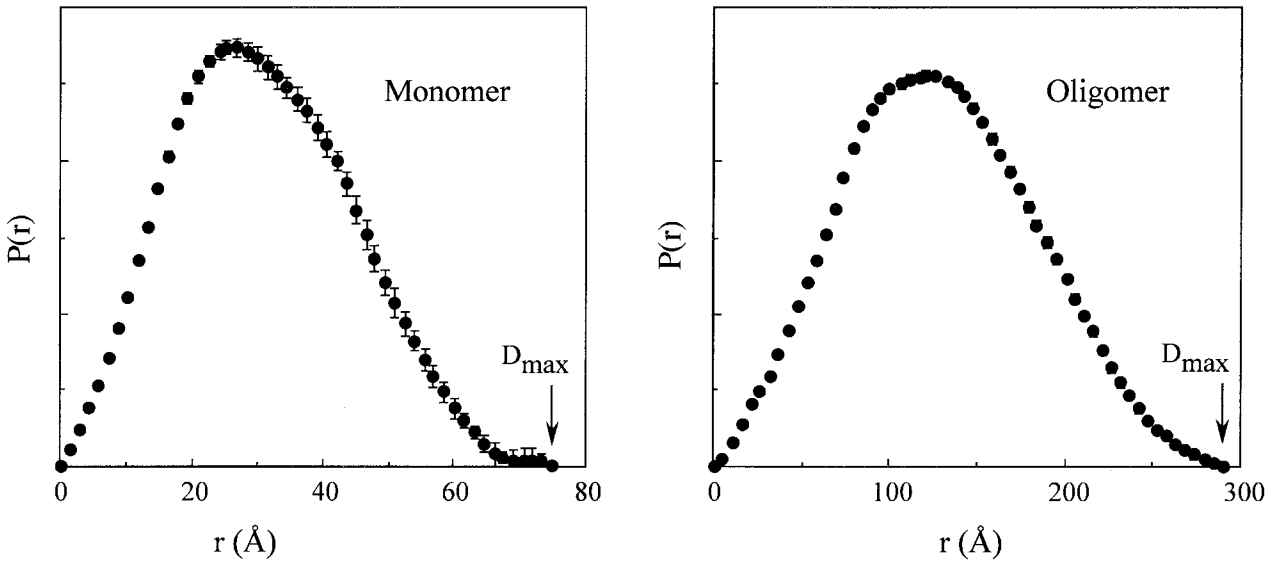

Fig. 2. The distance distribution function of the CEL-III monomer and oligomer.

and oligomer are summarized in Table 1. The forward scattering $I(0)/C$ is proportional to the molecular mass. Since the molecular mass of the monomer was determined as 47490 Da as described above, the molecular mass of the oligomer was estimated to be 1019 kDa. This value is much larger than that previously determined by SDS-PAGE (270 kDa) [20]. The D_{max} determined from the $P(r)$ function (Fig. 2) and Rg values also suggests that the oligomer molecule has a much larger size in solution than that observed by SDS-PAGE. Although our preliminary experiment suggests that the oligomer corresponds to a hexamer or a heptamer when compared by SDS-PAGE with some cross-linked CEL-III preparations (unpublished results), ultracentrifugal analysis indicates that this oligomer is much larger in solution in the absence of SDS, giving a sedimentation coefficient of 37S [20]. As revealed in the present study, the oligomer induced by lactose is estimated to be 1019 kDa from its $I(0)/C$ values, which corresponds to a 21-mer of the monomer protein. This indicates that the oligomer comprises three or four molecules of a smaller unit (hexamer or heptamer) which is resistant to SDS, held by a relatively weak interaction in aqueous solution.

Although the cross-section plot $\ln(I(S)\cdot S)$ vs. S^2 is theoretically valid only for a cylinder of infinite length, it could be applicable for the determination of the radius of gyration of the cross-section (Rc) in a $1.5 < 2\pi SRc < 2.8$ globular particle with an axial ratio of 1.5 (Fujisawa, T., unpublished data). Therefore, it can be expected to provide some clues to sectional information relating to CEL-III structures (Fig. 3). The $Ic(0)/C$, which is proportional to the unit-length mass are 10.5 and 57.4 for the monomer and the oligomer, respectively. This suggests that the oligomer is larger than the monomer by a factor of 5.5, which is about 6 in terms of cross-sectional density.

The molecular dimensions were also evaluated based on the simplest model. In the model building, the fitted ranges were automatically determined by the number of parameters to be determined. In the case of a two-axial ellipsoid or a solid cylinder, $S < 0.016$ Å^{-1} and $S < 0.004$ Å^{-1} for the monomer and the oligomer, respectively. Table 1 shows the parameters for the two-axial ellipsoid and cylindrical models obtained by least-square fit. As seen in Fig. 4, it is very difficult to distinguish between the ellipsoid and the cylindrical models in the fitted ranges mentioned above, whereas beyond the fitted region the latter seems better than the former. The dimensions of both models are consistent with the D_{max} values.

Since Rc uses larger scattering angle region, the comparison between the radius of the cylindrical model (R) and Rc may give further information relating to structure; R should equal to $\sqrt{2}Rc$ for a solid cylinder [26]. In the case of the monomer, $Rc = 15.1$ Å corresponds to $R = 21.4$ Å, which is close to the value of R determined from the least-square fit (20 Å) in the model. However, in the case of the oligomer, the cylindrical model radius gives $R = 88$ Å, which is rather smaller than the experimental data $\sqrt{2}Rc = 94.0$ Å. It could be rationalized if we assume a hole in the central axis and a density distributed off axis; Rc then becomes large. Interestingly, $Ic(0)/C$ shows that the cross-sectional density of the oligomer is six times as large as that of the monomer, suggesting that the oligomer consists of three or four units of hexamer associates with longitudinal

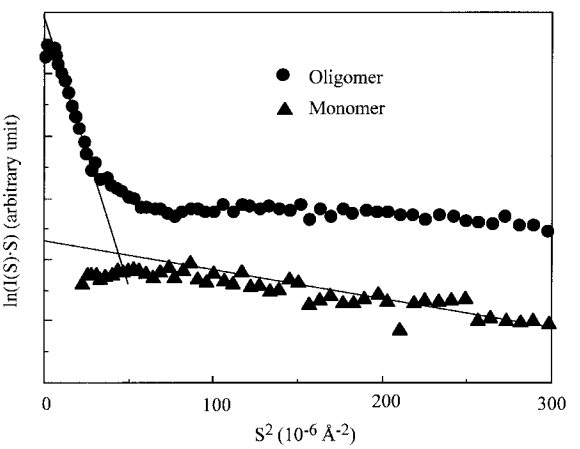

Fig. 3. Cross-section plot for the CEL-III monomer and oligomer. The fitted regions used for Rc determination were 8×10^{-6} Å$^{-2} < S^2 < 20\times10^{-6}$ Å^{-2}, 180×10^{-6} Å$^{-2} < S^2 < 300\times10^{-6}$ Å^{-2} for the oligomer and monomer, respectively.

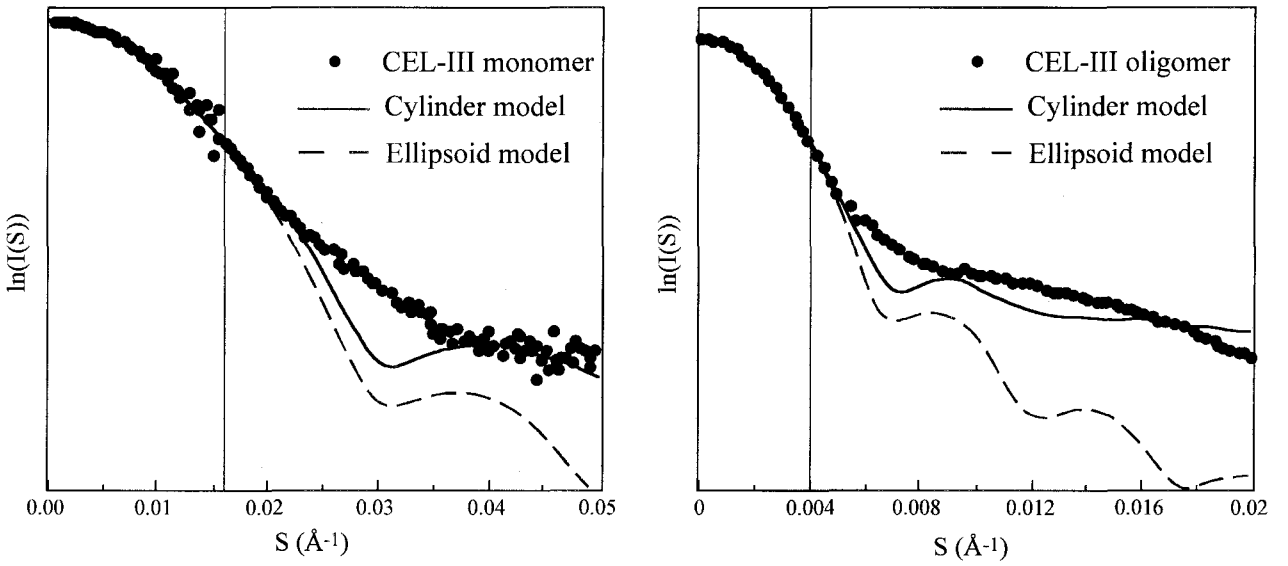

Fig. 4. Ellipsoid and cylindrical fittings to SAXS of the CEL-III monomer and oligomer. Vertical lines indicate the upper fitted region.

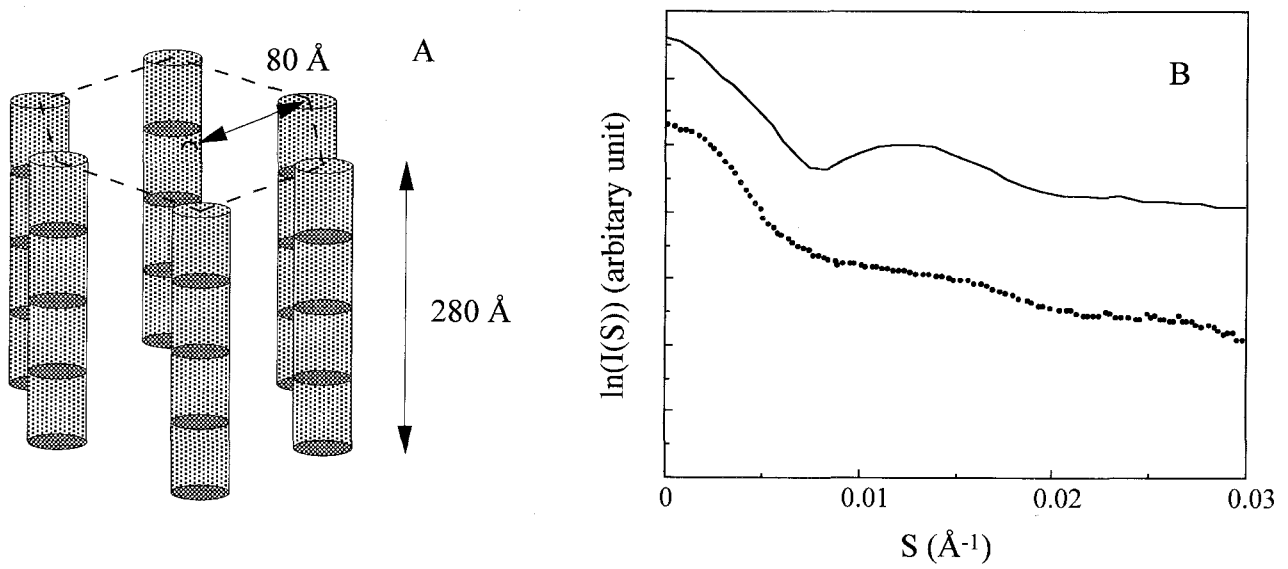

Fig. 5. The hexagonal model for the CEL-III oligomer. A: A simple model consisting of six rods positioned at the edges of a hexagon. One rod has a radius of 20 Å and a height of 280 Å. One side of the hexagon is 80 Å. B: Comparison of SAXS of the model (solid line) and experimental data (dotted line). The scattering characteristic, a ripple at 0.013 $Å^{-1}$ is well reproduced with this model.

direction. The D_{max} values are also consistent with that model. Since the monomer molecule can be approximated well with a solid cylinder with a radius of 20 Å and height of 75 Å, a very simple model is considered; six rods, each comprising three or four monomers associated in the longitudinal direction, are positioned at the edges of a hexagon. The scattering from this model structure is shown in Fig. 5, which shows a characteristic of CEL-III oligomer scattering profile, i.e. a ripple at 0.013 $Å^{-1}$. Such a hollow structure of the oligomer might also be associated with the ion-permeable pores which are formed in the erythrocyte membrane by CEL-III during the hemolytic process. More detailed structural analysis of the CEL-III oligomer applying the interference function of the monomers is under way.

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