Running title: Increased hemolytic activity of CEL-III mutants

**Note**

# Effects of amino acid mutations in the pore-forming domain of the hemolytic lectin CEL-III

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*Abbreviations*: $HC_{50}$, concentrations for 50% hemolysis; TBS, Tris-buffered saline; WT, wild-type

**Abstract**

The hemolytic lectin CEL-III forms transmembrane pores in the membranes of target cells. A study on the effect of site-directed mutation at Lys405 in domain 3 of CEL-III indicated that replacements of this residue by relatively smaller residues lead to a marked increase in hemolytic activity, suggesting that moderately destabilizing domain 3 facilitates formation of transmembrane pores through conformational changes.

**Keywords:** hemolysin; sea cucumber; lectin; pore-forming protein; site-directed mutagenesis

CEL-III is a hemolytic lectin isolated from the sea cucumber *Cucumaria echinata*. This lectin binds galactose-containing carbohydrates on the surface of cell membranes in a $Ca^{2+}$-dependent manner.[1)] CEL-III contains two carbohydrate-binding domains (domains 1 and 2), which adopt a ricin-type (R-type) lectin fold,[2)] also known as β-trefoil fold,[3, 4)] although they require $Ca^{2+}$ ions for binding to specific carbohydrates. Domains 1 and 2 are composed of three subdomains, each of which contains a carbohydrate-binding sites, except for subdomain 1β. Specific carbohydrates are recognized through coordinate bonds with $Ca^{2+}$ and *via* hydrogen bond networks around the residues lining these binding sites.[4)] On the other hand, one third of the C-terminal region of CEL-III corresponds to domain 3. Domain 3 contains a hydrophobic region, and is involved in the formation of transmembrane pores in targeted cell membranes. After binding to target cells *via* five carbohydrate-binding sites in domains 1 and 2, and inserting into the membrane through hydrophobic regions, CEL-III generates transmembrane pores by associating with itself, forming heptamers (Fig. 1).[5)] These pores can lead to

Fig. 1

hemolysis or cell death by disrupting the balance of ions across the cell membrane.[6] Our previous site-directed mutagenesis study, in which various residues throughout domain 3 were mutated to alanine residues,[7] identified some residues closely associated with the hemolytic action of CEL-III. Among them, Lys405 was one of the most conspicuous because its mutation to alanine led to a drastic increase in hemolytic activity. The mutant protein (K405A) showed a 360-fold increase in hemolytic activity, compared to wild-type (WT) protein. In order to elucidate the function of this residue in the hemolytic action of CEL-III, we have conducted a study further mutating this and related residues.

Mutations were introduced into the gene encoding CEL-III with a QuikChange site-directed mutagenesis kit (Agilent, Santa Clara, CA), using primers (33 nucleotides) and the CEL-III cDNA (DDBJ database accession number AB109017) as a template.[8] Plasmids containing the mutated genes were used to transform *Escherichia coli* BL21-codonplus (DE3)-RIPL cells (Novagen). Cells were cultured at 37°C in lysogeny broth media, on a shaking platform. Once the cell culture reached mid-log phase (optical density readings of 0.6–0.7, at 600 nm), protein expression was induced by the addition of isopropyl-$\beta$-D-thiogalactopyranoside to a final concentration of 0.4 mM. Cells were incubated for an additional 5 h at 37°C. The recombinant protein mutants were isolated in inclusion bodies following disruption of the cells by sonication. The inclusion bodies were solubilized in ‘solubilization buffer’ (0.2 M NaCl, 1 mM ethylenediamine tetraacetic acid, 6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 8.0), and the protein was refolded in ‘refolding buffer’ (0.4 M L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.1 M Tris-HCl, pH 8.0.[9] After dialysis against Tris-buffered saline (TBS; 0.15 M NaCl, 10 mM Tris-HCl, pH 7.5) containing 10 mM $CaCl_2$, the protein was purified by affinity chromatography on a lactose-Cellufine column

(1 × 3 cm).[6] The hemolytic activities of the CEL-III mutants were determined by monitoring the absorbance of a suspension of erythrocytes at 540 nm (which measures the release of hemoglobin) after mixing with the CEL-III mutants in 10 mM Tris-HCl (pH 8.5) containing 0.15 M NaCl and 10 mM $CaCl_2$ at 20°C for 1 h, as previously described.[6]

Since the mutant K405A showed a remarkable increase in hemolytic activity in our previous study,[7] several mutant proteins were prepared with different amino acid residues, (Ala, Ser, Glu, Arg, and Leu) at position 405 (K405A, K405S, K405E, K405R, and K405L, respectively), and their activity was examined. Among them, we were unable to refold K405L into a soluble form because of its strong tendency to auto-aggregate. This suggests that a leucine residue introduced at position 405 may have brought about an excessive change in the local environment inside domain 3, due to its hydrophobic side-chain, leading to the formation of the aggregates. On the other hand, the other CEL-III mutants were successfully refolded to soluble form. The refolded mutants were purified by affinity chromatography using a lactose-Cellufine column, which by itself indicates that they possessed intact carbohydrate-binding activity. Following elution from the affinity column using lactose, the CEL-III mutants were dialyzed against TBS to remove bound lactose, and used in a hemolytic assay.

Figure 2A compares the hemolytic activity of the various CEL-III mutants. Fig. 2
Their hemolytic activity was determined from the absorbance at 540 nm, due to hemoglobin release from rabbit erythrocytes, after 1 h incubation with the proteins. In accordance with our previous results,[7] K405A-CEL-III exhibited a much higher activity compared to WT-CEL-III. Concentrations for 50% hemolysis ($HC_{50}$) were 5.7 μg/ml for WT-CEL-III and 0.26 μg/ml for K405A-CEL-III, confirming enhanced activity by the K405A mutant. In addition to K405A, a remarkable increase in activity was also observed for

K405S ($HC_{50}$ = 0.36 μg/ml), whereas the other mutants, K405E and K405R, showed much lower activities ($HC_{50}$ = 51 μg/ml and 29 μg/ml, respectively). As seen in Fig. 3A, Lys405 is situated in the interior of domain 3 in the CEL-III monomer, forming an ionic bond with Asp371, contributing to the stabilization of the domain structure. Therefore, the mutation of Lys405 to residues with small, uncharged side-chains (K405A and K405S) may have partially destabilized the structure of domain 3 by disrupting this ionic bond. Since the hemolytic action of CEL-III proceeds *via* large conformational changes of domain 3 to form heptamers, such a 'loosening' partial disruption might conceivably facilitate heptamerization (and therefore promote pore formation) in target cell membranes. However, it is noteworthy that the ionic bond between Lys405 and Asp371 is still preserved in the heptameric form of native CEL-III,[5] suggesting that the enhancement of activity is brought about by destabilization of the local structure of the monomeric form, rather than by a loss of a particular bond between amino acid residues. This seems to be related to our previous observation that the hemolytic activity of CEL-III is also enhanced by mutation of three valine residues (Val341, Val343, and Val345) to alanine residues, which presumably lowers the stability of domain 3.[10] In contrast to K405A and K405S, decreased activity resulted in the case of mutations of K405 to relatively larger residues with charged side-chains (K405E and K405R). We speculate that the side-chains of glutamate and arginine may have caused excessive destabilization of domain 3, by introducing either an oppositely charged side-chain (K405E) or a larger side-chain (K405R). Moderate destabilization in domain 3 arising from K405A and K405S mutations appears likely to be the basis for the enhanced hemolytic action.

Fig. 3

We also examined the effect of substitutions of Asp371, the counterpart residue in the ionic bond with Lys405. As shown in Fig. 2B, all the Asp371

mutants exhibited reduced activities, including the alanine mutation (D371A), in contrast to the case for Lys405 mutants. D371A and D371R CEL-III mutants only achieved a maximum of 20% hemolysis, and D371K had almost no activity. As shown in the close-up view around the contact region between subunits in heptameric CEL-III (Fig. 3B), the Asp371 side-chain forms a hydrogen bond with the Gln300 side-chain in the adjacent subunit, in addition to an ionic bond with Lys405. This suggests that the mutations of Asp371 led not only to the disruption of the ionic bond with Lys405, but also to interactions with a neighboring subunit required for heptamerization. As seen in Figure 3B, there are three other residues (Asn369, Asp373, and Arg378) involved in interactions between adjacent subunits in that vicinity. This region is assumed to be particularly important during the early stage of heptamerization; it forms a prepore ring on the cell membrane, which then triggers extension of the 14-stranded β-barrel.[5] The importance of these residues during the heptamerization process, owing to their formation of hydrogen bonds and ionic bonds, has also been suggested by the complete loss of activity in the R378A mutant[7]—although Arg378 is fully exposed to aqueous solution in the original monomeric form (Fig. 3A). Taking these facts in to account, it seems reasonable to infer that the mutation of Asp371, which is expected to weaken the interaction between neighboring subunits, may significantly reduce the heptamerization efficiency needed for hemolytic action.

In contrast to the current results, a slightly higher activity of the D371A mutant compared with that of the WT was observed in our previous experiment.[7] Although the exact reason for this discrepancy is uncertain, it might be due to subtle differences in the folded states of the recombinant proteins that were prepared, including the WT protein. Besides R378A, K338A is another conspicuous mutant, showing an almost complete loss of hemolytic activity.[7] As shown in Fig. 3A, Lys338 is exposed to aqueous solution on the

surface of the protein in monomeric form, but it becomes relocated in the heptameric form to the end of the 14-stranded β-barrel, which is expected to be on the opposite side of the cell membrane. This suggests that this residue might play a particular role in β-barrel extension through interactions with the cell membrane. Investigation of the roles played by these specific residues would lead to further understanding of the mechanism of action of CEL-III, as well as other pore-forming proteins, which are widely distributed in different organisms.

## Author contribution

H. Unno, S. Goda, and T. Hatakeyama conceived and designed the experiments. T. Nagao and R. Masaki performed the experiments. T. Hatakeyama wrote the manuscript.

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## Figure Legends

**Figure 1.** Mechanism of hemolysis by CEL-III.

(A) CEL-III binds to Gal/GalNAc-containing carbohydrate chains present on target cell membranes *via* its five carbohydrate-binding sites. (B) Triggered by the binding to the carbohydrate chains, domain 3 of CEL-III releases from domains 1 and 2. (C) CEL-III heptamerizes to form a transmembrane pore composed of domain 3. Only two of the seven pore molecules are shown here in side view, for convenient viewing.

**Figure 2.** Hemolytic activity of the CEL-III mutants.

The hemolytic activity of mutants with different amino acid substitutions at (A) position 405 or (B) at position 371 was compared with that of WT-CEL-III. The assay was performed by incubating the proteins separately for 1 h at 20°C with rabbit erythrocytes in 10 mM Tris-HCl, pH 8.5, containing 10 mM $CaCl_2$. After centrifugation, the activities were determined by monitoring hemoglobin release from the erythrocytes at an absorbance wavelength of 540 nm. The highest hemolysis values obtained were used to define 100% activity.

**Figure 3.** Three-dimensional structures of a CEL-III monomer (PDB ID: 2Z48)[4)] and heptamer (PDB ID: 3W9T).[5)]

The regions around Lys405 of (A) monomeric and, (B) heptameric forms of CEL-III are depicted in close-up view. $Ca^{2+}$ (magenta) and $Mg^{2+}$ (yellow) are shown as spheres. Hydrogen bonds and ionic bonds are depicted as dashed lines. Bound carbohydrate molecules are omitted. These models were prepared using the program *PyMOL*.[11)]

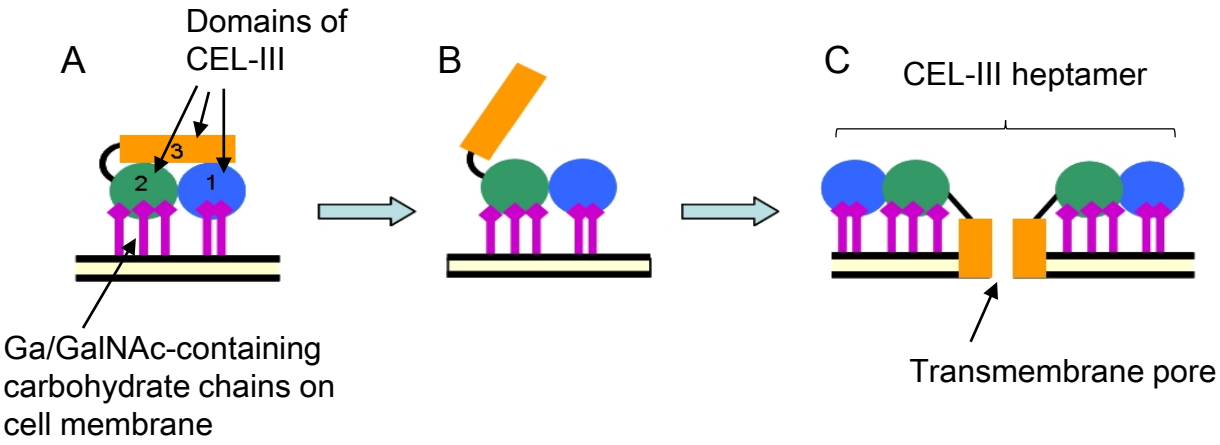
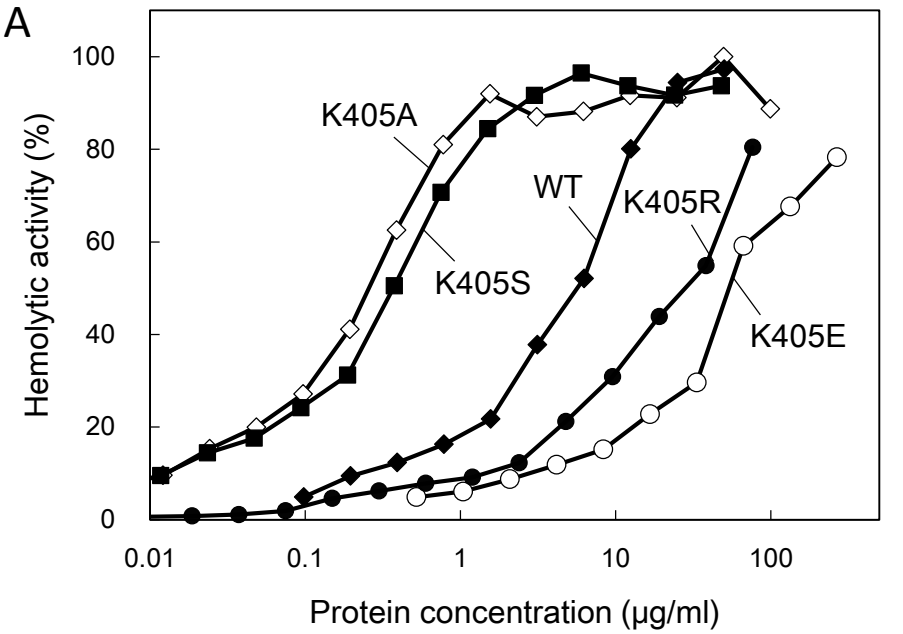

A
Domains of
CEL-III
3
2
1
Ga/GalNAc-containing
carbohydrate chains on
cell membrane
B
C
CEL-III heptamer
Transmembrane pore

Fig. 1


A
Hemolytic activity (%)
100
80
60
40
20
0
0.01
0.1
1
10
100
Protein concentration (μg/ml)
K405A
WT
K405R
K405S
K405E

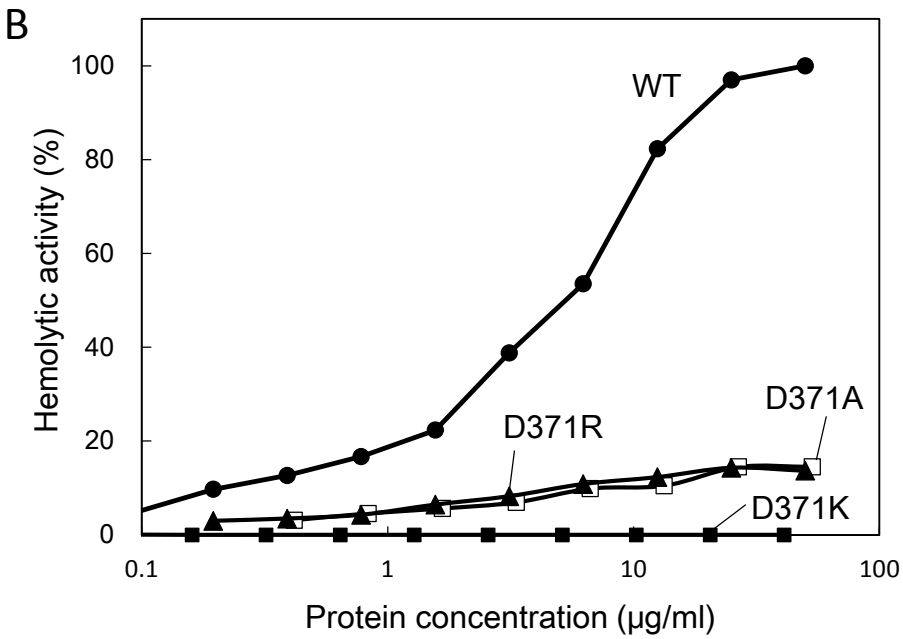

B
Hemolytic activity (%)
100
80
60
40
20
0
0.1
1
10
100
Protein concentration (μg/ml)
WT
D371A
D371R
D371K

Fig. 2

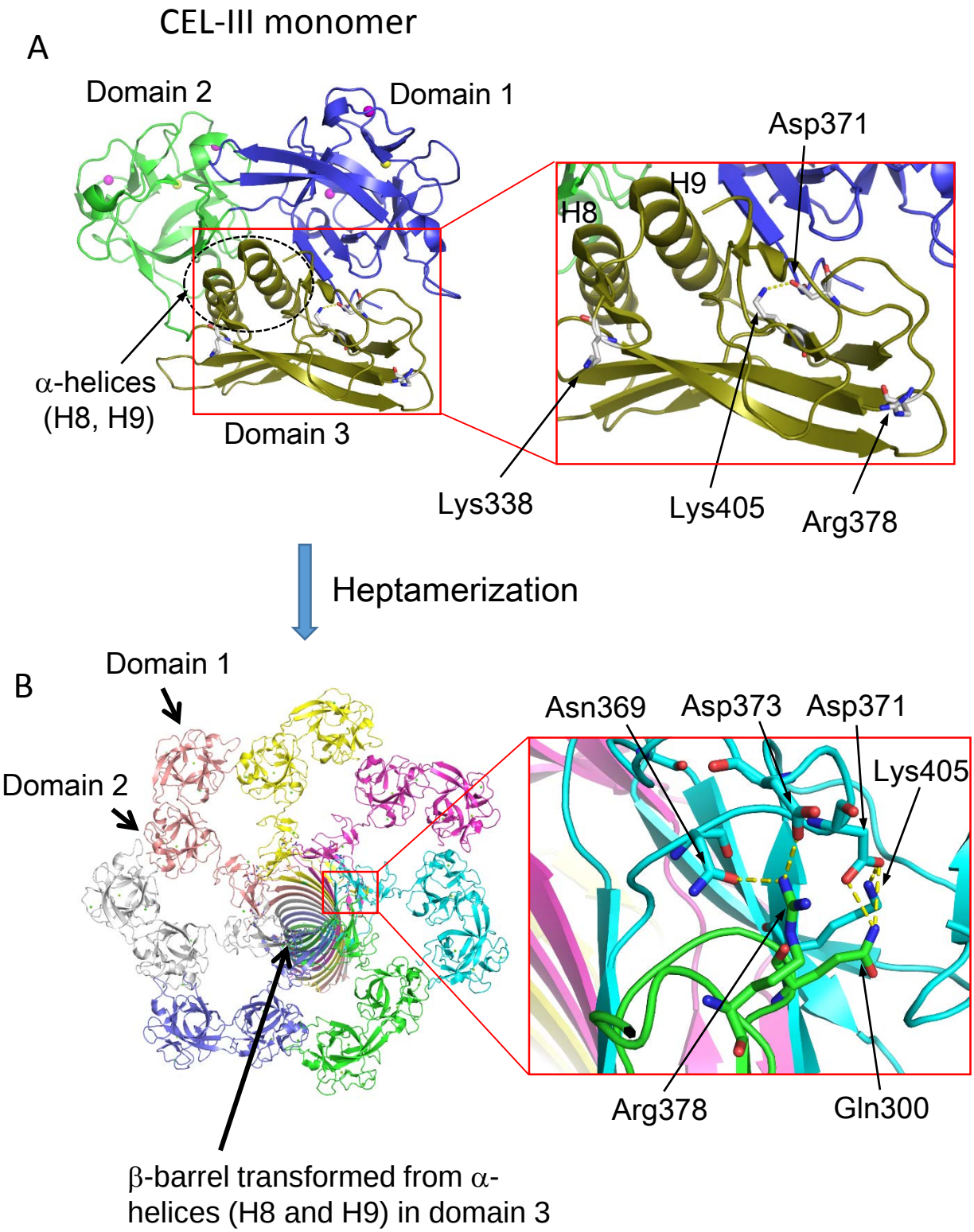

A
CEL-III monomer
Domain 2
Domain 1
Asp371
H9
H8
α-helices
(H8, H9)
Domain 3
Lys338
Lys405
Arg378
Heptamerization
B
Domain 1
Domain 2
Asn369
Asp373
Asp371
Lys405
Arg378
Gln300
β-barrel transformed from α-helices (H8 and H9) in domain 3

Fig. 3