Roles of the Valine Clusters in Domain 3 of the Hemolytic Lectin CEL-III in Its Oligomerization and Hemolytic Abilities

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Running title: Roles of valine clusters in hemolytic lectin CEL-III

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Abstract: The hemolytic lectin CEL-III and its site-directed mutants were expressed in *Escherichia coli* cells. Replacement of the valine clusters in domain 3 with alanine residues led to increased self-oligomerization in solution and higher hemolytic activity. The results suggest the involvement of these valine clusters in CEL-III oligomerization and hemolytic activity.

Key words: *Cucumaria echinata*, hemolysin, lectin, oligomerization, site-directed mutagenesis.

**ABBREVIATIONS**

EDTA = Ethylenediamine tetraacetate  
TBS = Tris-buffered saline  
CD = Circular dichroism  
GST = Glutathione S-transferase
INTRODUCTION

CEL-III is a Ca$^{2+}$-dependent Gal/GalNAc-specific lectin isolated from the sea cucumber *Cucumaria echinata*, which exhibits strong hemolytic and cytotoxic activities by forming ion-permeable pores composed of its oligomers in target cell membranes [1-4]. This lectin is composed of three domains, among which domains 1 and 2 function as carbohydrate-binding domains and domain 3 acts as an oligomerization domain [5-8]. Although domains 1 and 2 adopt β-trefoil folds similar to the B-chain of the toxic lectin ricin [9, 10], there is a conspicuous difference between these lectins since CEL-III contains five Ca$^{2+}$ ions at its carbohydrate-binding domains 1 and 2, which bind to galactose by forming coordinate bonds with its 3- and 4-OH groups [8]. On the other hand, domain 3 has a novel fold including two α-helices and one β-sandwich structure, and spontaneously oligomerizes by strong hydrophobic interactions following separation from domains 1 and 2 [6]. The two α-helices in domain 3 are situated at the interface of the three domains, and contain several hydrophobic amino acid residues, including two valine clusters [11]. Therefore, it seems highly likely that the α-helix region in domain 3 plays an important role in the oligomerization in target cell membranes. In fact, we previously found that synthetic peptides based on the sequence
of this α-helix region (residues 317-357) formed ion channels through the formation of β-sheet structure in lipid membranes [12]. Moreover, glutathione S-transferase (GST) fused with these α-helix region peptides showed self-oligomerization ability in solution [11]. These results suggest that the α-helix region has a strong tendency to oligomerize by forming β-sheet structure. In the present study, we expressed recombinant CEL-III (rCEL-III) and its site-directed mutants in *Escherichia coli* cells to further investigate the roles of the amino acid residues in domain 3, especially the two valine clusters as hydrophobic segments, in the oligomerization process.

**MATERIALS AND METHODS**

**Expression and Purification of rCEL-III**

Wild-type (WT) rCEL-III DNA was amplified by PCR using a CEL-III cDNA [5] as a template and the oligonucleotides 5’-CATATGCAAGTTTTGTGCACGAATCC-3’ and 5’-GGATCCTCAAATGTCCGTGCAGAAAG-3’ as forward and reverse primers, respectively. The amplified fragment was cloned into *E. coli* JM109 using the pGEM-T vector (Promega). The inserted gene was digested with the restriction enzymes NdeI and BamHI, and then ligated with the pET-3a vector (Novagen) that had previously
been digested with the same enzymes. The resulting plasmid containing WT-rCEL-III gene was used for transformation of \textit{E. coli} BL21(DE3)pLysS (Novagen). Protein expression was induced with 1 mM isopropylthiogalactoside, and the cells were incubated for an additional 5 h at 37°C. The expressed protein was obtained in inclusion bodies after disruption of the cells by sonication. Therefore, the inclusion bodies were solubilized in solubilization buffer (50 mM Tris-HCl pH 8.0, 0.2 M NaCl, 1 mM ethylenediamine tetraacetate (EDTA), 6 M guanidine hydrochloride) and the protein was refolded in refolding buffer (0.1 M Tris-HCl pH 8.0, 0.4 M L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.1 mM phenylmethylsulfonyl fluoride) [13]. After dialysis against Tris-buffered saline (TBS; 10 mM Tris-HCl pH 7.5, 0.15 M NaCl) containing 10 mM CaCl$_2$, the protein was applied to a GalNAc-Cellulofine column ($1 \times 3$ cm) [2] equilibrated with the same buffer. The active protein was eluted with TBS containing 20 mM EDTA, and further purified by gel filtration using a Superdex-200 column ($2.3 \times 60$ cm) in TBS. The purity of the resulting WT-rCEL-III was confirmed by SDS-PAGE. The genes for site-directed mutants of CEL-III with alanine substitutions for the valine residues at positions 322, 324, 326, 331, 333 and 335 (VA1, VA2 and VA3) (Fig. 1) were prepared by PCR with
the appropriate primers using a QuikChange mutagenesis kit (Stratagene) essentially as previously described [8]. Plasmid construction and the expression and purification of the mutant proteins were carried out using the same procedures described above for WT-rCEL-III.

**Circular Dichroism (CD) Spectroscopy**

Far-UV CD spectra of the proteins were measured in the range of 200-250 nm using a Jasco J-720 spectropolarimeter. The spectra were measured in a quartz cell of 1-mm path length at 20°C. All proteins were analyzed at a concentration of 0.1 mg/ml in TBS.

**Measurement of Hemolytic Activity**

The hemolytic activity of CEL-III was measured by the absorbance at 540 nm due to hemoglobin released from rabbit erythrocytes. Briefly, each protein sample was incubated with an erythrocyte suspension (5%, v/v) in buffer (10 mM Tris-HCl pH 8.5, 0.15 M NaCl, 10 mM CaCl₂) for 30 min and the suspension was centrifuged. Following cell lysis, the absorbance of the supernatant was measured at 540 nm. For 100%
hemolysis, Triton X-100 was added to a final concentration of 0.1% (v/v).

RESULTS AND DISCUSSION

Domain 3 has been assumed to be responsible for the self-oligomerization of CEL-III in target cell membranes because of the presence of a hydrophobic region [5] and its oligomerization tendency after limited digestion [6]. To investigate the roles of amino acid residues in domain 3, especially the two valine clusters as hydrophobic segments, in the self-oligomerization and hemolytic abilities, recombinant CEL-III proteins (Fig. 1) were expressed in *E. coli* cells. However, the expressed proteins were exclusively obtained in the insoluble fraction as inclusion bodies. Therefore, the inclusion bodies were collected and solubilized in solubilization buffer containing 6 M guanidine hydrochloride, followed by refolding of the proteins in the presence of 0.4 M L-arginine to prevent aggregation. The refolding buffer also contained 5 mM reduced glutathione and 0.5 mM oxidized glutathione to expedite the formation of intrachain disulfide bonds. The resulting WT-rCEL-III exhibited binding affinity for a specific carbohydrate, thereby allowing its efficient purification using a GalNAc-Cellulofine column (Fig. 2). The bound protein was eluted with EDTA, indicating that the binding
of the recombinant protein was Ca\textsuperscript{2+}-dependent. Mutant proteins with replacement of
the valine clusters by alanine residues (VA1, VA2 and VA3) were also expressed and
purified using the GalNAc-Cellulofine column (data not shown). These recombinant
proteins were further separated by gel filtration using a Superdex-200 column. As
shown in Fig. 3, WT-rCEL-III was mostly eluted at 70 min, corresponding to the
monomeric protein (47.5 kDa) by comparison with standard proteins, whereas the
mutant proteins (VA1, VA2 and VA3) exhibited increased ratios of high molecular mass
peaks corresponding to oligomeric proteins (>116 kDa) and the oligomerization was
most conspicuous for VA3.

Far-UV CD spectra of WT-rCEL-III and the mutant proteins are shown in Fig. 4A.
WT-rCEL-III exhibited a similar spectrum to native CEL-III (nCEL-III) isolated from
the body fluid of \textit{C. echinata}. However, the mutant proteins (VA1, VA2 and VA3)
produced spectra with increased negative values. Fig. 4B shows the difference CD
spectra between WT-rCEL-III and the mutant proteins. The spectrum for VA3 exhibited
a negative peak around 220 nm, which is characteristic of β-sheet structure, suggesting
that VA3 has a large increase in its β-sheet content [14]. A similar negative peak around
220 nm caused by an increased β-sheet structure was previously observed for the
oligomeric form of the domain 3 fragments produced by limited digestion of nCEL-III [6]. A smaller negative peak around 200 nm was observed in the difference spectrum for VA1, whereas no such peak was detected in the spectrum for VA2. Therefore, the intensity of the negative peak around 220 nm appears to be correlated with the extent of oligomerization seen in Fig. 3. These results suggest that the oligomerization proceeded with concomitant formation of β-sheets in domain 3.

The hemolytic activities of the recombinant proteins were examined using rabbit erythrocytes. Although WT-rCEL-III essentially adopted the correct structure as evidenced by its CD spectrum (Fig. 4A) and specific carbohydrate-binding ability (Fig. 2), its hemolytic activity was relatively weak compared with that of nCEL-III. Specifically, the concentrations of nCEL-III and WT-rCEL-III leading to 50% hemolysis were 0.4 and 40 µg/ml, respectively. These observations suggest that there may be subtle differences in the structures of the two proteins that affect their hemolytic activity, although the details remain unclear. On the other hand, the mutant protein VA2, in which the second valine cluster (residues 341, 343 and 345) was replaced by alanine residues, showed a 10-fold higher hemolytic activity (50% hemolysis at 4 µg/ml) than that of WT-rCEL-III (Fig. 5). Although the mutants containing mutations of the first
valine cluster (VA1) and both valine clusters (VA3) also exhibited higher activities than
WT-rCEL-III at concentrations below 25 µg/ml, the activities did not increase as much
as that of WT-rCEL-III with increasing protein concentration. This unusual behavior
was particularly significant for VA3, which demonstrated a maximum activity of only
26%. Since VA3 showed the strongest tendency for self-oligomerization among the
three mutants, it may have formed inactive oligomers at higher protein concentrations.
Once oligomerized in solution, CEL-III loses its ability to interact with the cell
membrane, although it remains active in carbohydrate binding [15].

In the present study, we have observed that mutations in the valine clusters in
domain 3 largely affect the oligomerization ability of CEL-III, probably by reducing the
stability of the α-helices in which the valine clusters exist, thereby promoting their
transition to β-sheets and consequent association of domain 3. However, as typically
seen in the case of VA3, an extremely high oligomerization tendency tended to lead to
the formation of less active proteins at higher concentrations. For the hemolytic activity,
it is important to form membrane-inserted oligomers, and this requires conformational
changes that induce simultaneous insertion and oligomerization of CEL-III in the
membrane. The enhanced hemolytic activity of VA2 compared with that of
WT-rCEL-III was probably due to its moderately high oligomerization tendency, which can be promoted by interaction with the cell membranes. The oligomerization mechanism accompanying the change in secondary structure of this protein is assumed to be shared by various pore-forming toxins of bacterial origin [16-20]. Elucidation of the oligomerization mechanism of CEL-III should therefore provide important clues regarding the mechanisms of such pore-forming toxins.

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Figure Legends

Figure 1. Positions of the valine clusters replaced by alanine residues in domain 3 of CEL-III. The three domains of CEL-III were shown as gray boxes. The hydrophobic region (residues 320-354) in domain 3 is indicated in black. The positions of the valine clusters in the sequences are enclosed in boxes. Residues are numbered according to native CEL-III, although the recombinant protein has an additional N-terminal methionine residue.

Figure 2. Affinity chromatography of WT-rCEL-III on a GalNAc-Cellulofine column. The protein was applied to a GalNAc-Cellulofine column (1 × 3 cm) equilibrated with TBS containing 10 mM CaCl₂. After washing the column, the adsorbed protein was eluted with TBS containing 20 mM EDTA. The fractions marked by the horizontal bar were pooled and examined by SDS-PAGE (right panel).

Figure 3. Gel filtration of the recombinant proteins on a Superdex-200 column. Recombinant proteins (WT, VA1, VA2 and VA3) were applied to a Superdex-200
column (2.3 × 60 cm) in TBS. *E. coli* β-galactosidase (116 kDa), bovine serum albumin (66 kDa) and bovine cytochrome c (12.5 kDa) were used as standard proteins, and their elution positions are indicated by arrows.

**Figure 4.** Far-UV CD spectra (A) and difference spectra (B) of native and recombinant CEL-III proteins. The difference CD spectra (B) were obtained by subtracting the mean residue ellipticity \( [\theta] \) values of WT-rCEL-III from those of VA1, VA2 and VA3.

**Figure 5.** Hemolytic activities of the recombinant CEL-III proteins. Hemolytic activity assays for the recombinant proteins (WT, VA1, VA2 and VA3) were carried out by incubating the proteins with rabbit erythrocytes in 10 mM Tris-HCl (pH 8.5) containing 0.15 M NaCl and 10 mM CaCl\(_2\) at 20°C for 30 min. After centrifugation, the activities were determined from the absorbances at 540 nm due to hemoglobin released from the erythrocytes.
Fig. 1
Fig. 2

Absorbance at 280 nm vs. Fraction number (2 ml)

20 mM EDTA/TBS

kDa

66
45
29
12.5
Fig. 3
Fig. 4
Fig. 5

Hemolytic activity (%) vs. Protein concentration (µg/ml)

- WT
- VA1
- VA2
- VA3