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Carbohydrate-Binding Properties of the Hemolytic Lectin CEL-III from the Holothuroidea *Cucumaria echinata* as Analyzed Using Carbohydrate-Coated Microplate

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The carbohydrate-binding properties of the hemolytic lectin CEL-III from the Holothuroidea *Cucumaria echinata* were studied using the microplate assay system which we have recently developed [Hatakeyama *et al.* (1996) *Anal. Biochem.* 237, 188-192]. When the binding of CEL-III to lactose covalently immobilized on a microplate was examined using colloidal gold solution, the binding was detected with as little as 1 μg/ml protein. Affinity of several carbohydrates to CEL-III was assessed by means of an inhibition experiment using the lactose-coated plate and it was found that *N*-acetylgalactosamine has the highest affinity for CEL-III, followed by lactose and lactulose. Examination of the binding of CEL-III to the lactose-coated plate at various pH values and temperatures revealed that the affinity is higher in the acidic pH region and at lower temperatures. From the Ca^{2+}-dependence profile for the binding of CEL-III to the lactose-coated plate, the apparent dissociation constant for Ca^{2+} was estimated to be 2.3 mM. These results suggested that the carbohydrate-binding properties of CEL-III are closely related to its hemolytic activity, although an additional interaction between the protein and the lipid bilayer, which is enhanced in the alkaline pH region, also seems to be necessary for its hemolytic action.

Key words: calcium, *Cucumaria echinata*, hemolysin, lectin, toxin.

CEL-III is a Ca^{2+}-dependent lectin from the Holothuroidea *Cucumaria echinata*, and exhibits strong hemolytic activity toward human and rabbit erythrocytes (*1*). This protein is a novel type of lectin with pore-forming activity, which leads to lysis of erythrocytes by colloid osmotic rupture of the cell membrane after its binding (*2*), as also occurs in the cases of pore-forming toxins of bacterial origin (*3-6*). Lysis of erythrocytes was effectively inhibited by GalNAc or galactose-containing carbohydrates, suggesting that hemolysis by CEL-III proceeds *via* binding to galactose- or GalNAc-containing receptors on the surface of erythrocytes. We have recently found that binding with specific carbohydrates, especially lactose, in the presence of a high concentration of NaCl at high pH, caused oligomerization of CEL-III in aqueous solution (*7*). The oligomerization was accompanied by a change in the secondary structure and a concomitant enhancement of the surface hydrophobicity of the protein. This suggests that binding to specific carbohydrate receptors on the erythrocyte surface may induce a conformational change of CEL-III to trigger its insertion into the cell membrane as an oligomer form, leading to the formation of ion-permeable pores in the membrane. Therefore, it appears very important to investigate the carbohydrate-binding nature of CEL-III in order to elucidate its oligomerization mechanism, which is closely related to the hemolytic process.

Here, we describe the carbohydrate-binding properties of CEL-III as examined using a microplate assay which we have recently developed (*8*). In this assay, the binding of the lectin to carbohydrates immobilized on a microplate, using divinyl sulfone, was measured with colloidal gold solution, which is very sensitive to the presence of protein.

MATERIALS AND METHODS

Materials—The *C. echinata* samples were generously provided by N. Ikeda (Fukuoka Fisheries and Marine Technology Research Center). Divinyl sulfone was purchased from Aldrich. Polystyrene microtiter plate and beads (amino-type) were from Sumitomo Bakelite (Tokyo). All the carbohydrates used were of the D-configuration.

Purification of CEL-III—CEL-III was purified from the body fluid of *C. echinata* using column chromatography on lactosyl-Cellulofine, GalNAc-Cellulofine, and Sephacryl S-200 as previously described (*2*). Chromatography was performed at 6°C. The purified protein was dialyzed against 10 mM Tris-HCl containing 0.15 M NaCl, pH 7.5 (TBS), and stored frozen at −30°C until use.

Protein Determination—Protein concentration was determined with bicinchoninic acid by the method given by Smith *et al.* (*9*) using bovine serum albumin as a standard.

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Abbreviation: TBS, Tris-buffered saline (10 mM Tris-HCl, 0.15 M NaCl, pH 7.5).

Microplate Assay for Carbohydrate-Binding Activity of CEL-III—The assay for carbohydrate binding activity was essentially performed as described previously (*8*). Immobilization of carbohydrates was carried out with a bifunctional cross-linking reagent, divinyl sulfone (*10*). To each microplate well having primary amino groups on the surface, 300 μl of divinyl sulfone (5%; v/v) dissolved in 0.5 M sodium carbonate buffer (pH 11) was added, and the plate was kept at 25°C for 1 h. It was washed with 0.5 M sodium carbonate buffer (pH 11), 300 μl of carbohydrate solution (10%; w/v) in the same buffer was added to each well, and the plate was incubated at 25°C overnight. The remaining vinyl groups were blocked with 1 M Tris for 1 h at room temperature, and the wells were washed with TBS. CEL-III (200 μl) in TBS containing 10 mM $CaCl_2$ was incubated in the wells coated with carbohydrate for 1 h at 6°C, then the wells were washed once with ice-cold TBS to minimize dissociation of the protein bound to the carbohydrates on the wells. Colloidal gold solution (200 μl) prepared as described previously (*8*) was then added to the wells, and after 30 min, the absorbance at 620 nm was measured as an indicator of the amount of bound protein, using a microplate reader (Immuno Mini NJ-2201, Intermed, Tokyo).

For determination of the temperature-dependence of the carbohydrate-binding activity of CEL-III, polystyrene beads (diameter: 6.35 mm) with amino groups on the surface were used instead of the microplate. Immobilization of lactose on the beads was done in the same manner as for the microplate, and the reaction was carried out in small siliconized glass test tubes. Siliconization was done by rinsing the test tubes in 5% dichlorodimethylsilane in chloroform, followed by baking at 180°C for 2 h (*11*).

RESULTS

Binding of CEL-III to a Microplate Coated with Lactose—Figure 1 shows the binding of CEL-III to a microplate coated with lactose by using divinyl sulfone. The binding of the protein was determined by measuring the absorbance at 620 nm arising from the colloidal gold complexed with the protein. The amount of the binding increased with increasing concentration of CEL-III, and reached the maximum with 5 μg/ml of the protein. The binding was competitively inhibited by 50 mM lactose, indicating a specific interaction of the protein with the immobilized lactose. The affinity of various carbohydrates for CEL-III was examined in a competitive inhibition experiment, as shown in Fig. 2 and Table I. In this experiment, the binding of CEL-III to the lactose-coated plate was measured in the presence of various free carbohydrates. GalNAc, lactose, and lactulose were found to be the most effective inhibitors, in agreement with the results of the inhibition experiment on hemolysis (Table I). Other galactose-related carbohydrates, such as galactose, melibiose, raffinose, methyl α-galactoside, and methyl β-galactoside showed moderate affinity for CEL-III. Methyl β-galactoside was found to have a higher affinity than methyl α-galactoside. In fact, melibiose (Galα1→6Glc) and raffinose (Galα1→6Glcα1→2βFru) showed relatively weak affinity compared with lactose (Galβ1→4Glc) and lactulose (Galβ1→4Fru). It appears that the C-6 hydroxyl group of galactose does not contribute to the binding, because fucose exhibited essentially the same affinity as galactose. Even nonspecific carbohydrates, such as glucose, mannose, and GlcNAc, showed some binding.

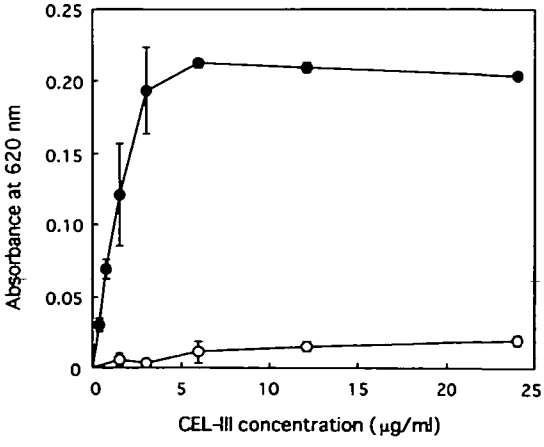

Fig. 1. **The binding of CEL-III to lactose-coated microplate wells.** Various concentrations of CEL-III (200 μl) in TBS containing 10 mM $CaCl_2$ were incubated in the microplate wells coated with lactose in the absence (●) or presence (○) of 50 mM lactose at 6°C. Bars represent the means±SD of three measurements.

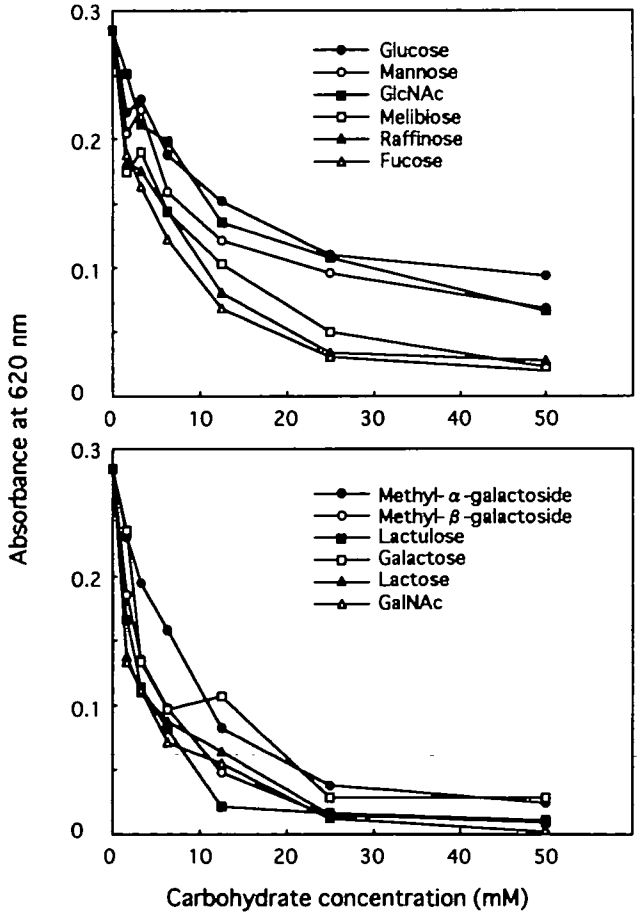

Fig. 2. **Inhibition of the binding of CEL-III to the lactose-coated plate by several carbohydrates.** CEL-III (40 μg/ml; 200 mμ) in TBS containing 10 mM $CaCl_2$ was incubated at 6°C in the wells in the presence of various concentrations of the indicated carbohydrates.

This is in contrast to the fact that glucose and mannose did not inhibit hemolysis by CEL-III (*1*) (Table I).

Effects of pH on the Carbohydrate-Binding of CEL-III—Figure 3 shows the pH-dependence profile for the binding of CEL-III to the lactose-coated plate. The binding of CEL-III to the plate was observed over a wide range of pH from 4 to 10, indicating that CEL-III is rather stable in terms of the binding ability in this pH region. Affinity of CEL-III was higher in the acidic pH region, and the highest binding was seen at pH 4, which was the lowest pH tested in this experiment. It is apparent that the increase in the binding at lower pH is not due to nonspecific adsorption of the protein on the plate caused by acid or alkaline denaturation, since 0.1 M lactose inhibited the binding of CEL-III almost completely.

Effects of Temperature on the Carbohydrate-Binding of CEL-III—The temperature dependence of the binding of CEL-III was measured using lactose-coated polystyrene beads. Since the surface of the beads has the same chemical nature as the microplate, lactose could be immobilized in the same procedure using divinyl sulfone. Individual beads were incubated with CEL-III at different temperatures then washed with the buffer, and bound lectin was detected with the colloidal gold solution. As shown in Fig. 4, the binding of CEL-III to the beads increased with lowering of the temperature, and the maximal binding was attained at around 10°C. Specificity of the binding was confirmed by the measurement in the presence of 50 mM lactose; only a little binding was observed in the presence of lactose.

Effects of Ca^{2+} on the Carbohydrate-Binding of CEL-

TABLE I. **Comparison of the carbohydrate concentration required for 50% inhibition of CEL-III-binding (K_i) to the lactose-coated plate with hemolysis by CEL-III in the presence of the carbohydrates at 6.3 mM.**

Carbohydrate	K_i[a] (mM)	Hemolysis[b] (%)
GalNAc	1.8	12.5
Lactose	2.0	18.8
Lactulose	2.1	15.6
Methyl β-galactoside	2.9	28.1
Methyl α-galactoside	6.2	39.1
Fucose	4.0	34.4
Galactose	4.2	40.6
Melibiose	5.5	46.9
Raffinose	4.7	56.3
Mannose	9.7	100
GlcNAc	13.0	—
Glucose	14.7	100

[a] K_i was calculated by nonlinear least-squares fitting using the equation: $A_{620} = A_{620max} - A_{620max} \cdot [carbohydrate]/([carbohydrate] + K_i)$, where A_{620max} represents the absorbance at 620 nm measured in the absence of competitive carbohydrate. [b] Hemolysis by CEL-III was measured using rabbit erythrocytes (*1*).

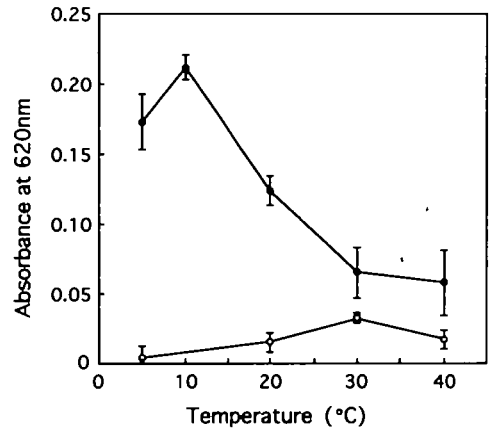

Fig. 4. **Temperature dependence of the binding of CEL-III to the lactose-coated plate.** CEL-III (24 μg/ml; 400 μl) in TBS containing $CaCl_2$ was incubated with lactose-coated polystyrene beads in the absence (●) or presence (○) of 50 mM lactose in siliconized glass test tubes at 6°C. After 1 h, the beads were washed with 1 ml of TBS and CEL-III bound to the surface of the beads was detected with 0.5 ml of colloidal gold solution.

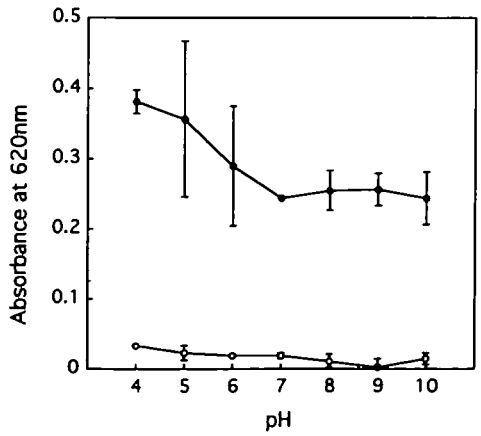

Fig. 3. **pH-dependence of the binding of CEL-III to the lactose-coated plate.** CEL-III (24 μg/ml; 200 μl) in buffers of different pH was incubated in the wells at 6°C in the absence (●) or presence (○) of 50 mM lactose. The buffers used were 10 mM sodium acetate (pH 4 and 5), 10 mM Bis-Tris-NaOH (pH 6 and 7), 10 mM Tris-HCl (pH 8), and 10 mM glycine (pH 9 and 10), each containing 0.15 M NaCl and 10 mM $CaCl_2$. Bars represent the means ± SD of three measurements.

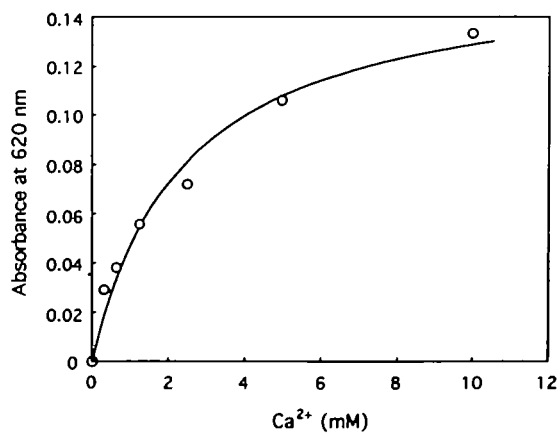

Fig. 5. **Ca^{2+}-dependence of the binding of CEL-III to the lactose-coated plate.** The binding of CEL-III (18 μg/ml; 200 μl) to the plate was measured after incubation in TBS containing various concentrations of $CaCl_2$ at 6°C. The curve was obtained from nonlinear least-squares fitting to the data using the equation: $A_{620} = A_{620max} \cdot [carbohydrate]/([carbohydrate] + K_d)$, where A_{620max} and K_d represent the maximum value of the absorbance at 620 nm and the apparent dissociation constant for the binding of Ca^{2+}.

III—Figure 5 shows the Ca^{2+}-dependence curve of the binding of CEL-III to the lactose-coated plate. The binding increased with increasing concentration of Ca^{2+}, and full activation was attained at about 10 mM. From the curve, the apparent dissociation constant (K_d) for the binding of Ca^{2+} to CEL-III was estimated to be 2.3 mM. This value is comparable to the Ca^{2+} concentration required for 50% hemolytic activity (about 1 mM) (*1*). This suggests that the Ca^{2+}-dependency of the hemolytic activity is closely related to the Ca^{2+} requirement of CEL-III for binding to the carbohydrate receptors on the erythrocyte surface.

DISCUSSION

In the previous paper, we suggested the usefulness of our microplate assay for quantitative evaluation of the interaction of lectins with various carbohydrates (*8*). This method could be widely applicable to various lectins with different carbohydrate-binding specificity, since any carbohydrate can be readily immobilized on the microplate by the same procedure. Furthermore, there is no need to label lectins with radioactive isotopes or marker enzymes. The sensitivity of this method is fairly high, and the assay can be carried out under various conditions, *e.g.*, at different pH values and temperatures.

When the microplate coated with lactose was used, the binding of CEL-III was detected with at least 1 μg/ml protein (Fig. 1). Comparable sensitivity of this assay was observed when other plant lectins, such as concanavalin A, wheat germ agglutinin, and *Ricinus communis* agglutinin (RCA_{120}) were examined (*8*). CEL-III was also effectively bound to the microplate coated with GalNAc, whereas effective binding was not observed when other carbohydrates, such as galactose, fucose, methyl α-galactoside, methyl β-galactoside, and lactulose, were immobilized on the microplate (data not shown). This may be related to the efficiency of immobilization of different carbohydrates on the microplate. The affinity of several carbohydrates to CEL-III was assessed by means of an inhibition experiment using lactose-coated microplates. Their affinity was in agreement with the inhibition profile for hemolytic activity of CEL-III (*1*), suggesting the importance of the binding of CEL-III to the carbohydrate receptors on the erythrocyte surface for its hemolytic action.

Upon the binding of lactose in the presence of a high concentration of NaCl at high pH, CEL-III forms an oligomer with an apparent molecular mass of 270 kDa that is the same size as the oligomer formed in the membrane of erythrocytes lysed by CEL-III (*7*). The oligomerization is accompanied by an alteration of the secondary structure of the protein as well as an enhancement of the surface hydrophobicity of the protein. Such a change in the three-dimensional structure of CEL-III is probably triggered by a local conformational change caused by the binding of lactose. Interestingly, the oligomerization of CEL-III could not be induced by the binding of GalNAc (*7*), even though GalNAc showed the highest affinity for CEL-III, and is also the strongest inhibitor of hemolysis (*1*). This suggests that GalNAc might be too small in size to induce the conformational change of the protein which is required for the oligomerization of the protein.

The binding of CEL-III to the lactose-coated beads at different temperatures indicated that the affinity is the highest around 10°C, and it markedly decreases with increasing temperature. At least in part, this may be related to the fact that CEL-III shows the highest hemolytic activity around 10°C. Under these conditions, CEL-III might readily interact with erythrocyte membrane irrespective of membrane fluidity, once it has bound to its carbohydrate receptors. A similar correlation between receptor-binding and hemolytic activities has also been reported in the case of *Staphylococcus aureus* α-toxin, in which both maximal binding of the toxin and maximal hemolysis were seen below 25°C and they were markedly reduced by raising the temperature (*12*).

The apparent K_d value for the binding of Ca^{2+} to CEL-III (2.3 mM) is comparable to the Ca^{2+} concentration required for 50% hemolytic activity (1 mM) (*1*), indicating that Ca^{2+} promoted hemolysis by activating the carbohydrate-binding activity of CEL-III. However, this value is fairly large compared with that for mannose-binding protein (about 0.1 mM), one of the most extensively studied C-type lectins (*13*). It is probable that CEL-III may not belong to the C-type lectin family. In fact, our preliminary sequence analysis of some peptides obtained from proteolytic digestion of CEL-III has suggested very limited homology with the C-type lectin family (unpublished data).

The affinity of CEL-III for lactose increased with decreasing pH, although almost no hemolytic activity was observed in the acidic pH region, and, on the contrary, the activity greatly increased with increasing pH in the alkaline region (*2*). Effective binding of CEL-III to the carbohydrate receptors in the acidic pH region was also suggested by the observation that CEL-III showed slightly higher hemagglutinating activity around pH 5-6 than at alkaline pH, when dextran was used as an osmotic protectant for erythrocytes (data not shown). Therefore, it is apparent that the binding of CEL-III to the carbohydrate receptors alone is not enough to trigger the hemolytic process; the subsequent interaction between the protein and the lipid bilayer, which could be facilitated in the alkaline pH region, also seems to be necessary.

As revealed in this study, there is a close correlation between the carbohydrate-binding and hemolytic activities of CEL-III, suggesting that the binding of CEL-III to the carbohydrate receptors on the erythrocyte surface, which concentrates the protein on the surface of erythrocytes, is essential for the hemolytic action. However, it appears that the binding of CEL-III to the receptors is not the sole factor governing the extent of hemolysis, as suggested by the pH-dependence of the lactose-binding activity. There should be an additional step in which the protein directly interacts with the lipid bilayer, involving a conformational change. Studies on the structural change of CEL-III following binding to the carbohydrate receptors should provide important clues for elucidation of the mechanism of its hemolytic action or membrane pore-forming ability.

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