**Note**

**Effects of Ca\(^{2+}\) on Refolding of the Recombinant Hemolytic Lectin CEL-III**

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**Abstract**

CEL-III is a hemolytic lectin isolated from *Cucumaria echinata*. Although recombinant CEL-III (rCEL-III) expressed in *Escherichia coli* showed very weak hemolytic activity compared with native protein, it was considerably enhanced by refolding in the presence of Ca\(^{2+}\). This suggests that Ca\(^{2+}\) supported correct folding of the carbohydrate-binding domains of rCEL-III, leading to effective binding to the cell surface and subsequent self-oligomerization.

**Key words**: lectin; hemolysis; refolding; calcium; carbohydrate-binding

CEL-III is a Gal/GalNAc-specific lectin isolated from the marine invertebrate *C. echinata* that exhibits strong hemolytic and cytotoxic activities by forming ion-permeable pores in the target cell membranes. This lectin is composed of three domains (domains-1, -2, and -3), each containing approximately 150 amino acid residues. Amino acid sequence and X-ray crystallographic analyses\(^{1,2}\) revealed that domains-1 and -2 (domains-1/2) are carbohydrate-binding domains that show similarity with the B-chain of the plant toxic lectin ricin,\(^{3,4}\) while domain-3 has a novel fold containing two \(\alpha\)-helices and one \(\beta\)-sandwich structure. Since domain-3 contains a hydrophobic region and also has a strong self-oligomerization tendency,\(^{5}\) it was assumed to function to form ion-permeable pores by oligomerizing in the target membrane. We have succeeded in the expression of recombinant CEL-III (rCEL-III) and its site-directed mutants in *E. coli* cells. However, their hemolytic activity was much lower than the native CEL-III (nCEL-III) purified from *C. echinata* body fluid.\(^{6}\) In the present study, we examined the effects of Ca\(^{2+}\) in the refolding process of rCEL-III to prepare the lectin with higher activity.

Expression of rCEL-III was done using plasmid pET-3a containing the rCEL-III gene in *E. coli* BL21(DE3)-pLysS cells, as described previously.\(^{7}\) The gene encoding recombinant domains-1/2 (Gln1–Asn294) was amplified by PCR using CEL-III cDNA\(^{8}\) as template and oligonucleotides 5’-CATATGCAAGTTTGTGGCA- CGAATCC-3’ and 5’-GGATCCAGTTTGTGGCATTGTCGCTGTG-3’ as forward and reverse primers respectively. The resulting DNA fragment was ligated with the pET-3a vector (Novagen) at restriction sites *NdeI* and *BamHI*. 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. The inclusion bodies were solubilized in solubilization buffer (50 mM Tris–HCl pH 8.0, 0.2 mM NaCl, 1 mM ethylenediamine tetraacetate, 6 M guanidine hydrochloride) and the protein was refolded in refolding buffer (0.1 mM Tris–HCl pH 8.0, 0.4 mM l-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.1 mM phenylmethylsulfonyl fluoride).\(^{8}\)

At this step, the refolding process was carried out in the presence and the absence of 10 mM CaCl\(_2\) to assess the effect of Ca\(^{2+}\). After dialysis of the refolded proteins in Tris-buffered saline (TBS; 10 mM Tris–HCl pH 7.5, 0.15 M NaCl) containing 10 mM CaCl\(_2\), the protein was purified by affinity chromatography and gel filtration, as previously reported.\(^{9}\)

We have observed that the rCEL-III produced by the refolding method showed much lower hemolytic activity than nCEL-III.\(^{10}\) The concentrations for 50% hemolysis were 0.4 µg/ml and 40 µg/ml for nCEL-III and rCEL-III respectively. This suggests that the refolding process had not been fully optimized, and should be improved to achieve a more active recombinant protein. In the present study, we examined the effect of Ca\(^{2+}\) added to refolding buffer on the activity of rCEL-III as well as domains-1/2. Figure 1 shows the hemolytic activity of nCEL-III and rCEL-III refolded in the presence and the absence of Ca\(^{2+}\) (rCEL-III(+Ca) and rCEL-III(−Ca) respectively). Hemolytic activity was measured using rabbit erythrocytes, and was expressed as the percentage of the absorbance at 540 nm of hemoglobin released from the erythrocytes.\(^{9}\) Similarly to our previous result, rCEL-III(−Ca) showed much lower activity than nCEL-III; the concentrations for 50% hemolysis were 0.2 µg/ml and 24 µg/ml for nCEL-III and rCEL-III(−Ca) respectively, while the activity of rCEL-III(+Ca) was considerably higher (2.6 µg/ml for 50% hemolysis) than that of rCEL-III(−Ca). This suggests that Ca\(^{2+}\) induced efficient refolding of rCEL-III to achieve a more active conformation. In the crystal structure of CEL-III/carbohydrate-complexes, Ca\(^{2+}\) ions are found in the cavities of the carbohydrate-binding sites in the five subdomains (1α, 1γ, 2a, 2β, 2γ) in domains-1/2.\(^{2}\)

These Ca\(^{2+}\) ions are coordinated with the oxygen atoms of nearby amino acid residues, stabilizing the subdomain

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**Abbreviations**: CD, circular dichroism; TBS, Tris-buffered saline; rCEL-III, recombinant CEL-III; nCEL-III, native CEL-III
structure composed of the β-trefoil fold\(^{10}\) (Fig. 2). The importance of Ca\(^{2+}\) is indicated by complete loss of the activity at very low concentrations of Ca\(^{2+}\)\(^{11}\) and in the presence of chelating reagents such as ethylenediamine tetraacetate. Therefore, the effects of Ca\(^{2+}\) on the folding of domains-1/2, was also examined using a recombinant protein consisting only of domains-1/2. Recombinant domains-1/2 showed only hemagglutinating activity without hemolysis because it is devoid of pore-forming domain-3. Hemagglutinating activity was measured using rabbit erythrocytes in TBS containing 10 mM CaCl\(_2\), as previously described.\(^{11}\) The hemagglutinating activity of domains-1/2 refolded in the presence of Ca\(^{2+}\) had structures more similar to the native protein, especially in domains-1/2, which is probably the reason for the higher activities of rCEL-III(+Ca) and domains-1/2(+Ca).

The carbohydrate-binding ability of CEL-III may be very important not only for its adsorption to cell surface through binding to specific carbohydrate chains, but also for subsequent conformational changes leading to the self-oligomerization process. We have found that binding of disaccharides containing a β-galactoside structure, such as lactose and lactulose, induces self-oligo-

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**Fig. 1.** Comparison of the Hemolytic Activities of rCEL-III(−Ca), rCEL-III(+Ca), and nCEL-III.

Hemolytic activity was measured using rabbit erythrocytes. The proteins were incubated with erythrocyte suspension (5%, v/v) in the buffer (10 mM Tris–HCl pH 8.5, 0.15 M NaCl, 10 mM CaCl\(_2\)) for 30 min at room temperature. Following cell lysis, the suspension was centrifuged and 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).

**Fig. 2.** Crystal Structure of the Carbohydrate-Binding Site of the CEL-III/GalNAc Complex.

The carbohydrate-binding site in subdomain 1y (residues 102–149) is shown. Amino acid residues involved in the binding of Ca\(^{2+}\) and GalNAc are indicated by stick model. Coordinate and hydrogen bonds are depicted as dashed lines.

**Fig. 3.** Comparison of the Far-UV CD Spectra of nCEL-III, rCEL-III(−Ca), and rCEL-III(+Ca) (A), and Those of Domains-1/2(−Ca) and Domains-1/2(+Ca) (B).

The spectra were measured using a Jasco J-720 spectropolarimeter with a quartz cell of 1-mm path length at 20°C. The samples were analyzed in TBS.
merization of CEL-III in aqueous solution at high pH and high salt concentration. This suggests that binding to specific carbohydrates is also important for CEL-III to trigger conformational changes leading to self-oligomerization, which could also occur on the target cell surface. In fact, X-ray crystallographic analysis recently revealed that binding of specific carbohydrates to CEL-III induced conformational changes not only around carbohydrate-binding sites, but also in domain-3. Taking into account these results, it is probable that promotion of the correct folding of domains-1/2 caused by interaction with Ca\(^{2+}\) leads to higher hemolytic activity through efficient carbohydrate-binding in domains-1/2 and following conformational changes in domain-3. Alternatively, it is also possible that Ca\(^{2+}\) directly affected the folding of domain-3 by nonspecific interactions, while the binding of Ca\(^{2+}\) to domain-3 has not been found in the crystal structure. The improved refolding method using Ca\(^{2+}\) should facilitate future site-directed mutagenesis studies of CEL-III through efficient evaluation of the roles of the various amino acid residues.

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References