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Characterization of Functional Domains of the Hemolytic Lectin CEL-III from the Marine Invertebrate *Cucumaria echinata*

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CEL-III is a Ca^{2+}-dependent, galactose/N-acetylgalactosamine (GalNAc)-specific lectin isolated from the marine invertebrate *Cucumaria echinata*. This lectin exhibits strong hemolytic activity and cytotoxicity through pore formation in target cell membranes. The amino acid sequence of CEL-III revealed the N-terminal two-thirds to have homology to the B-chains of ricin and abrin, which are galactose-specific plant toxic lectins; the C-terminal one-third shows no homology to any known proteins. To examine the carbohydrate-binding ability of the N-terminal region of CEL-III, the protein comprising Pyr1–Phe283 was expressed in *Escherichia coli* cells. The expressed protein showed both the ability to bind to a GalNAc-immobilized column as well as hemagglutinating activity for rabbit erythrocytes, confirming that the N-terminal region has binding activity for specific carbohydrates. Since the C-terminal region could not be expressed in *E. coli* cells, a fragment containing this region was produced by limited proteolysis of the native protein by trypsin. The resulting C-terminal 15 kDa fragment of CEL-III exhibited a tendency to self-associate, forming an oligomer. When mixed with erythrocytes, the oligomer of the C-terminal fragment caused hemagglutination, probably due to hydrophobic interaction with cell membranes, while the monomeric fragment did not. Chymotryptic digestion of the preformed CEL-III oligomer induced upon lactose binding also yielded an oligomer of the C-terminal fragment comprising six molecules of the 16 kDa fragment. These results suggest that after binding to cell surface carbohydrate chains, CEL-III oligomerizes through C-terminal domains, leading to the formation of ion-permeable pores by hydrophobic interaction with the cell membrane.

Key words: carbohydrate-recognition domain, *Cucumaria echinata*, hemolytic lectin, oligomerization, ricin.

Abbreviations: CRD, carbohydrate-recognition domain; EDTA, ethylenediamine tetraacetate; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophresis; PCR, polymerase chain reaction; Pyr, pyroglutamic acid; TBS, Tris-buffered saline; TBS-Ca, TBS containing 10 mM $CaCl_2$, PVDF, polivinylidene difluoride.

CEL-III is a Ca^{2+}-dependent and galactose/*N*-acetylgalactosamine (GalNAc)–specific lectin purified from the marine invertebrate *Cucumaria echinata* (*1*). This lectin exhibits strong hemolytic activity toward human and rabbit erythrocytes and cytotoxicity for some cultured cell lines (*2*). The hemolysis mechanism induced by CEL-III has been inferred as follows: (i) CEL-III binds to carbohydrate chains on the surface of the target cell membrane by its lectin activity. (ii) The conformation of the CEL-III molecule changes; simultaneously, CEL-III molecules oligomerize to form ion-permeable pores in the cell membrane. iii) Finally, erythrocytes are lysed by colloid osmotic rupture (*3*). Although the hemolytic mechanism of CEL-III resembles those of some bacterial toxins, such as α-hemolysin from *Staphylococcus aureus* (*4*) and aerolysin from *Aeromonas hydrophila* (*5*), its optimum temperature, pH and cell-surface receptor are significantly different. Receptors of CEL-III on the erythrocyte membrane have been identified to be neutral sphingoglycolipids, such as lactosyl ceramide, and globoside (*6*). Binding of CEL-III to carbohydrate chains induces its oligomerization not only in target membranes, but also in aqueous solution when incubated at high ionic strength and high pH (*3*). Because this type of oligomer hardly dissociates into monomers by treatment with reducing agents, SDS or denaturants, it seems that protein binding is stabilized by strong non-covalent interactions. The amino acid sequence of CEL-III has been determined from analyses of its cDNA and peptides produced by enzymatic digestion of the protein (*7*). Although CEL-III has no sequence similarity to any other Ca^{2+}-dependent lectin, including C-type lectins, the N-terminal two-thirds (Pyr1-Phe283) shows relatively low but definite sequence similarity to the B-chains of ricin (24%) (Fig. 1A) and abrin (29%),

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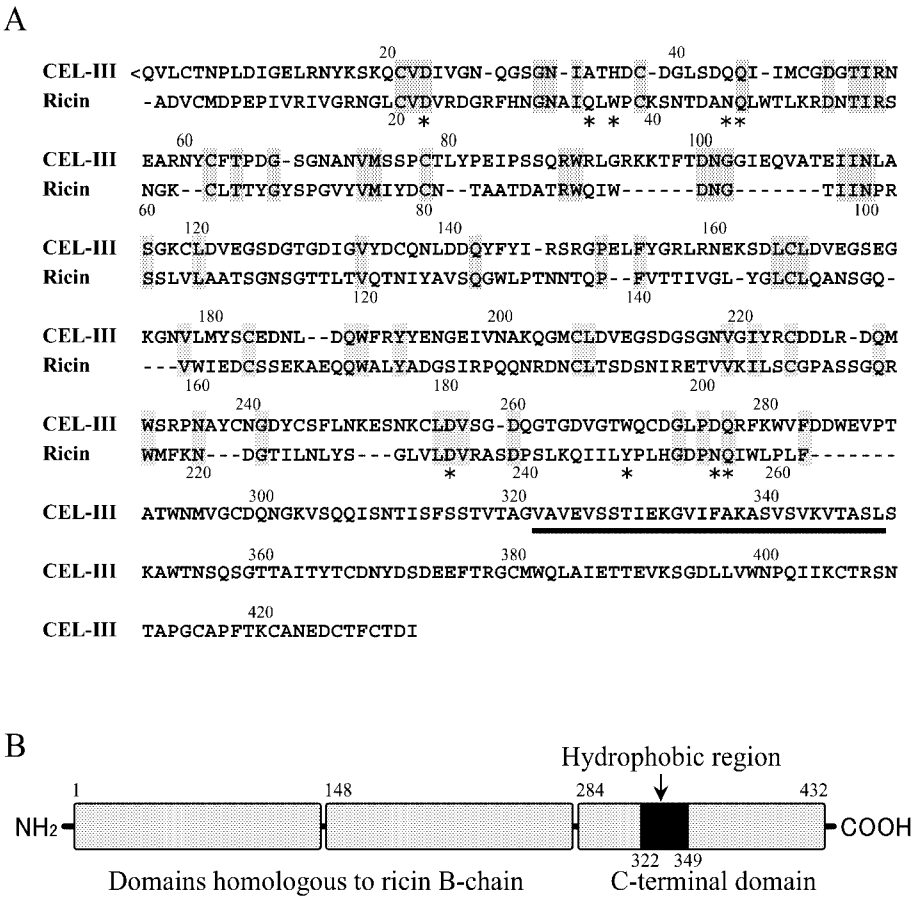

Fig. 1. **Comparison of the amino acid sequences of CEL-III and ricin B-chain (A) and a schematic drawing of the domain structure of CEL-III (B).** (A) Amino acids identical between the two proteins are shaded. The hydrophobic region, rich in Val residues, in the C-terminal domain is indicated by a horizontal bar. The residues involved in carbohydrate-binding of ricin B-chain are denoted by asterisks.

toxic plant lectins (*8*, *9*). Furthermore, the positions of eight cysteine residues in the B-chains of ricin and abrin are also conserved in the N-terminal portion of CEL-III, suggesting that the tertiary structures of these lectins are similar. On the other hand, the C-terminal one-third of CEL-III (Asp284–Ile432) shows no similarity to any known proteins. There is a hydrophobic segment around residues 322–349 in the C-terminal portion that might be involved in the interaction with target cell membranes. Therefore, it seems possible that the C-terminal region of CEL-III plays an important role in oligomerization and interaction with cell membranes after binding to the cell surface carbohydrate-chains *via* the N-terminal region. In this study, we prepared fragments containing the individual domains using recombinant DNA and protein chemical techniques, and investigated their roles in the hemolytic mechanism of the protein.

MATERIALS AND METHODS

Materials—CEL-III was purified from *C. echinata* body fluid as previously reported (*3*). Rabbit blood was obtained from Nippon Bio-Test Laboratories (Tokyo). Papain, trypsin, and chymotrypsin were purchased from Sigma. Lysyl endopeptidase and proteinase K were from Wako. All other chemicals were of analytical grade for biochemical use.

Protein Determination—Protein concentration was determined with bicinchoninic acid (*10*) using bovine serum albumin as a standard.

Expression of the N-Terminal Fragments of CEL-III in E. coli Cells—To obtain a cDNA fragment encoding the carbohydrate recognition domain (CRD) of CEL-III, PCR was performed using CEL-III cDNA (DDBJ database accession number AB109017) (*7*) as a template with the forward primer 5′-CATATGCAAGTTTTGTGCACGAATCCAC -3′ and reverse primer 5′-GGATCCTTAAAACACCCATTTGAATCGTTG-3′. The PCR products were ligated into the pGEM™-T Easy vector (Promega). After DNA sequence confirmation, the DNA fragment was excised by digestion with *Nde*I and *Bam*HI, and ligated into expression vector pET-22b, previously digested with the same enzymes. The resulting plasmid, pET-CRD, was introduced into *E. coli* BL21(DE3) and a recombinant protein was induced with 1 mM IPTG according to the supplier's instructions. After induction, the culture was incubated for an additional 6 h at 37°C. Cells were harvested and lysed by sonication in 30 mM Tris-HCl buffer (pH 7.5) containing 30 mM NaCl. The lysate was centrifuged at 8,000 ×*g* for 15 min and the pellet was collected and washed with 1 M sucrose, followed by 2% Triton X-100/10 mM EDTA. The inclusion bodies obtained were dissolved in 30 mM Tris-HCl (pH 8.0) containing 30 mM NaCl, 1 mM dithiothreitol (DTT), and 8 M urea. The protein solution was dialyzed against 30 mM Tris-HCl buffer (pH 8.0) containing 30 mM NaCl, 1 mM DTT, and 4 M urea for 24 h and finally against 10 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl and 10 mM $CaCl_2$. The recombinant protein was purified by affinity chromatography on a

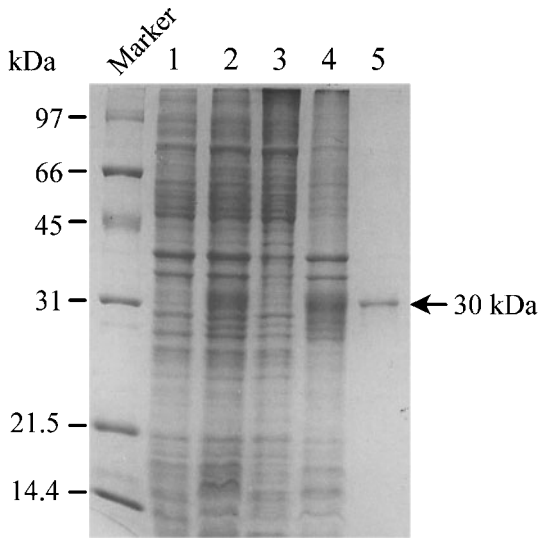

Fig. 2. **SDS-PAGE analysis of the proteins expressed in *E. coli* harboring pET-CRD.** Lane 1, before induction; lane 2, after induction by 1 mM IPTG; lane 3, supernatant of the cell lysate; lane 4, precipitate of the cell lysate; lane 5, purified recombinant protein. The arrow indicates the band of the recombinant protein.

GalNAc-Cellulofine column (0.8 × 10 cm) (*11*) pre-equilibrated with the same buffer.

Limited Digestion of CEL-III and Purification of the C-Terminal Fragments—CEL-III was digested with the four proteases, trypsin, chymotrypsin, lysyl endopeptidase, and proteinase K in 0.1 M NH_4HCO_3 solution at 30°C for 40 min using an enzyme to a substrate ratio of 1:50. To purify the 15 kDa C-terminal fragment, the tryptic digest was separated by affinity chromatography on a GalNAc-Cellulofine column (1 × 3 cm). The flow-through fraction, which contained the 15 kDa fragment, was then separated using gel filtration on a Sephadex G-50 column (1.5 × 90 cm) in 10 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl (TBS). The fragments separated on SDS-PAGE were blotted onto a polyvinylidene difluoride membrane (*11*), and N-terminal sequence analysis was performed using a PPSQ-1 or PPSQ-21 protein sequencer (Shimadzu).

Circular Dichroism (CD) Spectroscopy—CD spectra of the C-terminal fragment of CEL-III were obtained on a JASCO J-720 spectropolarimeter. After incubation at different temperatures in TBS, far-UV CD spectra of the C-terminal fragment were recorded in a 1 mm pathlength cell at 20°C.

Hemagglutination Assay—Serial twofold dilutions of the sample (50 μl) were mixed with the same volume of a 5% (v/v) suspension of rabbit erythrocytes in round-bottomed microtiter plate wells. The samples were incubated in TBS in the absence or presence of 10 mM $CaCl_2$, 10 mM lactose, or 10 mM EDTA. The extent of agglutination was examined visually after incubation for 1 h at room temperature.

Preparation of CEL-III Oligomers—CEL-III oligomers were prepared in aqueous solution according to the previously described method (*3*). In brief, CEL-III monomers were dissolved in 50 mM glycine/NaOH buffer (pH 10) containing 1 M NaCl and 10 mM lactose. After incubation at 25°C for 4 h, the resulting CEL-III oligomers were purified by gel filtration on a Sephacryl S-200 column. CEL-III oligomers bound to erythrocyte membranes were prepared by mixing 4% rabbit erythrocytes and 1 mg/ml CEL-III solution in TBS-Ca. After lysis was complete, the suspension was centrifuged, and the membrane pellet was washed twice with TBS-Ca containing 10 mM lactose. The CEL-III oligomers bound to the erythrocyte ghosts thus obtained were subjected to proteolytic digestion.

Proteolysis of the CEL-III Oligomer—The CEL-III oligomers formed upon lactose binding were digested in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM DTT and 10 mM EDTA. Papain digestion was performed at 37°C for 2 h using an enzyme to substrate ratio of 1:50; tryptic and chymotryptic digestions were performed identically to papain digestion in 50 mM Tris-HCl (pH 8.0). The resulting CEL-III oligomer peptides were purified by gel filtration on a Sephacryl S-200 column. The resultant fragments were treated with 30% formic acid and subjected to SDS-PAGE. Proteins were detected by staining with Coomassie Brilliant Blue R-250 or silver. The N-terminal amino acid sequence was determined by a PPSQ-1 or PPSQ-21 protein sequencer (Shimadzu).

RESULTS

Expression and Characterization of N-Terminal Fragments of CEL-III—Figure 1 shows a comparison of the amino acid sequences of CEL-III and the B-chain of ricin. The N-terminal two-thirds (residues 1–283) of CEL-III exhibits homology with the ricin B-chain, while the C-terminal one-third shows no homology to any known protein. Since the ricin B-chain contains two carbohydrate-binding domains in its polypeptide, it seems reasonable to infer that the N-terminal portion of CEL-III also constitutes two carbohydrate-binding domains (*7*). To assess this assumption, the recombinant protein corresponding to residues 1–283 of CEL-III was expressed in *E. coli* cells. As shown in Fig. 2, SDS-PAGE analysis of the proteins from induced *E. coli* cells indicated the production of a 30 kDa protein, which agrees well with the theoretical value (29,893 Da). The recombinant protein accumulated in cells as inclusion bodies (Fig. 2, lanes 3 and 4), which were dissolved in 8 M urea with 1 mM dithiothreitol; subsequently, the protein was refolded by the successive dialysis method. The recombinant protein was then subjected to affinity chromatography on a GalNAc-Cellulofine column. As seen in Fig. 3A, the recombinant protein was adsorbed to the column and eluted with 100 mM lactose, indicating that the protein has carbohydrate-binding ability. The recombinant protein also showed weak hemagglutinating activity (but not hemolytic activity) toward rabbit erythrocytes (Fig. 3B); agglutination occurred at 1.2 μg/ml protein, which corresponds to 0.7% of the activity of the native CEL-III. This may result from structural instability due to lack of the C-terminal region. In fact, the C-terminal deletion mutant of CEL-III, desC100, which is 49 amino acids longer than CRD at the C-terminal, has strong hemagglutinating activity the same as authentic CEL-III (Y. Kouzuma, unpublished data). Hemagglutination by the recombinant protein was abolished in the presence of lactose or EDTA, confirming

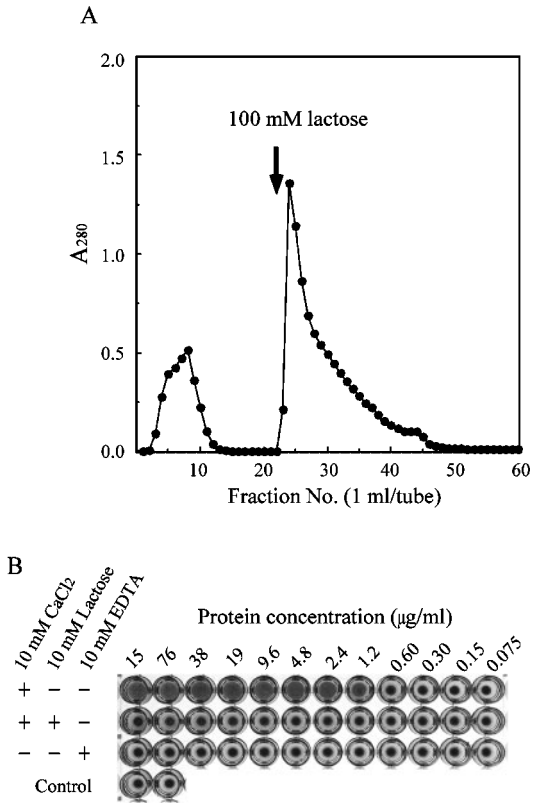

Fig. 3. **Carbohydrate-binding activity of the N-terminal fragment of CEL-III examined by affinity chromatography on GalNAc-Cellulofine (A) and hemagglutination assay (B).** (A) The refolded recombinant protein was subjected to affinity chromatography on a GalNAc-Cellulofine column (0.8 × 10 cm) equilibrated with 10 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$. The adsorbed protein was eluted with 100 mM lactose. (B) Hemagglutinating activity of the recombinant protein examined in the absence or presence of 10 mM $CaCl_2$, 10 mM lactose, or 10 mM EDTA. Control denotes samples without the addition of the recombinant protein.

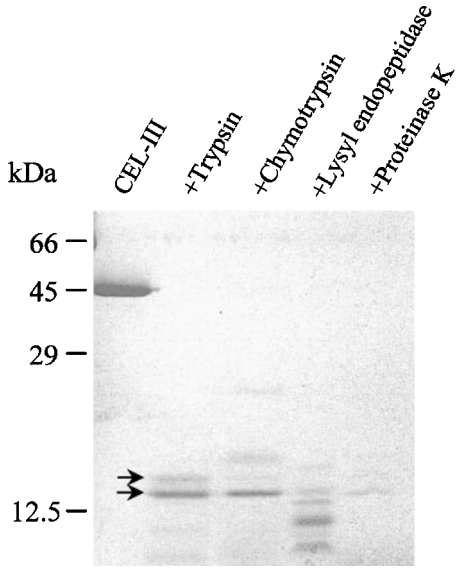

Fig. 4. **SDS-PAGE of the CEL-III fragments produced by digestion with several proteases.** Intact CEL-III and its fragments produced by digestion with trypsin, chymotrypsin, lysyl endopeptidase, and proteinase K were analyzed in a 12.5% gel. Arrows indicate 15 kDa and 17 kDa fragments derived from CEL-III.

that the activity depends on Ca^{2+}-dependent carbohydrate-binding ability.

Purification of the C-Terminal Fragment of CEL-III—We attempted to prepare the C-terminal fragment by limited proteolysis of native CEL-III because overexpression of the C-terminal region of CEL-III in *E. coli* cells was not successful. Figure 4 shows the results of the digestion of CEL-III by four proteases. The reaction was conducted in the absence of Ca^{2+} since Ca^{2+} makes CEL-III resistant to digestion, as reported previously (*12*). Among the proteases used in this experiment, trypsin produced two clear bands of 15 kDa and 17 kDa. These fragments were separated by affinity chromatography on a GalNAc-Cellulofine column (Fig. 5A). The flow-through fraction (**a**) contained a 15 kDa fragment and the adsorbed fraction (**b**) contained the 17 kDa fragment. Because the N-terminal sequence of the 17 kDa fragment was SRGPELFYGR, it was identified as a fragment beginning with Ser148, confirming that the CEL-III middle domain has carbohydrate-binding ability. The flow-through fraction (**a**) from the GalNAc-Cellulofine column was further separated on a Sephadex G-50 column (Fig. 5B). SDS-PAGE showed that flow-through fraction (**c**) from the Sephadex G-50 column contained a high-molecular-mass protein, and fraction **d** contained a 15 kDa fragment. Since the N-terminal sequence of fraction **d** was found to be WVFDDWEVPT, it was identified as the C-terminal fragment beginning with Trp281. On the other hand, the N-terminal sequence of fraction **c** was the same as that of fraction **d**, while some minor peptides beginning from Phe279 and Val304 were also observed. These results indicate that fraction **c** is an oligomerized form of the C-terminal fragment of CEL-III.

CD Spectra of the C-Terminal Fragment—As shown in Fig. 6, the monomeric form of the C-terminal fragment purified on a Sephadex G-50 column exhibited a far UV-CD spectrum with a negative peak around 200–205 nm. However, the oligomeric form of the fragment exhibited a very different spectrum showing a negative peak around 215–220 nm. This indicates that spontaneous oligomerization of the C-terminal fragments is accompanied by a drastic conformational change. Such a change in CD spectra was also observed when CEL-III oligomerized upon the binding of specific carbohydrates in solution (*4*), although the change was much smaller.

Hemagglutination by the C-Terminal Fragment of CEL-III—Figure 7 shows effects of the C-terminal fragment of CEL-III on rabbit erythrocytes. Although the monomeric form of the C-terminal fragment had no effect on erythrocytes (data not shown), the oligomeric form induced strong agglutination. Hemagglutination by the oligomeric form of the C-terminal fragment occurred without the addition of Ca^{2+}, while the activity was enhanced in presence of 10 mM Ca^{2+} and was completely abolished in the presence of 10 mM EDTA. This suggests that Ca^{2+}, tightly bound to the fragment, may be involved in the hemagglutinating activity. Hemagglutination was

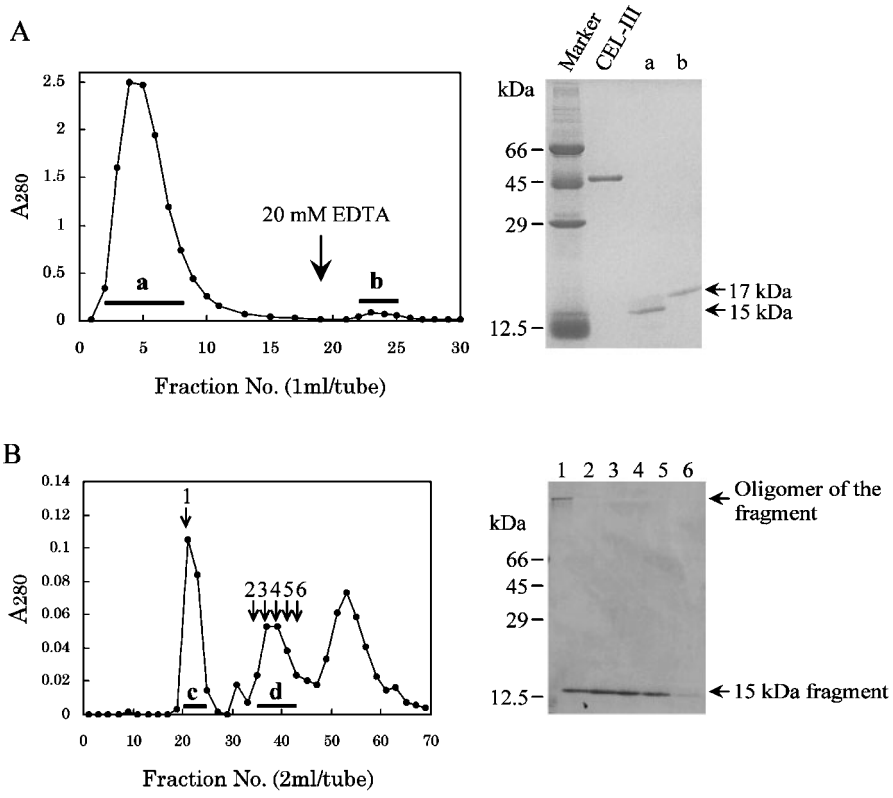

Fig. 5. **Separation of the tryptic digest of CEL-III on GalNAc-Cellulofine (A) and Sephadex G-50 (B) columns.** Flow-through fraction (**a**) eluted from the GalNAc-Cellulofine column was then applied to a Sephadex G-50 column. Fractions indicated with horizontal bars were pooled. (B) Vertical arrows indicate the samples examined by SDS-PAGE.

not inhibited by lactose, indicating that it is not caused by the carbohydrate-binding ability of the C-terminal fragment, but presumably by hydrophobic interactions between fragments and cell membranes. As described above, the CD spectra of the oligomeric and monomeric forms of the C-terminal fragment indicate considerably different secondary structures. It is possible that a conformational change accompanying oligomerization is closely related to the hemagglutinating action of the oligomer of the C-terminal fragment.

Proteolysis of the CEL-III Oligomer—Analysis of protease digests of preformed CEL-III oligomers further confirmed the importance of the C-terminal domain of CEL-III in the oligomerization process. CEL-III oligomerizes upon the binding of some disaccharides containing β-1,4-linked galactosides, such as lactose, lactulose, and *N*-acetyllactosamine, at high pH and high NaCl concentrations (*3*). This type of oligomer has characteristics similar to those of oligomers formed in the erythrocyte membrane during hemolysis. Figure 8 shows that protease-resistant large peptide fragments were produced when this oligomer was treated with papain, trypsin, or chymotrypsin. Since chymotrypsin generated the smallest fragment (91 kDa) with relatively high yield, this fragment was separated using a Sephacryl S-200 gel filtration column. The fragment showed no carbohydrate-binding ability when examined by affinity chromatography using a GalNAc-Cellulofine column (data not shown). Its N-terminal sequence was determined to be DDWEVPTAT after blotting onto the PVDF membrane. Therefore, the corresponding position was identified as Asp284 to Thr292 in the amino acid sequence of CEL-III. This position is very close to the cleavage site of trypsin as mentioned above, and also to the site cleaved by papain (Lys280-Trp281), which yielded the N-terminal carbohydrate-binding domain as reported in our previous paper (*7*). This indicates that the site around residue 280 is susceptible to several proteases, probably because this portion separates the N-terminal carbohydrate-binding domains from the C-terminal domain. To determine the number of polypeptides contained in the 91 kDa fragment, the fragment was treated with several reagents including 8 M urea, 7 M guanidine-HCl, and 30% formic acid. When treated with formic acid, the 91 kDa band disappeared and two smaller bands of about 16 kDa were newly formed (Fig. 8B), indicating that the fragment dissociated into constituent peptides. Therefore, the 91 kDa fragment of the CEL-III oligomer was assumed to be composed of six identical peptide fragments.

DISCUSSION

In the present study, the functional domains of CEL-III were isolated and their properties were examined to clarify their roles in the hemolytic mechanism of the protein. From the previously determined amino acid sequence (*7*), it was inferred that the N-terminal two-thirds contains two carbohydrate-binding domains that have homology with the B-chains of ricin and abrin. On the other hand, the C-terminal one-third shows no homology with any known protein. The C-terminal region was assumed to be involved in protein oligomerization and interaction with target cell membranes because of its hydrophobic region (residues 322–349). The results of affinity chromatography and hemagglutination assay (Fig. 3) confirmed that the N-terminal region (residues 1–283) has carbohy-

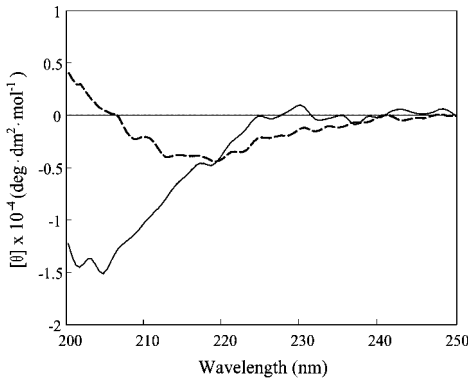

Fig. 6. **Far UV-CD spectra of the C-terminal fragment of CEL-III. Spectra were measured in a quartz cell with a 1 mm light path.** Spectra of the monomeric (solid line) and oligomeric (dashed line) forms of the C-terminal fragment (fractions **d** and **c** in Fig. 5B, respectively) were measured in TBS.

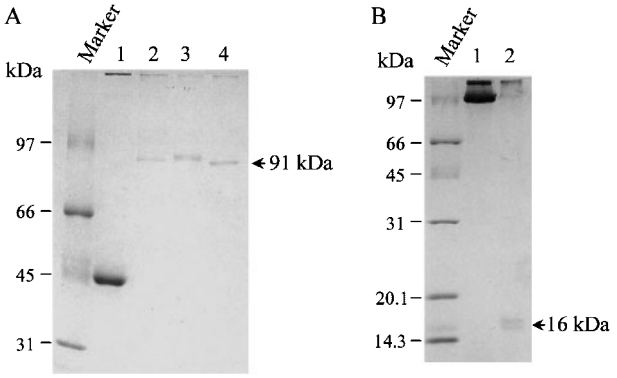

Fig. 8. **SDS-PAGE of protease digests of CEL-III oligomers.** (A) Proteolysis of the CEL-III oligomer: Lane 1, CEL-III monomer; lane 2, papain digest of the CEL-III oligomer; lane 3, tryptic digest; lane 4, chymotryptic digest. (B) Disassembly of the chymotryptic peptide of CEL-III. Purified chymotryptic peptides were treated with 30% formic acid for 30 min. After dialysis, the peptides were subjected to SDS-PAGE. Lane 1, chymotryptic large fragment of CEL-III; lane 2, formic acid-treated chymotryptic peptides.

drate-binding ability. Our previous data have already indicated that the proteolytic fragment of the N-terminal 12 kDa region has the ability to bind to a carbohydrate-immobilized column (*7*). In this study, the middle domain was obtained after limited proteolysis of CEL-III by trypsin; it was also shown to have carbohydrate-binding ability. Therefore, the N-terminal two-thirds contains two carbohydrate-binding domains.

CEL-III-induced hemolysis depends entirely on its carbohydrate-binding activity (*1*). Many amino acid residues in the ricin B-chain involved in carbohydrate-binding are conserved in CEL-III (*13*), suggesting that some of them may play similar roles in the CEL-III molecule. X-ray crystallographic study of the ricin-lactose complex indicated that five amino acid residues, Asp22, Gln35, Trp37, Asn46, and Gln47 in the N-terminal domain of the B-chain, and four amino acid residues, Asp234, Tyr248, Asn255, and Gln256 in the C-terminal domain, are involved in carbohydrate binding (*14*); the aromatic side chains of Trp37 and Tyr248 make stacking interactions with bound sugars, while other residues form hydrogen-bonds with hydroxyl groups in the sugars. In the sequence of CEL-III, the amino acid residues corresponding to those involved in the carbohydrate-binding of ricin B-chain, *i.e.*, Asp23, Ala34, His34, Gln44, Gln45 Asp256, Trp269, Asp276, and Gln277, are well conserved or at least replaced with similar amino acid residues (Fig. 1A). Mutation analyses of CEL-III CRD indicated that mutation of residues conserved between ricin B-chain and CEL-III, *i.e.*, Asp23, Gln44, Asp256, or Asp276, markedly reduces carbohydrate binding activity (Y. Kouzuma, unpublished data). This fact suggests that the carbohydrate binding mode of CEL-III CRD is very similar to that of ricin B-chain. On the other hand, there should be additional structural factors for Ca^{2+}-dependency of carbohydrate-binding ability. Little is known about the Ca^{2+}-binding manner of Ca^{2+}-dependent animal lectins except the canonical C-type lectins (*15*). Recently, a ricin-like domain was found in several proteins: the family F/10 xylanase from *Streptomyces olivaceoviridis* E-86 (*16–18*), EW1 lectin from earthworm *Lumbricus terrestris* (*19*), and human UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (*20*, *21*). However, there is no Ca^{2+}-dependency for carbohydrate-binding of these lectins. In this regard, CEL-III may be classified into a novel type of ricin-like protein family.

The N-terminal sequence of the monomeric form of the C-terminal fragment generated by tryptic digestion corresponds to the sequence beginning from Trp281. This position is between the second carbohydrate-binding domain and the C-terminal oligomerization domain that we inferred from amino acid sequence comparison. On the other hand, a considerable amount of the oligomeric form of the C-terminal fragment was also observed in flow-through fractions from gel filtration, indicating that a portion of the C-terminal fragments spontaneously oli-

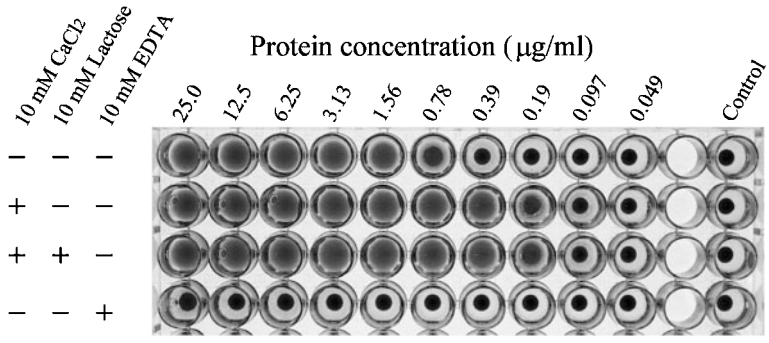

Fig. 7. **Interaction of the oligomeric form of the C-terminal domain fragments with erythrocytes as examined by hemagglutination assay.** The oligomeric form of the C-terminal fragments separated on a Sephadex G-50 column was serially diluted and mixed with rabbit erythrocytes in TBS with or without 10 mM $CaCl_2$, 10 mM lactose, and 10 mM EDTA. After 1 h, agglutination of cells was examined visually. Control represents samples without the addition of C-terminal fragments.

gomerized after cleavage by the protease. The CD spectra of monomeric and oligomeric forms of the C-terminal fragments differed considerably, indicating that oligomerization accompanies a conformational change in the fragment, as observed in the case of the oligomerization of the whole protein upon binding of lactose (*3*). The importance of the C-terminal domain in oligomerization was also supported by the results of limited hydrolysis of the CEL-III oligomers induced upon binding of lactose in solution. Chymotryptic digestion of the oligomer produced a 91 kDa fragment comprising only C-terminal fragments, indicating that C-terminal domains form a protease-resistant core in this oligomer. The number of polypeptides in this 91 kDa fragment is in good agreement with the number of polypeptide chains in the CEL-III oligomer as estimated from its size on SDS-PAGE (*3*).

It is also interesting that the oligomerized form of the C-terminal domain fragment induced hemagglutination. In contrast to hemagglutination caused by intact lectin (*1*), cell agglutination was not inhibited by lactose in this case. This suggests that the oligomerized form of the C-terminal domain interacts with cell membranes not by carbohydrate-binding activity, but probably based on its hydrophobic nature. In fact, we found that some synthetic 20mer peptides corresponding to the hydrophobic region in the C-terminal domain (residues 303–351) induced hemagglutination (T. Hatakeyama, T. Suenaga, S. Eto, T. Niidome, and H. Aoyagi, unpublished data). Our previous study indicated that CEL-III increases its surface hydrophobicity when forming an oligomer (*22*, *23*). Therefore, it is probable that increased surface hydrophobicity of CEL-III during oligomerization can be attributed to the association of its C-terminal domains. Probably, such an increase in hydrophobicity of the C-terminal domains is responsible for internalization of the CEL-III oligomer into a target cell membrane, leading to the formation of transmembrane pores. It is also noteworthy that hemagglutination induced by the C-terminal domain fragment was slightly enhanced by the addition of Ca^{2+}, and, in contrast, inhibited by EDTA. This suggests that there may be additional Ca^{2+}-binding sites in the C-terminal domain that aid in maintaining this domain structure.

As demonstrated in this study and predicted from amino acid sequence similarity with the B-chains of ricin and abrin, the N-terminal two-thirds of CEL-III is proved to contain two carbohydrate-binding domains. On the other hand, the C-terminal domain shows the ability to form oligomers resulting in increased hydrophobicity and interaction with the erythrocyte membrane. It is likely that these domains function cooperatively to induce hemolysis *via* the formation of transmembrane pores in target cells. We assumed previously that binding to cell-surface complex carbohydrates, such as lactosyl ceramide and globoside, triggers a conformational change in CEL-III to induce oligomerization in the lipid membrane (*6*). Although this study reveals the importance of the C-terminal domain in protein oligomerization, it remains unclear how binding to carbohydrate chains induces a conformational change in the C-terminal domain. Since we have obtained single crystals of CEL-III and its complex with lactose, X-ray crystallographic analysis in the future may provide further information concerning the carbohydrate-induced structural changes of this protein.

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