Effects of Chemical Modification of Carboxyl Groups in the Hemolytic Lectin CEL-III on Its Hemolytic and Carbohydrate-Binding Activities

Hiromiki Kuwahara, Takako Funada, Tomomitsu Hatakeyama,† and Haruhiko Aoyagi

Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Bunkyo-machi 1-14, Nagasaki 852-8521, Japan

Received November 8, 1999; Accepted January 31, 2000

Effects of chemical modification of carboxyl groups in the hemolytic lectin CEL-III on its activities were investigated. When carboxyl groups were modified with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and glycine methyl ester, hemolytic activity of CEL-III decreased as the EDC concentration increased, accompanied by reduction of oligomerization ability and hemagglutinating activity. However, binding ability of CEL-III for immobilized lactose was retained fairly well after modification, suggesting that one of two carbohydrate-binding sites might be responsible for such inactivation of CEL-III.

Key words: lectin; hemolysis; carboxyl group; chemical modification

Lectins are widely distributed among various animal tissues and body fluids, and play important roles in biological molecular recognition processes. Among four Ca$^{2+}$-dependent lectins isolated from a sea cucumber Cucumaria echinata, CEL-III has strong hemolytic activity, especially toward human and rabbit erythrocytes. Although CEL-III is a soluble monomeric protein (47.5 kDa) in solution, it forms membrane-associated oligomer corresponding to hexamer or heptamer, when incubated with erythrocytes, and leads to hemolysis. Formation of CEL-III oligomers in the cell membrane is assumed to be triggered by the conformational change of the protein after its binding to Gal- or GalNAc-containing glycolipids, such as lactosyl ceramide or globo-side. Therefore, it seems important to clarify the nature of carbohydrate-binding of CEL-III and its relationship with the hemolytic activity. We have previously found from a chemical modification study that amino groups in CEL-III were involved in the oligomerization process, but not important in its carbohydrate-binding and hemagglutinating activities.

To further discover the effects of modification of charged groups on the activities of CEL-III, we examined the modification of carboxyl groups in this study. In contrast to the case of amino groups, the modification of carboxyl groups led to considerable decrease in hemagglutinating activity as well as hemolytic activity, while the ability to bind to immobilized carbohydrate was only slightly affected. The results imply that hemolytic activity may depend on divalent binding of the protein to cell surface carbohydrate chains. CEL-III was purified from a protein extract of Cucumaria echinata, according to the method previously described. Protein concentration was calculated based on an absorbance value of 1.4 at 280 nm for 0.1% (w/v) protein solution, which had been measured with bicinchoninic acid by the method of Smith et al. using bovine serum albumin as a standard. The number of modified carboxyl groups were estimated using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager, PerSeptive Biosystems) in linear mode at an accelerating voltage of 25 kV. Bovine serum albumin was used as an external standard. Sinapinic acid was used as a matrix. Hemolytic activity of CEL-III was measured by the absorbance at 540 nm due to hemoglobin released from rabbit erythrocytes. The hemagglutination assay was done in the presence of dextran 4 as an osmotic protectant to prevent erythrocytes from lysis by CEL-III. The assay for carbohydrate-binding activity using lactose-coated microplates was essentially done as described previously.

Figure 1 shows the relationship of the number of carboxyl groups modified by EDC and glycine methyl ester, and hemolytic activity of the modified protein toward rabbit erythrocytes. As seen in this figure, hemolytic activity of modified CEL-III decreased as

† To whom correspondence should be addressed. Fax: +81-95-847-6749; E-mail: theta@net.nagasaki-u.ac.jp

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (to T.H.).

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline
concentration of EDC increased; hemolytic activity became less than 10% of that of native protein when 30 mM EDC was used, at which concentration about 34 carboxyl groups were estimated to be modified. This suggests that carboxyl groups are also involved in the hemolytic activity of this protein. The same modification reaction was done in the presence of 0.1 M lactose, a specific carbohydrate for CEL-III. However, the decrease in hemolysis was not significantly affected (data not shown).

Since formation of membrane pores by CEL-III oligomer is assumed to be the cause of hemolysis, we examined the change in oligomerizing ability of CEL-III in the erythrocyte membrane. After incubation of modified CEL-III with rabbit erythrocytes, its oligomer formed in the cell membrane was detected by immunoblotting analysis. As shown in Fig. 2A, the band around 270 kDa, corresponding to CEL-III oligomer, decreased as the modification by EDC proceeded, while the amount of CEL-III monomer was not significantly affected (Fig. 2B). This result indicated that modification of carboxyl groups inhibited oligomerization of CEL-III in the membrane, which is probably related to the decrease in the hemolytic activity. To discover the cause of such a decrease in oligomerization of CEL-III, hemagglutinating and carbohydrate-binding assays were done as shown in Fig. 3. Hemagglutinating activity (Fig. 3A) was measured in the presence of 30 mM dextran 4 as an osmotic protectant to prevent rupture of erythrocytes due to pore formation by CEL-III. As shown in this figure, hemagglutinating activity of the modified proteins decreased, showing a similar profile as that of hemolytic activity, suggesting that some carboxyl groups are also involved in the binding of CEL-III to

![Graph showing the number of modified carboxyl groups as a function of EDC concentration](image1)

**Fig. 1.** The Number of Modified Carboxyl Groups and Hemolytic Activity of Modified CEL-III as a Function of the Concentration of EDC.

Modification of the carboxyl groups of CEL-III was done by incubating the protein (0.5 mg/ml) with 1 mM glycine methyl ester and indicated concentrations of EDC in 20 mM phosphate-buffered saline, pH 5.8, for 15 min at 25°C. After dialysis against 10 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl (TBS), the protein was tested by the hemolytic assay. The number of succinyl groups was estimated by MALDI-TOF mass spectrometry based on the increase of the molecular mass of the modified protein due to introduced glycine methyl ester (mass of 71).

![Image showing immunoblotting analysis](image2)

**Fig. 2.** Immunoblotting Analysis of the Erythrocyte Membrane Treated with Modified CEL-III.

Rabbit erythrocyte suspension (5%, v/v) was incubated with CEL-III (0.5 mg/ml) modified using the indicated concentrations of EDC. After they were washed, the membranes were solubilized with the sample buffer for SDS-PAGE and used for SDS-PAGE (5% gel) and immunoblotting analysis (A). Detection was done using rabbit anti-CEL-III antibody and peroxidase-conjugated goat anti-rabbit IgG. The intensity of oligomer and monomer bands was expressed by densitometric analysis (B) using the computer program NIH Image (http://rnb.info.nih.gov/nih-image/).
Fig. 3. Changes in Hemagglutinating Activity and Carbohydrate-Binding Ability of CEL-III after Modification of Carboxyl Groups.

The hemagglutinating activity (A) was expressed as the titer that was the reciprocal of the highest dilution giving detectable agglutination. Solid-phase carbohydrate-binding assay (B) was done using a lactose-coated microplate25 with 0.5 mg/ml modified CEL-III. These assays were done in TBS-10 mM CaCl2. Open circles indicate the binding measured using the buffer containing 50 mM lactose.

erythrocytes, thus contributing to hemolysis. On the other hand, the ability to bind to immobilized carbohydrate indicated that the modification lead to only a slight decrease in the carbohydrate-binding ability (Fig. 3B). Even after modification with 40 mM EDC, nearly 50% of the binding remained, while the binding was considerably inhibited by lactose, confirming the specific interaction between the protein and immobilized lactose. This is consistent with the observation that a considerable amount of monomer was still bound to the erythrocyte membrane after modification (Fig. 2). One possible interpretation is that one of two carbohydrate-binding sites9 is still active even after modification, but divalent binding of CEL-III to the carbohydrate-chains on the cell surface is necessary to induce oligomerization of the protein as well as hemagglutination.

We have reported that succinylation of amino groups of CEL-III greatly reduced its hemolytic activity, while carbohydrate-binding activity was almost totally retained.40 In this study, similar reduction of hemolysis was observed when carboxyl groups were modified. However, in contrast to the case of modification of amino groups, modification of carboxyl groups considerably reduced hemagglutinating activity of the protein, and this may be closely related to the reduction of hemolytic activity. It cannot be excluded that modification of surface groups of the protein disturbed the interaction between protein molecules that is required for oligomerization. In fact, there was no significant protective effect of lactose in the experiments shown in Figs. 2 and 3 (data not shown), suggesting that the relevant carboxyl groups may not be located in the carbohydrate-binding sites. It is also possible that some carboxyl groups are involved in the binding of Ca\(^{2+}\), which is indispensable for carbohydrate-binding ability of CEL-III. Asp and Glu residues have already been shown to be the ligands of Ca\(^{2+}\), in the case of C-type lectins.9 Although CEL-III have recently been found not to be a C-type lectin,10 similar involvement of carboxyl groups in the binding of Ca\(^{2+}\) is very likely.

References


6) Hatakeyama, T., Murakami, K., Miyamoto, Y., and


