



Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga

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ABSTRACT

A sulfated polysaccharide, named fucoidan, from the marine alga *Cladosiphon okamuranus* is comprised of carbohydrate units containing glucuronic acid and sulfated fucose residues. Here we found this compound potently inhibits dengue virus type 2 (DEN2) infection. Viral infection was inhibited when DEN2, but not other serotypes, was pretreated with fucoidan. A carboxy-reduced fucoidan derivative in which glucuronic acid was converted to glucose did not inhibit viral infection. Elimination of the sulfated function group from fucoidan significantly attenuated the inhibitory activity on DEN2 infection with <1% fucoidan. DEN2 particles bound exclusively to fucoidan, indicating that fucoidan interacts directly with envelope glycoprotein (EGP) on DEN2. Structure-based analysis suggested that Arg323 of DEN2 EGP, which is conformationally proximal to one of the putative heparin binding residues, Lys310, is critical for the interaction with fucoidan. In conclusion, both the sulfated group and glucuronic acid of fucoidan account for the inhibition of DEN2 infection.

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Dengue virus is an envelope virus that causes human diseases, such as dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. There are four serotypes of dengue virus, type 1 (DEN1) to type 4 (DEN4), which have similar clinical manifestations and epidemiology in tropical and subtropical regions of the world where more than two billion people are at risk of infection [1–3]. The viruses are circulated and amplified by transmission to humans through *Aedes* mosquitoes [4]. The adsorption of viruses to the host cell surface is the initial and critical step for viral infection. Envelope glycoprotein (EGP) is a viral membrane protein involved in the early events of dengue virus infection, such as binding of the virus to the host cell surface and fusion between viral and host cell membranes [5,6]. EGP consists of three functional domains (domains I, II and III). Domain III is critical for virus adsorption to host cell receptors [7]. Recent X-ray crystallography and NMR studies demonstrated the three-dimensional structures of EGP or domain III of flaviviruses, dengue virus type 2, 3 and 4 [8–10]. Putative receptor molecules for dengue viruses have been reported, such as sulfated proteoglycans. Sulfated and non-sulfated carbohydrate molecules on the surface of host cells seem to be involved in the interaction with flaviviruses [11,12]. The receptor molecules used for binding and entry of dengue

viruses are apparently distinct between cell types and virus serotypes [13,14]. However, the molecular features of cellular receptors and the molecular mechanisms of virus entry have yet to be fully elucidated.

Dengue virus belongs to the family Flaviviridae the same family as Japanese encephalitis and yellow fever viruses, which are controlled by specific vaccinations. However, no licensed dengue vaccines or anti-dengue agents are clinically available.

Fucoidans are sulfated polysaccharides extracted from marine brown seaweeds that possess some biological activities similar to those of heparin [15,16]. Previous studies have shown that fucoidan mediates significant biological effects on mammalian cells. Particularly, fucoidans from brown seaweeds show anti-inflammatory and anti-coagulant activities [16]. These fucoidans show the anti-viral effects against infectious diseases, such as human immunodeficiency virus (HIV), herpes simplex virus and cytomegalovirus [17]. Recently, it was reported that sulfated polysaccharides from the red seaweeds and sulfated galactomannans from seeds of *Mimosa scabrella* inhibit *in vitro* and *in vivo* infection of flaviviruses, such as dengue and yellow fever viruses [18,19]. As these polysaccharides show no toxicity or irritation in humans, fucoidans may be useful as anti-viral agents as well as anti-coagulant and anti-inflammatory agents. However, there have been very few studies of relationships between the biological activities of fucoidans and molecular structures.

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The chemical structure of the *Cladosiphon* fucoidan has recently been described in detail [20,21]. The *Cladosiphon* fucoidan has one sulfate group for every two molecules of fucose. Furthermore, this fucoidan has one glucuronic acid residue for every six molecules of fucose as a branched chain.

In the present study, we examined the anti-dengue virus activity of fucoidan from the marine alga, *Cladosiphon okamuranus*. We also investigated molecular mechanisms of susceptibility of four serotypes of dengue virus to the fucoidan by structure-based analyses.

Materials and methods

Materials. Fucoidan and its derivatives were provided by Yakult Central Institute for Microbiological Research, Tokyo, Japan. These compounds were prepared as described previously [20]. All other chemicals were of the highest quality commercially available.

Cell culture and viruses. BHK-21 was cultured at 37 °C under 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) with 5% FBS. Dengue virus type 1 (DEN1), D1/Lao/03 strain, type 2 (DEN2), ThNH-7/93 strain (GenBank Accession No. U31949), type 3 (DEN3), D3/BDH02-01 strain (Accession No. AY496871), and type 4 (DEN4), ThD4-17/97 (Accession No. AY618989) were propagated in C6/36 cells as described previously [22].

Inhibition of virus infection by fucoidan and its derivatives. Virus infection was determined by focus-forming assay using BHK-21 cells as described previously [12]. BHK-21 cells were seeded onto 96-well plates and cultured for 24 h at 37 °C in DMEM supplemented with 2% FBS. After removal of medium, the virus–fucoidan premixtures were then inoculated for 2 h at 37 °C onto the cells. After washing with serum-free DMEM, overlay medium was added and plates were incubated at 37 °C for 43 h. Infectious foci were detected with human anti-dengue antisera, followed by HRP-conjugated goat anti-human immunoglobulin. Virus infectivity was determined as focus-forming units (FFU). The optimal titer of inoculated virus was predetermined such that more than 50 foci appeared per well.

Solid-phase virus-binding assay. The binding activities of DEN2 for fucoidan and its derivatives in solid-phase virus-binding assay were evaluated as described previously [23]. Briefly, fucoidan or its derivatives in phosphate-buffered saline were immobilized on wells of plastic plates (Universal BIND 1 × 8 Stripwell; Corning Inc., Corning, NY) by UV-crosslinking. After blocking with PBS containing 1% BSA, the plates were incubated for 1 h at 28 °C with virus solution. After washing, the plates were incubated for 1 h at 28 °C with human anti-dengue antiserum, followed by HRP-conjugated goat anti-human immunoglobulin. The complexes were detected by incubation with *o*-phenylenediamine. The absorbance was measured at 492 nm.

Preparation and cellular binding of DEN2 labeled with a fluorescence dye, DiO. A lipophilic dye, DiO (3,3'-dilinoleoyloxycarbocyanine perchlorate, FAST DiO™; Molecular Probes, Eugene, OR) was used to label virus particles as described previously [24]. All procedures for labeling of the virus were carried out without light. The virus (7.0×10^6 FFU/ml) was incubated at room temperature for 10 min in VP-SFM (Invitrogen, Carlsbad, CA) containing 6.4 μM FAST DiO. The labeling solution contained 8% PEG 6000 and 2.2% NaCl at the final concentration. The solution was then kept at 4 °C overnight. The labeled virus was sedimented (8700g) at 4 °C for 1 h and resuspended in PBS. The labeled virus was stored at –80 °C before use.

Direct binding activity of labeled dengue viruses to cultured cells was performed as follows. BHK-21 cells were seeded onto 96-well plates and cultured at 37 °C in DMEM supplemented with 10% FBS. After blocking with DMEM containing 2% BSA, the plates were incubated at 4 °C for 2 h in DMEM containing dengue virus

(10^6 FFU/ml). The bound virus was lysed at room temperature for 10 min with 1% Triton X-100 solution. Fluorescence was measured at 485 nm (excitation) and 535 nm (emission). The virus-binding activity was determined from the quantity of DiO associated with the cell surface.

Sequencing of dengue virus cRNA. A fragment of domain III of the DEN1 EGP gene was reverse transcribed and amplified from RNA extracted from the purified virus using *Taq* DNA polymerase, and the PCR products were directly sequenced.

Homology modeling. The Swiss-Model automated comparative protein modeling server (<http://swissmodel.expasy.org/SWISS-MODEL.html>) [25] was used for comparative structural analysis to model three structures of DEN1 (D1/Lao/03 strain), DEN2 (ThNH-7/93 strain) and DEN4 (ThD4-17/97) domain III onto those of DEN3 (PDB ID: 1UZG) [9], DEN2 (PDB ID: 1OKE) [6] and DEN4 (PDB ID: 2HOP) [10] proteins, respectively. DEN3 (D3/BDH02-01 strain) domain III is shown on the basis of the structure of PDB accession number 1UZG. VMD 1.8.6. OpenGL [26] tools running on UNIX were used to visualize all figures.

Results and discussion

Anti-dengue virus effects of fucoidan and its derivatives

Although previous studies indicated that sulfated polysaccharides from other natural sources showed anti-dengue virus activity, the molecular mechanisms of the inhibitory effects of these compounds have not been elucidated [18,19]. Fucoidan from the marine alga *C. okamuranus* was used for viral infection assay in the present study. This fucoidan was chosen as an anti-viral agent for the following reasons. Recently the carbohydrate structure has been defined well. The *Cladosiphon* fucoidan is comprised of a repeating unit of sulfated fucose and glucuronic acid residues [20]. In addition, its derivatives have been generated by chemical modifications [20], such as elimination of the sulfated group or reduction of carboxylic acid resulting in a desulfated derivative termed FD and a carboxy-reduced derivative termed FC, respectively (Fig. 1A). Fucan, a fucose polymer, was used for control experiments. Similar to other fucoidans, the *Cladosiphon* fucoidan mediates a variety of biological effects on mammalian cells. This fucoidan was more effective for healing of gastric ulcers than that from *Fucus vesiculosus* [21]. The *Cladosiphon* fucoidan also showed an inhibitory effect on the adhesion of *Helicobacter pylori* to carbohydrate ligands [27]. In dengue virus infection, the *Cladosiphon* fucoidan significantly inhibited DEN2 infection to BHK-21 cells in a dose-dependent manner (Fig. 1B). Treatment of the virus with 10 μg/ml fucoidan reduced the infectivity by 20% compared with that in untreated cells. The inhibitory activity of fucoidan is equivalent with that of heparin, which is a competitive entry inhibitor, as described previously [11,28]. Three types of fucoidan derivative were simultaneously examined for effects on infection of BHK-21 cells with DEN2. Sulfation is required for anti-dengue virus activity of glycan [28]. As expected, desulfation from fucoidan (FD or fucan) showed marked suppression of inhibitory activity (Fig. 1B). Interestingly, carboxy-reduction knocked out the effect of fucoidan against DEN2 infection. These findings strongly suggest that the glucuronic acid residue as well as sulfated fucose are essential for the inhibitory activity of fucoidan. Four dengue virus serotypes were premixed with fucoidan at various concentrations, and the premixtures were inoculated onto BHK-21 cells. The results are summarized in Table 1. The effects of fucoidan on DEN2 infection were much greater than those on the other dengue serotypes examined. Particularly, fucoidan did not inhibit DEN1 infection of BHK-21 cells under the experimental conditions used here. This observation strongly suggested that the

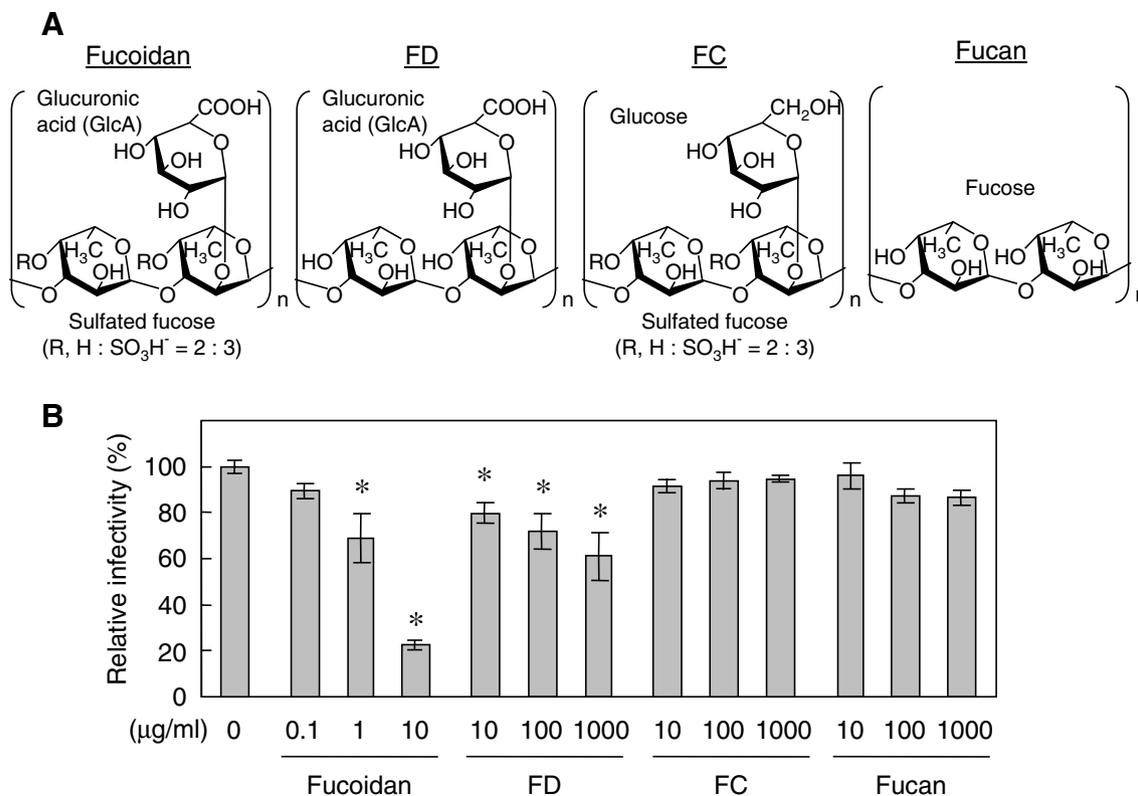


Fig. 1. Structures of fucoidan from *Cladosiphon okamuranus* and its derivatives used in this study. (A) Structures showing the repeating unit of polysaccharides. FC, a derivative prepared by reduction of the glucuronic acid residue in fucoidan; FD, a derivative obtained by elimination of the sulfated group in fucoidan; Fucan, fucose polymer. (B) Effects of fucoidan and its derivatives on DEN2 infection. Values indicate the averages of infectivity ratios of DEN2 with polysaccharides at the indicated concentrations relative to viral infection alone. Bars show standard deviation of triplicate measurements. Statistical significance was determined by *t*-test ($P < 0.01$). The results shown are representative data from three independent experiments.

Table 1
Effect of fucoidan on viral infection

Virus (serotype)	IC ₅₀ (µg/ml)
DEN1	>1000
DEN1	4.7
DEN1	500
DEN1	365

IC₅₀, concentration of 50% of inhibition. Infection experiments were performed as described in Materials and methods.

Inhibitory action of fucoidan on viral infection depends on distinction of EGP structures based on amino acid residues that may influence interaction of the virus with fucoidan.

Binding activity of DEN2 to fucoidan and its derivatives

To clarify the structural determinant responsible for the interaction with DEN2, we examined DEN2 binding to fucoidan and its derivatives by solid-phase virus-binding assay. Previously, we established a direct binding assay with carbohydrate molecules for influenza viruses [23]. In the present study, we applied the assay for determination of the direct binding dynamics of fucoidan to DEN2. Fig. 2A shows the results of solid-phase virus-binding assay. The virus particles showed significant binding activity to native fucoidan immobilized on plastic plates in a dose-dependent manner, but did not bind to other derivatives. This observation clearly indicated that both glucuronic acid and sulfated fucose residues were involved in the interaction with DEN2.

In addition, we examined the effects of fucoidan and its derivatives on the direct binding of DEN2 to BHK-21 cells. In accordance

with a previous report by van der Schaar et al. [24], the virus particles were labeled with the fluorescent probe, DiO. The labeled DEN2 was used for determination of cellular binding activity. Fig. 2B shows binding of the labeled virus to the cells in the presence or absence of compounds. Although fucoidan marginally inhibited the cellular binding of DEN2, the inhibition was dose-dependent. Heparin as a positive control also showed inhibition of virus binding. In comparison to infection experiments, the inhibitory activity of the virus binding was apparently lower than expected (see Fig. 1B). As fucoidan is thought to inhibit virus binding to the cells in a competitive manner, lower activity may be observed when fucoidan was used at the same concentration as in the infection experiments. On the other hand, fucoidan derivatives such as FD or FC did not inhibit binding of DEN2 to BHK-21 cells. This result strongly suggested that both glucuronic acid and sulfated fucose residues accounted for inhibition of the virus binding to BHK-21 cells. Taken together with the findings of infection experiments, both glucuronic acid and sulfated fucose residues of the *Cladosiphon* fucoidan appear to critically affect the interaction of DEN2 with cellular receptors.

Sequencing and modeling analyses of dengue virus types 1–4

Of the dengue virus serotypes, the DEN2 strain ThNH-7/93 was highly susceptible to the *Cladosiphon* fucoidan. The inhibitory effect of fucoidan on ThNH-7/93 infection was almost 100-fold greater than that on infection by DEN3 or DEN4 strains (Table 1). Another strain, DEN1 (D1/Lao/03), showed no susceptibility to fucoidan under our experimental conditions. To elucidate the molecular basis of susceptibility of the *Cladosiphon* fucoidan, the

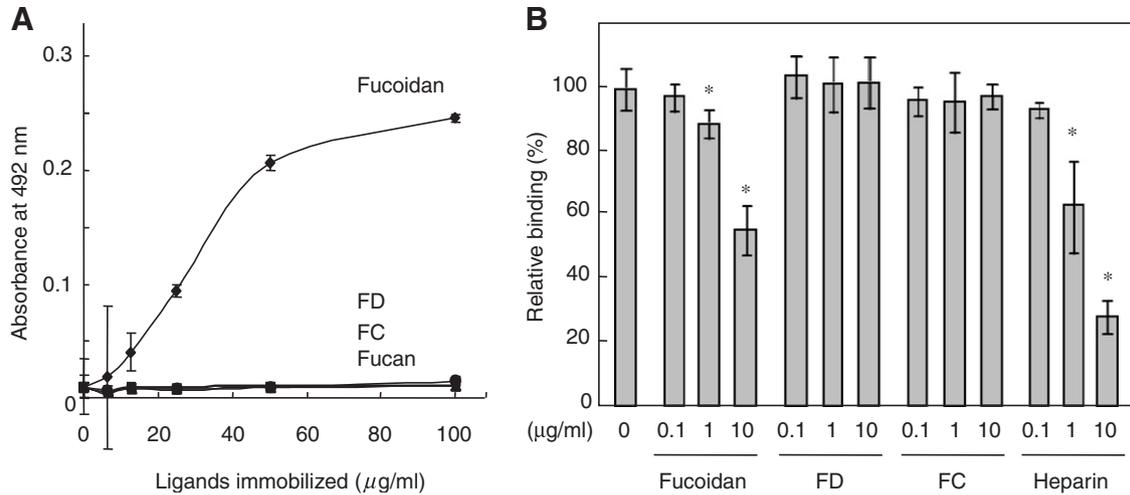


Fig. 2. Functional interaction of fucoidan with DEN2 particles. (A) DEN2 binding to fucoidan and its derivatives immobilized on plastic plates. Wells were coated with fucoidan (diamonds), FD (squares), FC (triangles), and fucan (circles) at the indicated concentrations. Values indicate the averages of viruses bound to each sample. Bars show standard deviation of triplicate measurements. The results shown are representative data from three independent experiments. (B) Inhibitory activity of fucoidan on DEN2 binding to BHK-21 cells. Values indicate the averages of cellular binding ratios of DEN2 with polysaccharides at the indicated concentrations relative to virus alone. Bars show standard deviation of triplicate measurements. FC, a derivative prepared by reduction of the glucuronic acid residue in fucoidan; FD, a derivative obtained by elimination of the sulfated group in fucoidan. Heparin was used as a positive control. Statistical significance was determined by *t*-test (*P* < 0.01). The results shown are representative data from three independent experiments.

nucleotide sequence of DEN1 EGP was examined and multiple-alignment analysis of the amino acid sequences of the proteins of the four-serotype strains was performed (Fig. 3A). Basic amino acid

residues at positions 295 and 310 are critically involved in the interaction with sulfated glycosaminoglycans such as heparin and highly sulfated heparan sulfate, as described previously [29].

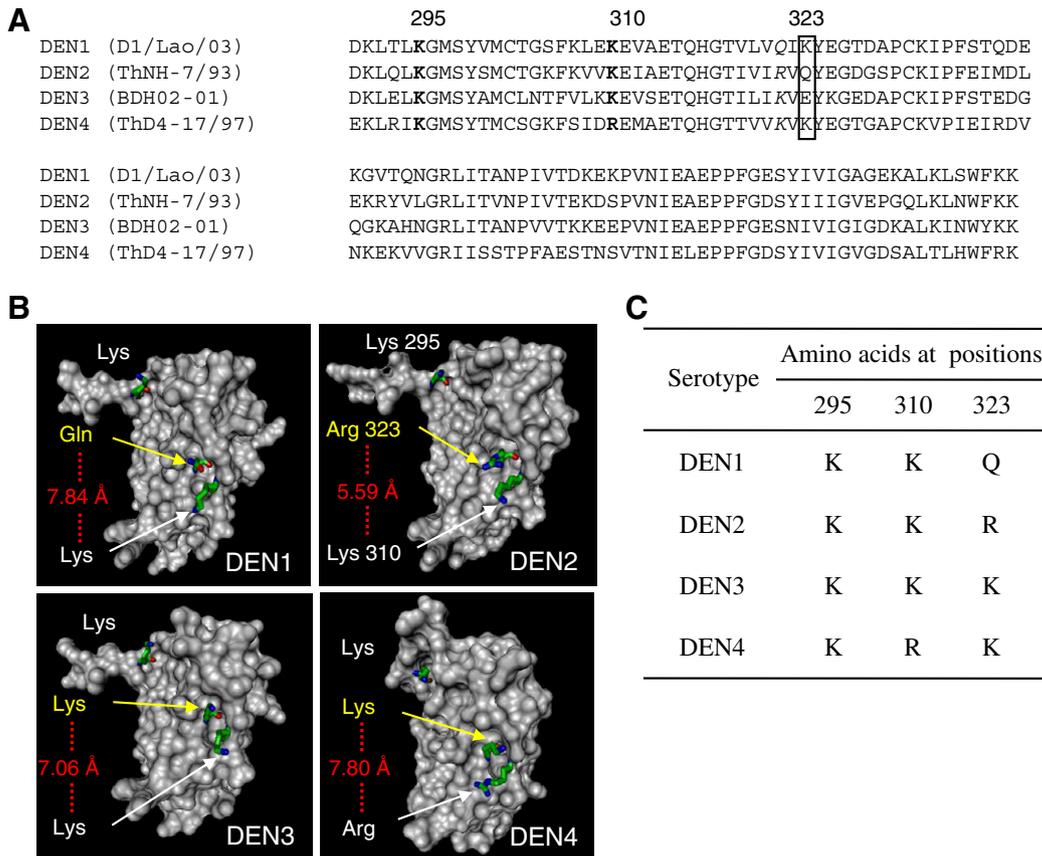


Fig. 3. Prediction of basic amino acid residues responsible for the interaction of domain III with fucoidan. (A) Alignment of amino acid sequences of domain III of envelope glycoprotein among dengue virus types 1–4. Basic amino acid residues 295 and 310 of DEN2 and the corresponding residues of other serotypes are shown in bold. Amino acid residue 323 (boxed) may be critical for the interaction with fucoidan. (B) The structures of domain III are shown on the basis of the three-dimensional structures. Distances between nitrogen atoms of side chains of Lys310 (Arg310 in DEN4) and Arg323 in DEN2 (Gln323 in DEN1, and Lys323 in DEN3 and DEN4) are shown as red dotted lines. (C) Summary of consensus amino acids at positions 295, 310 and 323 of domain III of the four serotypes. Search of protein database was carried out using protein query of each serotype domain III by blastp algorithms.

Both basic amino acid residues (Lys295 and Lys or Arg310) were conserved in all strains examined. The results of our infection and binding experiments clearly demonstrated distinct susceptibilities to fucoidan among dengue serotypes. Fucoidan strongly inhibited ThNH-7/93 infection and binding to BHK-21 cells. DEN 3 and 4 strains were moderately susceptible to fucoidan. On the other hand, fucoidan did not affect D1/Lao/03 infection. In addition, our structure-based experiment demonstrated that glucuronic acid residues are one of the critical determinants for fucoidan function. These findings strongly suggest that some basic amino acid residues on ThNH-7/93, but not DEN1 strain, may account for susceptibility to fucoidan. One putative candidate amino acid was identified in the EGP domain III region between the four-serotype strains. As the positive charge of arginine is greater than that of lysine, the basic amino acid residue at position 323 may contribute to the interaction of the virus with the glucuronic acid residue of fucoidan. Substitution of arginine (or lysine) to glutamine at position 323 in the EGP domain III may diminish the interaction of D1/Lao/03 with glucuronic acid (carboxylic acid) residues. The locations of three amino acid residues at positions 295, 310, and 323 in domain III were estimated in homology-modeled structures of four-serotype strains generated based on the domain III structures reported previously [8–10]. In Fig. 3B, the overall structures of the domains are similar. The amino acid sequence of domain III of D3/BDH02-01 is identical to that of DEN3 protein (PDB accession number 1UZG). Therefore, other domain III proteins were modeled on the basis of the three-dimensional structures, as reported previously. In all structures, the amino acid residue at position 323 is located between residues at 295 and 310. The distance between Arg323 and Lys310 in DEN2 domain III is closer to those between Lys323 and Lys310 or Arg310 in DEN3 or DEN4, respectively. This prediction also suggested that the packed positive charges on domain III enhance the interaction with negative charges of fucoidan. Protein database search by blastp algorithms demonstrated that the three amino acid residues at positions 295, 310 and 323 of domain III were conserved among all strains of each serotype registered (Fig. 3C), meaning that the amino acid residue at position 323 accounts for susceptibility of dengue virus serotypes to fucoidan. Taken together, these observations strongly suggest that not only substitution of Arg (or Lys) to Gln at position 323 but also the distance from position 310 may cooperatively contribute to the susceptibility of dengue virus to fucoidan.

In the present study, we determined unique carbohydrate determinants involved in anti-dengue virus activity of fucoidan from the marine alga *C. okamuranus*. Using structure-based analyses, we also elucidated the molecular mechanisms of the susceptibilities of four dengue virus serotypes to the fucoidan. Information on these carbohydrate residues may facilitate the development of effective anti-dengue virus agents.

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