HIGH MANNOSE-BINDING LECTIN WITH PREFERENCE FOR THE CLUSTER OF α1-2 MANNOSE FROM THE GREEN ALGA BOODLEA COACTA IS A POTENT ENTRY INHIBITOR OF HIV-1 AND INFLUENZA VIRUSES
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Running title: Antiviral activity of high mannose-binding green algal lectin

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The complete amino acid sequence of a lectin from the green alga Boodlea coacta (BCA), which was determined by a combination of Edman degradation of its peptide fragments and cDNA cloning, revealed that (1) B. coacta used a noncanonical genetic code (where TAA and TAG codons encode glutamine rather than a translation termination), and that (2) BCA consisted of 3 internal tandem-repeated domains, each of which contains the sequence motif similar to the carbohydrate-binding site of GNA (Galanthus nivalis agglutinin)-related lectins. Carbohydrate-binding specificity of BCA was examined by a centrifugal ultrafiltration-HPLC assay using 42 pyridylaminated oligosaccharides. BCA bound to high mannose-type N-glycans but not to complex type, hybrid type, core structure of N-glycans or oligosaccharides from glycolipids. This lectin had exclusive specificity for α1-2 linked mannose at nonreducing terminal. The binding activity was enhanced as the number of terminal α1-2 linked mannose substitutions increased. Mannobiose, mannotriose, and mannopentaose, were incapable of binding to BCA. Thus, BCA preferentially recognized the nonreducing terminal α1-2 mannose cluster as a primary target. As predicted from carbohydrate-binding propensity, this lectin inhibited the HIV-1 entry into the host cells at a half maximal effective concentration of 8.2 nM. A high association constant (3.71 × 10^8 M^-1) of BCA with the HIV envelope glycoprotein gp120 was demonstrated by surface plasmon resonance analysis. Moreover,
BCA showed the potent anti-influenza activity by directly binding to viral envelope hemagglutinin against various strains including a clinical isolate of pandemic H1N1-2009 virus, revealing its potential as an antiviral reagent.

High mannose-binding lectins are found from various taxonomy, animals, plants, algae, and bacteria (1). In plant, mononcot mannose-binding lectins form a large family of structurally and evolutionary related lectins, which recently referred to as GNA (Galanthus nivalis agglutinin)-related lectins, most of which bind to high mannose glycans (2). Legume lectin family is another large group of plant lectins that includes the various high mannose-binding lectins (3). Importantly, some of these high mannose-binding plant lectins exhibit a strong anti-HIV activity but others are weak or completely inactive (4, 5). Such different biological activities virtually depend on the diverse carbohydrate-specificities of these plant lectins. Apart from the plant lectins, high mannose-binding cyanobacterial (blue-green algal) or eukaryotic algal lectins are a group of promising compounds for antiviral agents because of their unique oligosaccharide binding nature and physicochemical characteristics (6, 7). For example, HIV-inactivating proteins such as CV-N from Nostoc ellipsosporum (8, 9), scytovirin from Scytonema varium (10), MVL from Micrystis viridis (11), and OAA from Oscillatoria agardhii (12) are high mannose-binding cyanobacterial lectins. These prokaryotic lectins share the common structural features, an internal multiplication of the amino acid sequences. Three-dimensional structures of these lectins commonly exhibit characteristic domain swapping (6). However, they are structurally independent from each other in their amino acid sequences. Although many high mannose-binding lectins have been demonstrated to exhibit anti-HIV activity (13), cyanobacterial or eukaryotic algal lectins are the most-potent compounds so far reported compared with other plant lectins, because they inhibit HIV replication with half maximal effective concentration (EC_{50}) values in the low nanomolar to picomolar range (6). Red algal lectin griffithsin (GRFT) from Griffithsia sp. is the most-potent inhibitor of HIV multiplication, which displays the picomolar activity (14). Although red algal lectins share the common features (eg, low molecular weight, cation-independent hemagglutination, and no binding propensity for monosaccharide), GRFT exceptionally binds to monosaccharides such as glucose or mannose (14). Red algal lectin ESA-2 from Eucheuma serra is structurally and evolutionarily related to cyanobacterial lectin OAA (15). Both lectins exclusively recognize high mannose-type N-glycans with extremely high affinity (association constant (K_A) = ~10^8 M^{-1}) but do not recognize monosaccharides or small oligomannoses (12, 15). They also inhibit the HIV entry into the host cells with
EC₅₀s of low nanomolar range by directly binding to envelope gp120 (12).

In addition to inhibiting HIV, some high mannose-binding lectins (eg, CV-N) show a broad range of antiviral activity against influenza virus (16), Ebola virus (17), human herpes virus 6 (18), and hepatitis C virus (HCV) (19). GRFT has been demonstrated to inhibit cytotoxic effects of the corona virus that causes severe acute respiratory syndrome (SARS) (20). Currently, emergence of 2 influenza virus strains, swine-origin influenza virus (H1N1-2009) and the highly pathogenic avian influenza virus (H5N1), has become a global threat to public health. Therefore, new anti-influenza agents are in great demand to confront the emergence of highly pathogenic mutants that acquired the ability to transmit human to human.

Previously, a novel lectin (BCA, previously declared as boonin) was isolated from the green alga *Boodlea coacta*, and its biochemical features were partially characterized (21). Interestingly, hemagglutination activities of BCA were strongly inhibited by glycoproteins with high mannose-type N-glycan, but not by the monosaccharides tested. Here we efficiently obtained high-purity BCA using the yeast mannan-Cellulofine affinity column, and clarified the full-length sequence of BCA by protein sequencing and cDNA cloning. Detailed oligosaccharide-binding specificity of BCA and its antiviral activity against 2 global viruses, HIV and influenza virus, were evaluated.

**EXPERIMENTAL PROCEDURES**

*Materials*—The specimen of *B. coacta* was collected on the coast of Kagoshima, Japan. The algal sample was immediately transferred to the laboratory, washed, lyophilized, and ground on a ball mill to a powder. The powdered alga, which had been kept at -20°C, was used for purification of the lectin BCA. A small portion of the alga was stored at -20°C in RNAlater (Life Technologies, CA, USA) until used for the RNA extraction. Lysylendopeptidase (Lys-C) and Endoproteinase Asp-N were obtained from Takara Bio (Kyoto, Japan). Pyridylaminated (PA-) oligosaccharides were prepared as described previously (15).

The following viruses were kindly provided by Dr. T. Sakaguchi (Hiroshima University, Japan): A/WSN/33 (H1N1), A/PR8/34 (H1N1), A/FM/1/47 (H1N1), A/Kyoto/1/81 (H1N1), A/Bangkok/10/83 (H1N1), A/Beijing/262/95 (H1N1), A/Aichi/2/68 (H3N2), A/Udorn/72 (H3N2), A/Philippines/2/82 (H3N2), and B/Ibaraki/2/85. A clinical isolate of H1N1-2009 virus, A/Oita/OU1 P3-3/09, was generously provided by Dr. A. Nishizono (Oita University, Japan). Recombinant glycosylated HIV-1 IIIB gp120 (baculovirus) was purchased from ImmunoDiagnostics (MA, USA). Influenza viruses were grown in the chorioallantoic fluid of 10-day-old chicken eggs. Madin-Darby canine kidney
(MDCK) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin.

Purification of B. coacta Lectin—The powdered alga (25 g) was stirred at 4°C overnight with 10 volume (w/v) of 20 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl (PBS). The mixture was centrifuged at 13,000 × g for 30 min, and the supernatant was recovered. The residues were extracted once more with 150 ml of PBS in the same way. To both extracts combined, solid ammonium sulfate was added to attain a final concentration of 75% saturation. The mixture was kept overnight at 4°C and centrifuged at 13,000 × g for 30 min. The resulting precipitate was dissolved in a small amount of distilled water and then dialyzed thoroughly against PBS. After the nondialyize was centrifuged at 13,000 × g for 30 min, the supernatant was collected as a salting-out fraction.

A 3 ml-portion (23.2 mg protein) of the salting-out fraction was applied to a yeast mannan-immobilized column (10 × 100 mm, Vt=7.85 ml, 1.19 mg ligand/ml gel) equilibrated with PBS. The affinity column was prepared as described previously (22). The column was thoroughly washed with 20 mM phosphate buffer (pH 7.0) containing 1 M NaCl, and then eluted with absolute ethylene glycol. The flow rate of 0.2 ml/min was maintained during the chromatography. Fractions of 1 ml were collected and measured for absorbance at 280 nm and for hemagglutination activity. The active factions showing the hemagglutination activity (>25) were pooled, thoroughly dialyzed against distilled water, and further applied to a YMC PROTEIN-RP column (6.0 × 250 mm) (YMC, Kyoto, Japan) equilibrated with 10% acetonitrile in 0.05% trifluoroacetic acid (TFA). The column was washed with the starting solvent, and then eluted at a flow rate of 1.0 ml/min by a linear gradient (10% - 70%) of acetonitrile in 0.05% TFA. The eluate was monitored by absorption at 280 nm and hemagglutination activity. The active fractions were pooled and dialyzed against distilled water.

Analytical Methods—Protein concentration was quantitated by the Lowry method (23) using bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24) was performed using a 15% (w/v) gel. Staining for carbohydrate was carried out using the G. P. Sensor, a carbohydrate-detection kit (J-OIL MILLS, Tokyo, Japan), as described previously (25), except that fetuin was used as a reference glycoprotein.

Hemagglutination Assay—Hemagglutination assay was performed using a 2% (v/v) suspension of trypsin-treated rabbit erythrocytes, as described previously (21). Briefly, rabbit blood preparation was washed 3 times with 50 volume of saline, and the packed cells were suspended in saline to give
a 2% (v/v) suspension of native erythrocytes. One tenth volume of 0.5% trypsin in saline was added to a 2% native erythrocyte suspension and the mixture incubated at 37°C for 60 min. After washing 3 times with saline, a 2% trypsin-treated erythrocyte suspension was prepared in saline. Hemagglutination activity was expressed as a titer, the reciprocal of the highest 2-fold dilution exhibiting positive hemagglutination, or as a minimum hemagglutination concentration (MAC), the protein concentration of the highest lectin dilution exhibiting positive hemagglutination.

**Enzymic Digestion and Separation of Peptides**—BCA was subjected to S-pyridylethylation according to the method described previously (15). Two hundred µg of S-pyridylethylated (PE-) BCA was digested with Lys-C (enzyme/substrate = 1/100 (w/w)) in 50 mM Tris-HCl (pH 8.5) at 37°C for 24 h. Asp-N digestion (enzyme/substrate = 1/100 (w/w)) was performed using the same amount of PE-BCA in 50 mM Tris-HCl (pH 7.5) at 37°C for 18 h. For the isolation of peptide fragments, each digest was separated by reverse-phase HPLC on a TSKgel ODS-120T column (4.6 × 250 mm) (Tosoh, Tokyo, Japan) with a linear gradient (5% - 65%) of acetonitrile in 0.1% TFA.

**Amino Acid Sequence Analysis**—The N-terminal amino acid sequences of intact protein and peptides generated by enzymic digestion were determined by an automated-protein sequencer (Applied Biosystems 477A) connected to the 3-phenyl-2-thiohydantoin (PTH) amino acid analyzer (120A) (Applied Biosystems, CA, USA).

**Molecular Weight Determination of Protein and Peptides**—The molecular weights of native BCA, PE-BCA, and peptide fragments were determined by Finnigan LCQ electron spray ionization (ESI)-mass spectrometry (MS) (Finningan, CA, USA).

**cDNA Cloning of BCA**—Total RNA was extracted from the RNAlater-treated alga using the Plant RNA Isolation Reagent (Life Technologies). mRNA purification from the total RNA was performed using a NucleoTrap mRNA Purification Kit (Macherey-Nagel, Düren, Germany). Full-length cDNAs were synthesized from 200 ng of mRNA using a GeneRacer Kit (Life Technologies) according to the manufacturer’s instruction. The first polymerase chain reaction (PCR) for rapid amplification of the cDNA 5’end (5’RACE) was initiated by adding each 0.2 µl of 10-fold diluted synthesized cDNA to 8 tubes of a 9.8 µl solution containing 6 pmol of GeneRacer_5’_Primer, 50 pmol of a degenerated primer BCA_5’_RACE_R1 designed from the partial BCA sequence (see Supplementary Table 1 for the primer sequences), 1 µl of 10 × Blend Taq buffer (Toyobo, Osaka, Japan), 2 nmol each of
dNTP, and 0.25 U Blend Taq DNA polymerase (Toyobo). The reaction with T Gradient Thermocycler (Biometra, Göttingen, Germany) consisted of denaturation at 94ºC for 3 min, followed by 35 cycles consisting of denaturation at 94ºC for 30 sec, annealing at gradient temperature of 50ºC to 64ºC (2ºC increments) for 30 sec, and extension at 72ºC for 1 min, and the final extension step at 72ºC for 5 min. The PCR products in 8 tubes were pooled and then diluted to 100-fold. The nested PCR was performed by the same method, except that 0.2 µl of the dilution was used as a template and 2 pmol of GeneRacer_5’_Nested_Primer and 50 pmol of a degenerated primer BCA_5’_RACE_R2 as a primer pair (Supplementary Table 1). Nested PCR products were subcloned into pGEM-T Easy vector (Promega, WI, USA). Cycle sequencing reaction was performed using a BigDye Terminator Cycle Sequencing Kit Ver. 3.1 and ABI 3130xl DNA sequencer (Life Technologies). 3’RACE was performed in the same way as 5’RACE as described above, except the use of 2 pmol each of GeneRacer_3’_Primer and BCA_F1 (Supplementary Table 1) designed from the sequence obtained by the 5’RACE. At last, following the manufacturer’s instruction, the full-length BCA cDNA was amplified using a high-fidelity DNA polymerase KOD FX Neo (Toyobo) and a primer pair of BCA_5’_End_F and BCA_3’_End_R (Supplementary Table 1), which were designed from the 5’ and 3’ terminal sequences of BCA cDNA obtained by 5’ and 3’RACE, and subcloned into pCR-Blunt II-TOPO vector (Life Technologies).

**Sequence Data Processing**—Homologous sequences were identified with the basic local alignment search tool (BLAST) program. The internal tandem repeat regions were compared with each other using ClustalW2 (26). Signal peptide region was predicted with SignalP 3.0 (27).

**Centrifugal Ultrafiltration-HPLC Method**—The oligosaccharide-binding activity of BCA was determined using a centrifugal ultrafiltration-HPLC method as described previously (15). Briefly, 90 µl of 500 nM BCA in 50 mM Tris-HCl, pH 7.0, and 10 µl of 300 nM PA-oligosaccharide were mixed and kept at room temperature for 1 h. Subsequently, unreacted PA-oligosaccharides were recovered by centrifugation (10000 × g, 30 sec) with Nanospin Plus (Gelman Science, MI, USA). An aliquot of the filtrate was applied to TSKgel ODS-80Tm column (4.6 × 150 mm, Tosoh) and eluted with 10% methanol in 0.1 M ammonium acetate buffer at a flow rate of 1.0 ml/min in a column oven (40ºC). The eluate was monitored at an excitation wavelength of 320 nm and an emission wavelength of 400 nm and then unbound PA-oligosaccharide (Unbound) was quantified. The amount of bound PA-oligosaccharide (Bound) was obtained by following formula:
$O_{\text{bound}} = O_{\text{added}} - O_{\text{unbound}}$, where $O_{\text{added}}$ represents the amount of added PA-oligosaccharide determined from the filtrate of reaction solution without a lectin. The binding activity ($O_{\text{bound}}/O_{\text{added}}$) was calculated as a ratio of the amount of bound PA-oligosaccharide to that of added.

**Anti-HIV Activity of BCA—In vitro** evaluation of anti-HIV activity of BCA was performed by a colorimetric assay as described previously (28). Briefly, 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT, Sigma-Aldrich, MO, USA) was used to detect the viability of both HIV-1 (HTLV-IIIB strain) and mock-infected MT-4 cells in the presence of a test compound at various concentrations.

**Interaction of BCA with gp120**—Direct interaction of BCA with the HIV envelope glycoprotein gp120 was analyzed by surface plasmon resonance (SPR) analysis using a BIAcore 2000 system (GE Healthcare, Buckinghamshire, UK) as described previously (12), except that the recombinant glycosylated HIV-1 IIIB gp120 (baculovirus, ImmunoDiagnostics) was immobilized to give 300 resonance units (RU) on a carboxymethylated dextran-coated sensor chip (CM5, GE Healthcare). The data were fit globally to a simple Langmuir 1:1 binding model with local $R_{\text{max}}$ (maximum response) using BIAevaluation 3.1 software.

**Anti-Influenza Activity of BCA—Evaluation** of anti-influenza activity was performed by the neutral red (NR) dye uptake assay. Various concentration of the lectin was prepared with DMEM containing 20 µg/ml trypsin in a 96-well microplate. To each well, virus was added as a multiplicity of infection of approximately 0.001 infectious particles per cell. After incubating at 37°C for 48 h, 100 µl of NR dye (150 µg/ml in DMEM) was added and further incubated for 2 h. NR dye incorporated into the cells was extracted by the addition of 100 µl of 1% acetic acid/50% ethanol. The color intensity of the dye absorbed by, and subsequently eluted from the cells, was measured at 540 nm with a microplate reader (1420 multilabel counter, PerkinElmer, MA, USA) as a factor of surviving from the virus infection.

**Immunofluorescence Microscopy**—Immunofluorescence staining was performed to visualize and evaluate BCA inhibition of influenza virus infection as described previously (29). Briefly, MDCK cells grown on cover glass were infected with A/Udorn/72 at a multiplicity of infection of approximately 0.001 infectious particles per cell, in the presence or absence 1 µM BCA in DMEM containing 10 µg/ml trypsin. After 24 h post infection, the infected cells were fixed and visualized by incubating with mouse monoclonal anti-hemagglutinin antibody (HyTest, Turku, Finland) at 37°C for 1 h followed by with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Anticorps Secondaires, Compiègne, France).
France) at 37°C for 1 h. The cells were mounted using Vectashield with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and were observed under a fluorescence microscope (OLYMPUS BX51, Olympus, Japan).

**ELISA Assay**—Direct interaction between BCA and viral hemagglutinin was analyzed using an enzyme-linked immunosorbent assay (ELISA) as described previously (29). BCA (5 µg/ml) in carbonate buffer (pH 9.6) was immobilized on ELISA plates (BD Biosciences, Bedford, MA). Following washing with phosphate buffered saline, pH 7.4, containing 0.1% Tween20 (PBST), the wells were blocked with 3% skim milk for 1 h at 37°C. After further PBST washing, the wells were incubated with various concentrations of an influenza vaccine preparation (Astellas, Tokyo, Japan) enriched for virus hemagglutinin for 1 h at 37°C. After washing with PBST, the wells were incubated with mouse anti-hemagglutinin monoclonal antibody (HyTest) for 1 h at 37°C followed by incubation with horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (GE Healthcare) for 1 h at 37°C. After washing with PBST, 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, Saint-Louis, MI) was added. The reaction was stopped using TMB stop reagent (Sigma-Aldrich) and absorbance at 450 nm was measured using a microplate reader (1420 multilabel counter, PerkinElmer). The same ELISA assay was performed to test the inhibitory effect of yeast mannan on the interaction between BCA and hemagglutinin, except that yeast mannan was added to the plate coated with BCA prior to incubation with hemagglutinin (3 µg/ml).

**RESULTS**

**Purification of BCA**—The lectin of *B. coacta* was extracted with buffer and effectively recovered as a precipitate with 75%-saturated ammonium sulfate (Supplementary Table 2). Total hemagglutination activity of the precipitate was higher than that of the extract, implying that some unknown inhibitor(s) coexisting in the extract, that form complexes with the lectin as seen in some plants (30), may be removed during the procedure by salting-out. In affinity chromatography of the ammonium sulfate-precipitate, the active component was adsorbed to the column and eluted as a single peak with ethylene glycol, although some activity was also detected in non-adsorbed fraction (Fig. 1A). The major active peak eluted with ethylene glycol gave a single protein band of 14 kDa, whereas the nonadsorbed fraction gave several protein bands including 16 kDa (Fig. 1B). The protein yield of the major active peak was 4.2 mg from 25 g of the powdered alga (Supplementary Table 2). The active peak was further purified by reverse-phase chromatography on an YMC PROTEIN-RP
column (Fig. 1C). The finally purified lectin thus obtained was named BCA and used for further examination. The molecular masses of BCA and PE-BCA determined by ESI-MS were 13812 and 13919 and resembled that estimated by SDS-PAGE. The difference in molecular masses of BCA and PE-BCA suggests that the lectin protein contains a single cysteine residue in the molecule. BCA was negative for carbohydrate-staining, indicating that it has no sugar moiety (data not shown).

**Nucleotide and Amino Acid Sequences of BCA** —To elucidate the complete amino acid sequences of BCA, cDNA cloning for BCA was performed. The full-length cDNA encoding BCA was isolated by 5′ and 3′ RACEs using the degenerated primers designed based on the partial sequences determined by Edman degradation of PE-BCA and its peptide fragments. The cloned nucleotide sequence of BCA and its deduced amino acid sequence are shown in Fig. 2A. The deduced amino acid sequence was almost comparable in the partial sequences determined by Edman degradation, as shown in Fig. 2B. The peptide fragments produced by proteolytic digestions (Lys-C and Asp-N) of PE-BCA were separated by reverse-phase HPLC and designated L1-L16 and A1-A15 (data not shown). The N-terminal amino acid sequences and molecular masses of these fragments were determined as well as PE-BCA, which resulted in elucidation of the partial sequences that correspond to 118 amino acid residues of the lectin molecule (Fig. 2B). In the sequencing, some peptides (A1, A4, A5, L4, and L12) were generated by nonspecific cleavage. The 30 N-terminal amino acid sequence of PE-BCA was determined as GAFQ(K)AISGESGYLSHAFAKIWLQNYQGL, at the forth residue of which 2 amino acids, glutamine and lysine, were identified, suggesting the presence of the isoforms. Comparison of cDNA and peptide sequences of BCA elucidated that the general stop codons of TAA and TAG, but not TGA, encoded glutamine residues (Q56 and Q132) (Fig. 2A), as reported for some proteins in several other species of green algae (31-33). Thus, BCA cDNA was composed of 1023 bp containing 117 bp of 5′ untranslated region (UTR), 363 bp of 3′ UTR, and 540 bp of open reading frame (ORF), which encoded 180 amino acids including signal peptide of 19 residues (1-19 aa), 3 internal tandem-repeated domains (20-144 aa), and C-terminal region of 36 residues (145-180 aa) (Fig. 2B). The 3 tandem-repeated domains, each of which consisted of 40-43 residues, showed sequence identity of 47.6% to 62.8% to each another (Fig. 3A). No homologous gene candidates that show high sequence similarities with BCA were found by *in silico* search. BCA showed no significant sequence similarity with other high mannose-type glycan-binding lectins such as CV-N, OAA, and ESA-2. Strikingly, however, BCA partially possessed the carbohydrate-binding motif of GNA-related
lectins, despite having no overall sequence similarity. The sequence alignment of both BCA and GNA shows obvious disruption of GNA-subdomain III in BCA, whereas the sequences of BCA at GNA-subdomain I and II were partially conserved (Fig. 3B).

Carbohydrate Binding Specificity of BCA—The carbohydrate binding specificity of BCA was investigated by a centrifugal ultrafiltration-HPLC method. Fig. 4 shows the structure of PA-oligosaccharides used in this study. Of the 42 kinds of PA-oligosaccharides tested, BCA selectively recognized 9 carbohydrate structures (oligosaccharide Nos. 14-22) that are categorized into high mannose-type N-glycans (Nos. 13-24) as shown in Fig. 5. The complex type N-glycans (Nos. 1-12), hybrid type N-glycans (Nos. 25-27), the core structures of N-glycans (Nos. 28 and 29), and oligosaccharides from glycolipid (Nos. 30-37) were incapable of binding to BCA. The binding ability of BCA to high mannose-type N-glycans differed depending on the structure of branched carbohydrate moiety. BCA showed the preference for the oligosaccharides bearing terminal α1-2 linked mannose(s), and the activity was increased in proportion to the increased number of α1-2 substitutions at nonreducing end. This observation was evident from the fact that BCA bound completely to the oligosaccharides (Nos. 15 and 19) bearing 3 fully exposed α1-2 linked mannoses (Figure 5). As for the oligosaccharides having 2 terminal α1-2 mannoses, 2 close proximate α1-2 mannoses were likely to be preferred for BCA compared with 2 sterically distant α1-2 mannoses, because BCA completely bound to oligosaccharide No. 20, whereas the activities for oligosaccharide Nos. 16 and 21 were somewhat decreased (about 80%). Activity toward oligosaccharide No. 17 was much lower (25.4%) despite having 2 terminal α1-2 linked mannoses. With the oligosaccharides having only 1 terminal α1-2 mannose (Nos. 14, 18, and 22), the binding activity of BCA was further decreased showing the activity around 30% - 47%. In contrast, this lectin did not interact with high mannose-type N-glycans that are devoid of terminal α1-2 mannose as seen in oligosaccharide Nos. 13, 23, and 24. These results indicate that primary targets of BCA are the terminal α1-2 linked mannose(s). Moreover, no interaction was observed in mannobioses (Nos. 38-40), mannotriose (No. 41), and mannopentaose (No. 42) that are the main constituents of branched moiety of high mannose-type N-glycans. Interestingly, even α1-2 linked mannobiose (No. 38) lacks the binding ability to BCA. The results suggest that BCA exclusively recognize nonreducing terminal α1-2 linked mannose and the clustering of those residues might contribute to enhance BCA affinity.

Anti-HIV Activity of BCA—In vitro evaluation of potent anti-HIV activity of BCA was determined by the conventional MTT assay using MT-4 cells. BCA inhibited
the HIV-1 infection dose-dependently, with an EC₅₀ of 8.2 nM (Fig. 6A). Cell viability was not affected up to 100 nM, the highest dose in this experiment.

Direct Interaction of BCA with gp120—To evaluate the molecular basis of anti-HIV activity of BCA, we tested the direct interaction of BCA with a recombinant HIV envelope glycoprotein gp120 by SPR analysis. As shown in Fig. 6B, BCA dose-dependently bound to the gp120 immobilized on a sensor chip CM5. From the kinetic analysis, the association constant (Kₐ) of BCA-gp120 interaction was calculated to be 3.71×10⁸ M⁻¹.

Anti-Influenza Virus Activity of BCA—The anti-influenza virus activity of BCA was investigated with respect to inhibiting replication in MDCK cells by the NR dye uptake assay. Ten influenza A virus strains including laboratory-adapted strains and 1 influenza virus B strain were tested for their BCA sensitivity (Fig. 7A). As shown in Table 1, BCA showed antiviral activity for most of influenza virus strains tested, even though the EC₅₀ varied dependent on the strains. Philippines/2/82 (H3N2) was the most sensitive strain to BCA. Interestingly, BCA showed stronger inhibition against H3N2 subtypes at EC₅₀s of 18.8-74.2 nM, whereas it was much weaker for H1N1 subtypes showing EC₅₀s of 79.3-1590.2 nM. The clinical isolates of recent pandemic strain, swine-origin influenza virus, A/Oita/OU1 P3-3/09 (H1N1) was also susceptible to BCA, although the degree of susceptibility (EC₅₀ of 820 nM) was much lower compared with the H3N2 strains. A laboratory-adapted strain, A/PR8/34(H1N1), was the only strain that is insensitive to BCA. This lectin was also effective against influenza B strain B/Ibaraki/2/85 at a moderate level.

Immunofluorescence Microscopy—To explore the mechanism of inhibition of influenza virus infection by BCA, the presence of viral antigen in the infected cells was observed in the presence or absence of BCA using immunofluorescence microscopy. After 24 h post infection with A/Udorn/72 (H3N2), viral antigens were detected with the specific anti-hemagglutinin antibody. Fig. 7B shows that BCA efficiently inhibited influenza virus entry into the cells whereas the virus invaded and proliferated in the host cells in the absence of BCA. The viral ion channel (M2 protein) inhibitor, Amantadine, did not prevent virus invasion into the cells.

Direct Interaction of BCA with Influenza Viral Hemagglutinin—To examine whether BCA directly binds to the oligosaccharide on enveloped glycoprotein (hemagglutinin), ELISA assays were performed using an influenza vaccine preparation, which contains hemagglutinin of A/California/7/09 (H1N1), A/Victoria/210/09 (H3N2), and B/Brisbane/60/08. As shown in Fig. 7C (left panel), obvious binding of hemagglutinin to the immobilized BCA was demonstrated. In
contrast, hemagglutinin did not bind to a reference glycoprotein (yeast mannan). The interaction between hemagglutinin and BCA was significantly inhibited by the presence of yeast mannan bearing high mannose glycans (Fig. 7C, right panel), indicating that BCA actually binds to the hemagglutinin through high mannose glycans.

DISCUSSION

High mannose-binding algal lectins are ones of the expected and promising compounds for anti-HIV or antiviral agents such as microbicides, as they exhibit exclusive specificity toward certain oligosaccharide(s) on the virus’ surface, with high affinity, thereby inhibiting virus entry into the host cells. Here, we report that BCA is the first HIV- and influenza-virus–inhibiting protein from the green algae, which shows strict specificity for high mannose oligosaccharide but unprecedented mode of oligosaccharide recognition. We also elucidated the primary structure of BCA and its distinctive structural features.

Several green algal species belonging to the orders Dasycladales and Siphonocladales, which B. coacta belongs to, have been found to use noncanonical genetic code where TAA and TAG encode glutamine instead of translation termination (31-33) as well as oxymonads (34, 35), diplomonads (36, 37), and some ciliates (38, 39). We found that BCA cDNA uses the noncanonical genetic code as shown in Fig. 2. To confirm that B. coacta itself uses the unusual genetic code, the full-length cDNA encoding the translation elongation factor-1 α (EF-1α), which was found to use the noncanonical genetic code in some green algae (33), was cloned from B. coacta (data not shown; DDBJ/EMBL/GenBank accession No. AB604604). However, we did not find TAA and TAG codons in its ORF. Then, we nonspecifically surveyed other genes that may use the unusual genetic code in the full-length cDNA pool derived from B. coacta (see Supplementary Fig. 1 for details). As a result, we found another gene, ribosomal protein L37a, to use the noncanonical genetic code in B. coacta (Supplementary Fig. 1; DDBJ/EMBL/GenBank accession No. AB604605). Although we did not find TAA and TAG codons in putative ORF of almost all genes cloned in this experiment, they used the TGA codon as a termination, but not TAA and TAG, as well as B. coacta EF-1α gene. Thus, it is strongly suggested that B. coacta uses noncanonical genetic code, and the algal species using the unusual genetic code may exist in the order Siphonocladales.

The molecular mass (13812) of BCA determined by ESI-MS was distinct from the calculated one (17561) from the deduced amino acid sequence of BCA cDNA, which consisted of 161 amino acids excluding signal peptide sequence. Concerning this discrepancy, the molecular mass (13812) calculated from the portion
(20-144 aa) of 3 tandem-repeated sequences well coincides with the determined mass by ESI-MS. In the sequence analyses of the peptide fragments of PE-BCA, none of fragments derived from the C-terminal region (145-180 aa) deduced from BCA cDNA was obtained. This suggests that the precursor of BCA may be posttranslationally modified for its C-terminal truncation as seen in the other plant lectins (40) including GNA-related lectin family (41, 42), resulting in synthesis of the mature BCA consisting of repeated domains alone. The occurrence of three internal repeats and both N- and C-terminal potential truncation regions of BCA led us to survey the similarity of this protein to GNA, which also consists of three subdomains, signal peptide and C-terminal propeptide. Interestingly, BCA has sequence motifs similar to the carbohydrate-binding site of GNA-related lectins. Nevertheless, overall sequence similarity was quite low between BCA and GNA-related lectins at undetectable level by the normal homology search. It should be noted that both signal peptide and the C-terminal propeptide of GNA are necessary for trafficking to the vacuole (42). Moreover, the importance of C-terminal propeptide of GNA to temporary inactivate the carbohydrate-binding ability in the endoplasmic reticulum have been proposed (42). The presence of a putative vacuolar targeting motif of BCA and partially conserved GNA-like carbohydrate-binding site suggest the similar physiological role(s) of BCA.

GNA-related lectins have been distinguished either as single-domain lectins with an exclusive specificity towards mannose/oligomannosides or as two-domain lectins which acquired a diverse carbohydrate specificity (2). From the aspects of simple monomeric structure consisting of a single GNA-like domain and the strict specificity for high mannose oligosaccharides of BCA, this lectin more closely resembles to the single-domain GNA-related lectins. Of the three subdomains with consensus amino acid sequences (QDNVY), each of which corresponds to characteristic mannose-binding sites of GNA-related lectins, putative carbohydrate-binding sites of BCA were partially conserved at subdomain I and II of GNA-related lectins, but it underwent a significant change at subdomain III. This might reflect the different specificity of BCA from other typical GNA-related lectins but the mode of oligosaccharide recognition of BCA should be clarified by the structural analyses of BCA-oligosaccharide complexes. It is noteworthy that BCA has been predicted to have three α-helices within its monomeric molecule, each located in tandem repeat domains as deduced from the secondary structure (Supplementary Fig. 2). This suggests that the three-dimensional structure of BCA might be distinct from the typical GNA-related lectins, which exhibit a β-prism structure built up of three subdomains, each consisting of four strands of antiparallel β sheets (43). Moreover, it is likely that plant GNA-related lectins may
have evolved through the process unrelated from BCA, because prokaryotic proteins with the GNA domain(s) share a high sequence similarity with plant GNA-related lectins (44, 45) but not with BCA. Thus, the biosynthetic process of this algal lectin is an interesting target to be further investigated, including the identification of its C-terminal amino acid.

Cyanobacterial lectins, CV-N (*N. ellipsosporum*) and scytovirin (*S. varium*), show the strong anti-HIV activity at EC<sub>50</sub>s of picomolar level. A red algal lectin, GRFT is the strongest HIV entry inhibitor with the EC<sub>50</sub> of 40 pM and has broad-spectrum activity against various HIV clades (14). The cyanobacterial lectin, OAA (*O. agardhii*) and the red algal lectin, ESA-2 (*E. serra*), which are members of a new lectin family recently discovered, also inhibit HIV entry into the cells at EC<sub>50</sub>s of nanomolar levels (12). BCA inhibits the HIV replication at an EC<sub>50</sub> of 8.2 nM with the stronger activity than OAA and ESA-2. All of these lectins inhibit HIV infection, based on the same mechanism that is through the recognition of high affinity binding to the high mannose glycans of gp120 on the virus surface. Recent studies have shown that the pattern of glycosylation of HIV viral particles depends largely on the derived host cell lines rather than the strain difference and affects the HIV infectivity (46). Therefore, susceptibility of different host-derived HIV strains to BCA remains to be clarified. Besides the reason of glycomic distinctions in viral particles, the relative difference of antiviral potency among lectins might be somewhat ascribed on their diversified mode of high mannose oligosaccharide recognition.

Fig. 8 shows the schematic diagram of high mannose oligosaccharide recognized by BCA. This lectin primarily targets the α1-2 linked mannose at nonreducing end and clustering of the α1-2 mannose significantly increase the binding affinity for BCA. It is likely that BCA recognizes the nonreducing terminal α1-2 linked mannose(s) rather than the internal one, because the binding activity of BCA was not altered between oligosaccharide No. 16 and 21, in which the only structural difference is the presence of additional α1-2 linked mannose at D1 terminal (Fig. 4). This is also applicable in comparison between oligosaccharide No. 14 and 18, where internal α1-2 mannose at D1 arm did not affect the binding activity.

It is known, for instance, that high mannose-binding proteins such as human HIV-neutralizing antibody 2G12 or CV-N prefer the D1 and D3 arm rather than the D2 arm (47, 48). Actinohivin, an anti-HIV lectin from the actinomycete, which binds high mannose oligosaccharide, prefers the nonreducing terminal α1-2 mannose at D1 arm, and shows the highest affinity for the oligosaccharide having a combination of D1 and D3 α1-2 mannoses (49). MVL specifically recognizes 4-5 units of the oligomannose core (50), whereas scytovirin prefers the D3 arm of Man<sub>3</sub>GlcNAc<sub>2</sub> (51). Both ESA-2 and OAA prefer the exposed α1-3 mannose in the D2 arm (12, 15). In
contrast to these lectins, BCA did not show preference for certain arms, but rather, showed the preference for clustering of α1-2 mannose residues at the nonreducing terminal regardless of the attached arm position. This unique oligosaccharide-binding property of BCA, the exclusive recognition of the α1-2 mannose cluster, might be explained somewhat by ‘cluster effect’ of multivalent ligands. Multivalent ligands sometimes display significant increases in functional affinity for lectins. For instance, the hepatic Gal/GalNAc receptor on the surface of mammalian hepatocytes has been shown to discriminate the cluster of galactose residues effectively (52). The number of Gal residues/cluster and the distance between the Gal residues are critical determinants of the binding affinity. Studies on the effect of multivalent presentation of mannose ligands for ConA showed the dramatic enhancement in functional affinity of mannose-containing polymers relative to the monovalent derivatives (53). Thus, in biological systems, binding affinity and specificity of lectins can sometimes be enhanced by using the multivalent saccharide ligands, resulting in the successful recognition in many biological events such as cell-cell interactions. Unfortunately, however, the biological ligand(s) of BCA have not been identified like other algal lectins. Very recently, 1 of the 2 oligomannose binding sites of cyanobacterial lectin MVL has shown to have a catalytic activity to cleavage of chitin fragments to GlcNAc (54). Therefore, it would be important to survey catalytic activity of algal lectins including BCA to gain the insight into their biological significance.

The mechanisms by which high mannose-binding lectins inhibit HIV adsorption to its target cells are also applicable for other enveloped viruses. Indeed, CV-N showed wide variety of antiviral activity for enveloped viruses such as influenza viruses, Ebola virus, human herpes virus 6, and HCV (16-19). The emergence of the recent pandemic strain, swine-origin influenza virus (H1N1-2009) led us to address the potential anti-influenza virus activity of BCA, to explore the possibility as a novel antiviral agent. BCA showed potent anti-influenza activity against most of all influenza virus strains tested, except a laboratory-adapted strain PR/8/34 (H1N1). The recent pandemic strain, A/Oita/OU1 P3-3/09 (H1N1), was also inactivated by BCA, but the sensitivity was much lower (EC50 of 800 nM) compared with other sensitive strains. It has been reported that the pandemic H1N1 viruses, which have only a single N-glycosylation sequons on the head of hemagglutinin, were resistant to the innate immune proteins of collectin superfamily, whereas the recent seasonal H1N1 possessing 3 to 4 sequons were sensitive to these proteins (55). The degree of glycosylation of viral hemagglutinin would be a key determinant for BCA sensitivity because the strain PR/8/34, which is devoid of N-glycosylation sequons, was resistant to
BCA. Interestingly, the inhibitory activities of BCA for H3N2 subtypes were relatively higher than for H1N1 subtypes. It seems that H3N2 subtypes are more sensitive to α1-2 linked mannose-binding lectins than H1N1 subtypes, because this tendency was also observed for CV-N. CV-N has been reported to neutralize H3N2 subtype (A/Sydney/05/97) completely whereas H1N1 subtype (A/Beijing/262/95) is still infectious by the same CV-N treatment (16). Similarity in virus-inactivating profiles between BCA and CV-N might be primarily ascribed on the selective recognition of α1-2 linked mannose unit.

As for influenza viruses, the hemagglutinin of glycoprotein appears to be a potential target for antiviral agents because certain glycosylation sites on hemagglutinin of influenza A viruses are highly conserved and show site-specificity of attached glycans (56). It has been demonstrated that hemagglutinin 1 (HA1) subunit has a high mannose oligosaccharide at site 65, which contains almost exclusively Man9GlcNAc2, near the receptor binding site.

As the number of amino acid sequences that encode potential N-glycosylation site, referred to as sequons, in human influenza A hemagglutinin H3 subtype HA1 has increased over time, more recent strains might be more susceptible to lectins. This increase in the number of sequons is only found in the human H3 subtype of influenza A virus, and not in H1 subtype (57). In this connection, we have observed the increased BCA sensitivity to Philippines/2/82 (H3N2) compared with the earlier isolated strain, Aichi/2/68 (H3N2). Similarly, H3N2 strains have become more sensitive to human surfactant protein D (SP-D) over time, in accordance with the increase of attached glycans on hemagglutinin (58). Hartshorn and his associates showed that sensitivity of H3N2 to SP-D increased with multiplication of glycosylation site, and the extent was greater when the glycosylation site was introduced in close proximity to the receptor-binding site (58). Further experiments will be needed to verify our results using more-recent H3N2 strains, because the newly introduced glycosylation site does not always have high mannose oligosaccharides with α1-2 linked mannose unit; in other words, increased sequons might not directly link to the BCA sensitivity.

Effective viricidal agents are continuously high demand because the vaccine supply is sometimes not on time. Antibody-based medicine might have some defects as the virus mutates with high frequency by antigenic drift or antigenic shift. Furthermore, they usually could not fully be active against the different subtypes such as H3N2 or H1N1. At this point, carbohydrate moieties on the virus surface may be alternative good targets for medicinal treatment because they exist in most influenza strains, especially in more-recent strains with high abundance. Some virus strains have been developing an increased
number of glycosylation sites to evade antibody pressures by changing antigenicity (59). Therefore, it is advantageous to use lectins prophylactically, as they universally inactivate a wide range of virus strains and different subtypes. Further evaluation of BCA safety concerning cytotoxicity and inflammatory activity should be required, because some lectins such as CV-N show various side effects such as mitogenic activity and stimulation in the production of a wide variety of cytokines (60).

REFERENCES


**FOOTNOTES**

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1The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank databases under the accession numbers AB604330, AB604604, and AB604605 for *B. coacta* lectin BCA, EF-1α, and ribosomal protein L37a genes, respectively.

2The abbreviations used are: BCA, *Boodlea coacta* agglutinin; BLAST, basic local alignment search tool; CV-N, cyanovirin-N; DAPI, 4’,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle medium; ESA, *Eucheuma serra* agglutinin; ESI-MS, electron spray ionization-mass spectrometry; FITC, fluorescein isothiocyanate; GNA, *Galanthus nivalis* agglutinin; GRFT, *Griffithsia* sp. lectin; HA1, hemagglutinin 1; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; Lys-C, lysylendopeptidase; MAC, minimum hemagglutination concentration; MDCK cell, Madin-Darby canine kidney cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; MVL, *Microcystis viridis* lectin; NR, neutral red; OAA, *Oscillatoria agardhii* agglutinin; ORF, open reading frame; PA, pyridylaminated; PBS, phosphate buffered saline; PBST, PBS
containing 0.1% Tween20; PCR, polymerase chain reaction; PE, pyridylethylated; RACE, rapid amplification of the cDNA end; RU, resonance units; SARS, severe acute respiratory syndrome; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SP-D, surfactant protein D; SPR, surface plasmon resonance; TFA, trifluoroacetic acid; THA, total hemagglutination activity; TMB, 3,3′,5,5′-tetramethylbenzidine; UTR, untranslated region.

FIGURE LEGENDS

Fig. 1. Purification of BCA. (A) Affinity chromatography on a yeast mannan-Cellulofine column of the ammonium sulfate-precipitate. A 3 ml-portion (23.2 mg protein) of the precipitate was applied to a yeast mannan-immobilized column (10 × 100 mm, 1.2 mg ligand /ml gel) equilibrated with 0.15 M NaCl in 20 mM phosphate buffer. The column was washed with 1 M NaCl in the buffer and then eluted with absolute ethylene glycol. Fractions of 1 ml were collected and measured for absorbance at 280 nm and for hemagglutination activity. The active fractions denoted by a bar were pooled, and thoroughly dialyzed for further examination. (B) SDS-PAGE at purification steps of BCA. The B. coacta extract (lanes 1 and 2), the precipitate with ammonium sulfate (lanes 3 and 4), the eluates with ethylene glycol (lanes 5 and 6) and the nonadsorbed fractions from the column (lanes 7 and 8) were applied under both reducing and nonreducing conditions (odd and even lanes). Twenty-five µg of protein was loaded in each lane. The position of BCA in the SDS-PAGE gel is represented by a black arrowhead. M, molecular weight standards. (C) Reverse-phase HPLC on a YMC PROTEIN-RP column of the active peak (denoted by a bar in Fig. 1A) obtained by affinity chromatography. The column was washed with 10% acetonitrile in 0.1% TFA, and then eluted with a linear gradient (10% → 70%) of acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min. The eluate was monitored by absorbance at 280 nm. The active fractions represented by a bar were recovered.

Fig. 2. Nucleotide and amino acid sequences of BCA. (A) Nucleotide and deduced amino acid sequences of the cDNA encoding BCA. The stop codon TGA is shown as an asterisk. Glutamine residues coded by TAA and TAG codons are represented in bold. The italicized and nonitalicized numbers represent the positions of nucleotides and amino acids. The nucleotide sequence data appear in the DDBJ, EMBL, and GenBank databases under Accession No. AB604330. (B) The amino acid sequence of BCA. The partial amino acid sequences obtained by Edman degradation of PE-BCA and its peptide fragments (represented with arrows) are compared with the complete amino acid sequence deduced from the BCA cDNA. The peptide fragments produced by cleavage of PE-BCA with Lys-C or Asp-N were separated by
reverse-phase HPLC. Amino acids identified by sequential Edman degradation are indicated by solid lines, whereas unidentified amino acids are indicated by dashed lines. The numbers above the sequence represent the position of amino acids. Numeric values below the lines indicate the molecular masses of peptides determined by ESI-MS, whereas the values in parentheses indicate calculated ones from the sequences. Two peptides were included in the fraction of L8 (L8-1 and -2). L, Lys-C peptides; A, Asp-N peptides. The putative signal peptide and C-terminal propeptide are shown as the underlined sequence and the double underlined sequence, respectively. The signal peptide region was defined with SignalP 3.0.

Fig. 3. Structural features of BCA. (A) The primary structure of BCA. The sequence alignment of repeated domain was generated with ClustalW2. Identical residues are shaded. (B) Comparison of amino acid sequences between BCA and GNA. The alignment was carried out using ClustalW2 and then manually adjusted. The structural motif of three mannose-binding sites of GNA and corresponding sequences in BCA are represented in bold. The subdomain numbers of GNA are indicated above each binding site. Signal peptide and C-terminal propeptide regions are represented in lower-case letter.

Fig. 4. The structures of PA-oligosaccharides used in this study. Open circle, galactose; open square, N-acetylgalactosamine; closed diamond, glucose; closed square, N-acetylglucosamine; closed circle, mannose; open triangle, fucose; open pentagon, xylose.

Fig. 5. Binding activities of BCA to PA-oligosaccharides. Binding activity was expressed as a ratio (%) of the amount of a bound oligosaccharide to that of an added oligosaccharide. The assay was performed in duplicate for each PA-oligosaccharide and the activity is expressed as the average value from duplicate assays. The assays were reproducible without any significant difference.

Fig. 6. Anti-HIV-1 activity of BCA. (A) Determination of in vitro anti-HIV-1 activity of BCA in MT-4 cells. Anti-HIV-1 activity was determined using a colorimetric (MTT) method. HTLV-IIIB strain was used as a virus strain of HIV-1. White and black circles indicate mock-infected (○) or HIV-1 infected (●) cells with BCA. EC<sub>50</sub> and EC<sub>90</sub> values of BCA for the inhibition of HIV-1 infection were 8.2 nM and 13.2 nM. The assay was performed in triplicate, and the activity was expressed as the average value from triplicate assays. (B) Interaction of BCA with a recombinant HIV envelope glycoprotein gp120. The interaction was analyzed by surface plasmon resonance (SPR). Each sensorgram represents the BCA binding to gp120 on a sensor chip CM5. Ninety µl of BCA solutions (15.6, 31.3, 62.5, 125 and 250 nM) were injected
into the flow cells at 30 µl/min for 3 min. The response in resonance units (RU) is plotted against time (s). Binding kinetics of the interaction between BCA and gp120 were calculated by fitting the data to Langmuir model for 1:1 binding. $k_a$, association rate constant; $k_d$, dissociation rate constant; $K_A$, association constant; $K_D$, dissociation constant.

Fig. 7. Anti-influenza activity of BCA. (A) Determination of in vitro anti-influenza activity of BCA in MDCK cells. Assay was performed by NR dye uptake assay, where the cell viability was measured after 48 hours incubation with influenza viruses. The assay was performed in duplicate, and the activity was expressed as the average value from duplicate assays. Closed square, A/FM/1/47 (H1N1); open square, A/Kyoto/1/81 (H1N1); closed circle, A/Bangkok/10/83 (H1N1); open circle, A/Beijing/262/95 (H1N1), closed triangle, A/Oita/OU1 P3-3/09 (H1N1); open triangle, A/WSN/33 (H1N1); closed diamond, A/Aichi/2/68 (H3N2); open diamond, A/Udorn/72 (H3N2); closed star, A/Philippines/2/82 (H3N2); open star, B/Ibaraki/2/85. (B) Inhibition of influenza virus entry into MDCK cells by BCA. MDCK cells were infected with A/Udorn/72 (H3N2) in the presence or absence of 1 µM BCA. Amantadine (1 mM) was utilized as a reference. After 24-h infection, viral antigens in the infected cells were detected by the specific antibody against viral hemagglutinin under a fluorescence microscope. Nuclei within the cells were stained with DAPI (200× magnification). (C) Interaction of BCA with an influenza envelope glycoprotein hemagglutinin. The interaction was analyzed using an ELISA assay. BCA or a reference glycoprotein, yeast mannan, was immobilized onto the plate and incubated with an influenza vaccine which contains hemagglutinin from a mixture of influenza viruses: A/California/7/09 (H1N1), A/Victoria/210/09 (H3N2), and B/Brisbane/60/08. The bound hemagglutinins were detected by the specific anti-hemagglutinin antibody as described in “Experimental Procedures”. To examine the inhibitory effect of yeast mannan on interaction between BCA and hemagglutinin, the plate coated with BCA was incubated with yeast mannan prior to the incubation with influenza vaccine (3 µg/ml) and assayed in the same way as above.

Fig. 8. Schematic representation of oligosaccharide recognized by BCA. The nonreducing terminal α1-2 mannose cluster is strongly recognized by BCA.
Table 1. *In vitro* activity of BCA against various influenza strains

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<tr>
<th>Virus</th>
<th>Strain</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Antiviral index*</th>
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<tr>
<td><strong>Influenza A</strong></td>
<td>PR8/34 (H1N1)</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>FM/1/47 (H1N1)</td>
<td>1,590.22 ± 3.85</td>
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<td></td>
<td>Kyoto/1/81 (H1N1)</td>
<td>79.34 ± 7.00</td>
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<td>Bangkok/10/83 (H1N1)</td>
<td>106.47 ± 8.05</td>
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<td>96.52 ± 13.25</td>
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<td>819.58 ± 8.33</td>
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<td>Philippines/2/82 (H3N2)</td>
<td>18.80 ± 0.95</td>
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<td><strong>Influenza B</strong></td>
<td>Ibaraki/2/85</td>
<td>81.82 ± 9.82</td>
<td>&gt; 44.3</td>
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IC<sub>50</sub> value of BCA was determined with MDCK cells as > 3623.2 (nM).

*Antiviral index represents the ratio of IC<sub>50</sub> to EC<sub>50</sub>.*
Fig. 1
Fig. 2

A

ATTATCGTCAGCTGCTGATAGCTGAATAATGATTCTAATTATTTCATTTAATATTCATATGAATATGAT
CTCTGGAAAAATTGGCACATACCTTACTACCTACCTATTTTATTCTATTTTCTATTTTTGTAATATAGAAGGATATG

M 1

AAAGCCGTGATGATTGCATCTCCTACTACACTCACCCAAAAATTTTTGTTGAGTCGCCGCGGCGGACA

K T V D C I A L L C T L L C G V R G G A 21

TTCACACTGCTGCTGATAGCTGAATAATGATTCTAATTATTTCATTTAATATTCATATGAATATGAT
CTCTGGAAAAATTGGCACATACCTTACTACCTACCTATTTTATTCTATTTTCTATTTTTGTAATATAGAAGGATATG

L4 1053.7 (1054.2)

L6 1150.8 (1151.2)

F Q A I S G E S G K Y L S H A F A K I W L Q N G Y Q G L G E A W D I Q Y F G N N 61

NVIAMHAKGGGEETTTLSSAFAEG 81

AAAGATTGTCCTCAAAAAGGGAGTGCAGGGAATATAGATGAAATAATACGATGAGTGTCGTCG

K I W L Q K G V Q G D G E K W K Y E W V 101

GGGACCGTTACGTGCTCTACCTGTGTTTGGGGCGGCGGCGGCGGATATACCTGGCAAGCCGCCCT

G N Y V L H C L G G E T G Y T L S A 121

TTCACACTGCTGCTGATAGCTGAATAATGATTCTAATTATTTCATTTAATATTCATATGAATATGAT
CTCTGGAAAAATTGGCACATACCTTACTACCTACCTATTTTATTCTATTTTCTATTTTTGTAATATAGAAGGATATG

A4 1213.0 (1213.4)

A5 855.5 (855.9)

L6-1 1390.0 (1390.5)

L9 986.7 (886.9)

L8-1 1390.0 (1390.5)

L L 986.7 (786.8)

AFSKVLQGKGDGKVLYENKGEVGVNSDSSSLAPEVLFQAOQIPEGLEDPPKLTI S 180

B

1

MKTVDCIA LLLCTT LC G V R G G A F Q A I S G E S G K Y L S H A F A K I W L Q N G Y Q G L G E A W D I Q Y F G N N

N-terminal sequence of intact protein 120

NVIAMHAKGGGEETTTLSSAFAEG 81

AAAGATTGTCCTCAAAAAGGGAGTGCAGGGAATATAGATGAAATAATACGATGAGTGTCGTCG

K I W L Q K G V Q G D G E K W K Y E W V 101

GGGACCGTTACGTGCTCTACCTGTGTTTGGGGCGGCGGCGGCGGATATACCTGGCAAGCCGCCCT

G N Y V L H C L G G E T G Y T L S A 121

TTCACACTGCTGCTGATAGCTGAATAATGATTCTAATTATTTCATTTAATATTCATATGAATATGAT
CTCTGGAAAAATTGGCACATACCTTACTACCTACCTATTTTATTCTATTTTCTATTTTTGTAATATAGAAGGATATG

A4 1213.0 (1213.4)

A5 855.5 (855.9)

L6-1 1390.0 (1390.5)

L9 986.7 (886.9)

L8-1 1390.0 (1390.5)

L L 986.7 (786.8)

AFSKVLQGKGDGKVLYENKGEVGVNSDSSSLAPEVLFQAOQIPEGLEDPPKLTI S 180

121

L8-2 672.6 (673.8)

L10 1631.4 (1631.7)

A12 881.3 (881.0)
Fig. 3

A

20 ---GAFQAI...GKYLSHAF-KIWLQNGYQGCEAWDIYF--- 59
60 NNVI...HAKGE...TSLHAFGKIWLQKVGCQGDGEKVNYNGN 102
103 -NGYV...HCLG...TSLHAFSKWLQKHQGDEVLYENKG 144

B

Subdomain III
BCA mktvdcialllcrgvrgGAFQAI...GKYLSHAFAKIWLQNGY---QGCEAWDIYF--- 58
GNA makasllilaiflgvitscisdNILYGETLSTGEFLNYGSFVFIMQEDONLVLYDYV--- 59

Subdomain II
BCA GNNVIAHMAGGEETTLHAFGK...LQKVGCQGDGEKVEWVNGYVHLCLGGETGYTL 118
GNA -DKPIWATNTGG-------LSRSCFLS-NO...NLYVNP...KPIWASNTGGQNGN--- 106

Subdomain I
BCA SHAFSKVWLQKDGEVLYENKGqv...dsleeapevl...aqgipegledppkt 178
GNA ----YVCIL-----QKDRNVIYG-TDRWATGTHTGLv...appsekytagkiklvtak 157

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GNA -- 157
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**Oligosaccharide from glycolipid**

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**Oligomannose**

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Legend:
- ○ Gal
- ● Man
- ◆ Glc
- □ GlcNAc
- ○ GalNAc
- △ Fuc
- ○ Xyl
Fig. 7

A

![Graph showing inhibition of infection (%) vs BCA (nM) for various strains of influenza virus.](image)

B

<table>
<thead>
<tr>
<th>Condition</th>
<th>Image</th>
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<tbody>
<tr>
<td>Control (Mock infected)</td>
<td><img src="image" alt="Mock infected control with FITC and DAPI" /></td>
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<tr>
<td>A/Udom/72</td>
<td><img src="image" alt="A/Udom/72 control with FITC and DAPI" /></td>
</tr>
<tr>
<td>1 μM BCA + A/Udom/72</td>
<td><img src="image" alt="1 μM BCA + A/Udom/72 control with FITC and DAPI" /></td>
</tr>
<tr>
<td>1 mM Amantadine + A/Udom/72</td>
<td><img src="image" alt="1 mM Amantadine + A/Udom/72 control with FITC and DAPI" /></td>
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</tbody>
</table>

C

![Graph showing absorbance at 450nm vs Hemagglutinin concentration (μg/ml) and Yeast mannan concentration (μg/ml).](image)
High mannose-binding lectin with preference for the cluster of α1-2 mannose from the green alga *Boodlea coacta* is a potent entry inhibitor of HIV-1 and influenza viruses.

Yuichiro Sato, Makoto Hirayama, Kinjiro Morimoto, Naoki Yamamoto, Satomi Okuyama and Kanji Hori

*J. Biol. Chem.* published online April 1, 2011

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