A Novel Core Fucose-specific Lectin from the Mushroom Pholiota squarrosa

A novel core fucose-specific lectin from the mushroom *Pholiota squarrosa**

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Background: Fuc-α1-6 oligosaccharide has a variety of biological functions.
Results: Purification of a novel Fucα 1-6-specific lectin from the mushroom *Pholiota squarrosa*.
Conclusion: The lectin binds only to core α1-6 fucosylated *N*-glycans and not to the other types of fucosylated oligosaccharides.
Significance: The lectin will be a promising tool for analyzing the biological functions of α1-6 fucosylation.

SUMMARY

Fucα1-6 oligosaccharide has a variety of biological functions and serves as a biomarker for hepatocellular carcinoma because of the elevated presence of fucosylated α-fetoprotein (AFP) in this type of cancer. In this study, we purified a novel Fucα1-6-specific lectin from the mushroom *Pholiota squarrosa* by ion-exchange chromatography and affinity chromatography on thyroglobulin-agarose. The purified lectin was designated as PhoSL (*Pholiota squarrosa* lectin). SDS-PAGE, MALDI-TOF mass spectrometry, and *N*-terminal amino acid sequencing indicate that PhoSL has a molecular mass of 4.5 kDa and consists of 40 amino acids (NH_2-APVPVTKLVCDGDTYKCTAYLDF GDGRWVAQWDTNVFHTG-OH). Isoelectric focusing of the lectin showed bands near *p*I 4.0. The lectin activity was stable between pH 2.0 and 11.0 and at temperatures ranging from 0 to 100°C for incubation times of 30 min. When PhoSL was investigated with frontal affinity chromatography using 126 pyridylaminated oligosaccharides, it was found that the lectin binds only to core α1-6 fucosylated *N*-glycans and not to other types of fucosylated oligosaccharides, such as α1-2, α1-3, and α1-4 fucosylated glycans.

Furthermore, PhoSL bound to α1-6 fucosylated AFP but not to non-fucosylated AFP. In addition, PhoSL was able to demonstrate the differential expression of α1-6 fucosylation between primary and metastatic colon cancer tissues. Thus, PhoSL will be a promising tool for analyzing the biological functions of α1-6 fucosylation and evaluating Fucα1-6 oligosaccharides as cancer biomarkers.

Fucose is a monosaccharide that is found on glycoproteins and glycolipids in vertebrates, invertebrates, plants, and microorganisms. Fucosylation comprises the transfer of a fucose residue to oligosaccharides and glycoproteins and is one of the most important oligosaccharide modifications involved in cancer and inflammation (1). Fucosylation is divided into several types, including α1-2, α1-3, α1-4, and α1-6 fucosylation. Among them, α1-6 fucosylation, which is referred to as core fucosylation, is a cancer biomarker for hepatocellular carcinoma (HCC) because of the elevated presence of α1-6 fucosylated AFP (AFP-L3) in this type of cancer. The α1-6 fucosylation of glycoproteins is catalyzed by α1-6 fucosyltransferase (FucT8), which transfers an L-fucose residue to the reducing terminal *N*-acetylglucosamine on *N*-glycans via an α1-6-linkage (2). This oligosaccharide structure can be detected by lectin affinity electrophoresis using *Lens culinaris* agglutinin (LCA), which has an affinity to core-fucosylated mono- and bi-antennary *N*-glycans (3-5). Therefore, the detection of AFP-L3 by this method has been clinically used to make a differential diagnosis of HCC from liver cirrhosis (6-8). LCA can be used for affinity chromatography, but using it for lectin blot analysis to evaluate cellular fucosylation can be difficult because of its low sugar-binding specificity. Conventionally, in addition to LCA, other commercially available core fucose-binding lectins, such as *Pisum sativum* agglutinin (PSA) (9), *Aleuria aurantia* lectin (AAL) (10-13), *Narcissus pseudonarcissus* agglutinin (NPA), *Vicia faba* agglutinin (VFA) (14-16), and *Aspergillus oryzae* lectin (AOL) (12,17,18) have been used in studies on glycobiology. However, most fucose-binding lectins recognize any type of fucosylation, and LCA binds not only to fucose but also to mannose residues in *N*-glycans (3). AOL has been reported to be α1-6 fucose-specific, but in fact also binds alpha1-2 fucose residues in lectin microarrays and in lectin frontal chromatography (19). Therefore, there is a real need for novel α1-6 fucose-binding lectins with a strict binding specificity.

In the course of our continued screening for new mushroom lectins (20,21) (unpublished data), we found lectin activity for core fucose in the extracts of the mushroom *Pholiota squarrosa* and succeeded in the purification of a core fucose-binding lectin from the mushroom. Here, we describe the isolation, characterization, and biological activity of this core fucose-binding lectin.

EXPERIMENTAL PROCEDURES

Materials — Fruiting bodies of *Pholiota squarrosa* were collected from Tochigi, Fukushima, and Miyagi prefectures, Japan, and identified by HyphaGenesis Inc. (MEX-1083). The fruiting bodies were frozen upon collection and stored at –20ºC. DEAE Sepharose Fast Flow was purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK). Butyl-Toyopearl and TSK-GEL G3000SWXL were purchased from Tosoh (Tokyo, Japan). MALDI-TOF mass spectra were acquired on an AutoFlex (Bruker Daltonics Inc., Billerica, MA). Erythrocytes were products of Nippon Biotest Laboratories Inc. (Tokyo, Japan) and Biotest AG (Landsteinerstr, Dreieich). All of the sugars and glycoproteins used for the hemagglutinating inhibition tests and ELISA were purchased from Nacalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Calbiochem (La Jolla, CA), and Sigma-Aldrich Co. (St. Louis, MO). Pyridylaminated (PA)-oligosaccharides for FAC analysis were purchased from Takara Bio

Inc. (Shiga, Japan) and Masuda Chemical Industries Co., Ltd. (Kagawa, Japan). HiTrap NHS-activated Sepharose was purchased from GE Healthcare UK Ltd. Stainless steel empty miniature columns (inner diameter, 2 mm; length, 10 mm; bed volume, 31.4 μl) were obtained from Shimadzu Co. (Kyoto, Japan). The Huh-7D12 cell line was purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). AFP from human cord serum was a product of SCIPAC Ltd. (Sittingbourne, UK). *N*-Glycosidase F was purchased from Roche Applied Science (Mannheim, Germany). Human serum samples for the study were prepared by KAC Co., Ltd. (Kyoto, Japan) with informed consent from the patients. The *Lens culinaris* agglutinin lectin affinity HPLC column (LA-LCA: 0.46 × 15 cm), biotinylated LCA, -AAL were products of J-Oil mills, Inc. (Tokyo, Japan).

Preparation of Affinity Adsorbent — Thyroglobulin and anti-human AFP antibody NB-011 (Nippon Biotest Laboratory Inc.) were conjugated to HiTrap NHS-activated Sepharose according to the manufacture's protocols.

Purification of PhoSL — All of the procedures were carried out at 4°C. After defrosting, the fruiting bodies of *P. squarrosa* were homogenized and then extracted overnight with 10 mM Tris-HCl buffer (pH 7.2) containing 0.1% (v/v) sodium sulfite. The homogenate was centrifuged at 8,500 × *g* for 20 min, and the resultant supernatant was applied to a DEAE-Sepharose column (2.5 × 5 cm) equilibrated with the same buffer. After unbound materials were washed with the buffer, the bound fraction was desorbed with a linear gradient elution of NaCl (0, 0.05, 0.1, 0.2, 0.5, and 1 M) in the same buffer. The lectin-containing fraction eluted with 0.2 M NaCl was concentrated by ultrafiltration and lyophilized. The lyophilized fraction was re-dissolved in PBS, and applied to a column of thyroglobulin-agarose (2.5 × 15 cm) equilibrated with PBS. The column was exhaustively washed with the same buffer, and the adsorbed lectin was desorbed with 0.2 M ammonia. The eluate was immediately neutralized with 1 M HCl, dialyzed extensively against distilled water, and lyophilized. Approximately 2.7 mg of PHOSL was obtained from 100 g of the fresh fruiting bodies.

SDS-PAGE — SDS-PAGE (PhastGel Gradient 10–15 and Highdensity) was performed according to the method of Laemmli (22). Samples were heated in the presence or absence of 2-mercaptoethanol for 5 min at 100°C. Gels were stained with Coomassie Brilliant Blue. The molecular mass standards, XL-Ladder Broad (APRO life Science Institute, Inc., Tokushima, Japan) and smart peptide protein standard (GenScript USA Inc. Piscataway, NJ), were used. Gels were also stained with Glycoprotein Staining Kit and GelCode Phosphoprotein Staining Kit (Thermo Fisher Scientific Inc., Waltham, MA) for glycosylated and phosphorylated proteins, respectively.

Gel Filtration for Estimation of Molecular Mass — Gel filtration by HPLC was carried out on a TSK-gel G3000SWXL column (7.8 × 300 mm) at 25°C in 20 mM phosphate buffer (pH 7.4) containing 20% acetonitrile at a flow rate of 0.5 ml/min. Fractions were collected by monitoring the absorbance at 280 nm. The molecular mass was calibrated with standard proteins (Sigma-Aldrich Co.).

Isoelectric Focusing — Isoelectric focusing was performed on a PhastGel IEF gel (pH 3–9) using Phastsystem (GE Healthcare UK Ltd.). The pI standards were purchased from GE Healthcare UK Ltd.

N-*Terminal Sequence Analysis* — The *N*-terminal amino acid of the intact protein was analyzed on a PPSQ-21A protein peptide sequencer (Shimadzu Co.).

Bioinformatics Analysis — A sequence homology search was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Peptide Synthesis — A peptide possessing the sequence determined by *N*-terminal sequence analysis (NH_2-APVPVTKLVC DGDTYKCTAYLDFGDGRWVAQWDTNVF

HTG-OH) was synthesized chemically by Toray Research Center (Tokyo, Japan). The crude synthesized peptide was purified by reverse-phase HPLC using an ODS column (Wakosil-II 5C18HG, 2 × 25 cm) (Wako Pure Chemical Industries, Ltd.) with a linear gradient of 10–90% CH_3CN/0.05% TFA in H_2O at a flow rate of 1 ml/min. The effluent was monitored at 215 nm. The solvent was removed by evaporation at room temperature, leaving behind the peptide as a residual powder.

Thermostability, pH Stability, and Metal Cation Requirements — The thermostability and pH stability of the lectin were examined as described previously (23-26). Briefly, samples in PBS (0.1 mg/ml) were heated for 30 min at 4, 30, 40, 50, 60, 70, 80, or 100°C, cooled on ice, and titrated. In addition, the samples in PBS were heated for 0.5, 1, 2, 3, 6, or 12 h at 4, 60, 80, or 100°C, cooled on ice, and titrated. The pH stability of the lectin was measured by incubating the samples in different buffers (0.1 mg/ml) for 12 h at 4°C, dialyzed against PBS, and titrated in PBS. The following buffers were used: 50 mM glycine-HCl buffer (pH 2.0, 3.0), 50 mM sodium acetate buffer (pH 4.0, 5.0), 50 mM sodium phosphate buffer (pH 6.0, 7.0), 50 mM Tris-HCl buffer (pH 8.0), and 50 mM glycine-NaOH buffer (pH 9.0–11.0). To examine the metal cation requirements for hemagglutination by the lectin, the sample (0.1 mg/ml) was incubated in 10 mM EDTA for 1 h at room temperature, dialyzed against PBS, and titrated. Afterwards, 0.1 M metal cation ($CaCl_2$, $FeCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$) was added to the demetalized lectin, and the solution was incubated for 1 h at room temperature and titrated.

Solubility — The solubility of the lectins, PHOSL and LCA, was measured by incubating each sample in various buffers (1 mg/ml) for 12 h at 4°C, monitoring absorbance at 280 nm, and titrating in PBS. The following buffers were used: PBS, 10 mM Tris-HCl buffer (pH 7.4), 10 mM sodium phosphate buffer (pH 7.4), 10 mM potassium phosphate buffer (pH 7.4), 10 mM sodium citrate buffer (pH 7.4), and 50 mM veronal buffer (pH 8.6).

Hemagglutination and Inhibition Assay — The hemagglutinating activity of lectin was determined using a 2-fold serial dilution procedure using intact erythrocytes. Briefly, 20 μl of a lectin-containing solution was added to well 1st of a 96-well microtiter plate with U-shaped wells (Greiner), and a 2-fold serial dilution in PBS was made down the plate. Thereafter, 20 μl of a 3% solution of erythrocytes was added to each well, and hemagglutination was allowed to proceed for 1 h at room temperature. The hemagglutination titer was determined as the reciprocal of the highest dilution in which hemagglutination was observed. Results were interpreted as follows: tight button = negative; spread red blood cells = positive.

Inhibition was expressed as the minimum concentration of each sugar or glycoprotein required to inhibit hemagglutination of titer 4 of the lectin using rabbit erythrocytes. Briefly, ten microliters of each sugar- or glycoprotein-containing solution was added to well 1st of a 96-well microtiter plate with U-shaped wells (Greiner), and a 2-fold serial dilution in PBS was made down the plate. Next, 10 μl of titer 4 of the lectin dissolved in PBS was added to each well, and the reaction was allowed to proceed for 1 h at room temperature. Twenty μl of a 3% solution of erythrocytes was added to each well, and hemagglutination was allowed to proceed for 1 h at room temperature. The hemagglutination inhibitory concentration of each sugar was expressed as the reciprocal of the highest dilution in which inhibition of hemagglutination was observed

FAC Analysis — The principle and protocol of frontal affinity chromatography (FAC) analysis have been described previously (27-29). Briefly, the lectin and the peptide were each dissolved in 0.2 M $NaHCO_3$ containing 0.5 M NaCl (pH 8.3) and coupled to a HiTrap NHS-activated Sepharose. After washing and deactivation of excess active groups by 0.5 M Tris containing 0.5 M NaCl (pH 8.3), the lectin- or peptide-immobilized Sepharose beads were suspended in 10 mM Tris-HCl buffer, pH 7.4, containing 0.8% NaCl (TBS); the slurry was packed into a stainless steel column (2.0 × 10 mm) and connected to the FAC-1 machine, which had been specially designed and manufactured by Shimadzu Co. The amount of immobilized protein was determined by measuring the amount of uncoupled protein in the washing solutions by the method of Bradford (30). The flow rate and the column temperature were kept at 125 μl/min and 25°C, respectively. After equilibration with TBS, an excess volume (0.3 ml) of PA-glycans (3.8 or 4.5 nM) was successively injected into the columns by an auto-sampling system. Elution of PA-glycans was monitored by measuring fluorescence (excitation and emission wavelengths, 310 and 380 nm, respectively). The elution front relative to that of a standard oligosaccharide (PA-05, Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-PA), i.e., $V - V_0$, was then determined. V is the elution volume of each PA sugar. PA-05, which has no affinity to the lectin, was used for the determination of V_0. For obtaining the effective ligand content (B_t) of each lectin column, concentration-dependence analysis was performed using varying concentrations (0.5–10 μM) of PA-403 [GlcNAcβ1-2Manα1-3(Galβ1-4GlaNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6) GlcNAc-PA] (Masuda Chemicals, Takamatsu, Japan). Woolf-Hofstee plots were made using the $V - V_0$ values, and the Bt values were thus obtained. Next, using the Bt values for each lectin column, K_d values for a series of glycans were calculated from the equation $K_d = Bt/(V - V_0)$, if $K_d >> [A_0]$. In this study, binding affinity is discussed using an association constant (K_a), where K_a is the inverse of K_d ($K_a = 1/K_d$).

Cell Culture and Purification of AFP-L3 — Huh7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) containing 10% fetal bovine serum (Life Technologies Corporation, Carlsbad, CA) and 1/100 penicillin-streptomycin solution (×100) (Wako Pure Chemical Industries, Ltd.) at 37°C and 5% CO_2. The cultured supernatant was centrifuged at 8,500 × *g* for 20 min and filtered. The resultant supernatant was applied to a column of anti-human AFP antibody NB-011 (Nippon Biotest Laboratories Inc.)-immobilized Sepharose (5 ml) equilibrated with PBS. After unbound materials were washed with the buffer, the bound fraction was desorbed with 0.1 M glycine-HCl buffer (pH 3.0). The eluant was immediately neutralized with 1 M Tris-HCl (pH 9.0). The fractions were concentrated and applied to an LA-LCA column (0.46 × 15 cm) equilibrated with 50 mM Tris-H_2SO_4 (pH 7.2). The column was exhaustively washed with the same buffer, and the adsorbed AFP was eluted by a linear gradient of methyl α-mannoside (0 to 0.2 M) in the buffer. The eluant was dialyzed extensively against distilled water, ultrafiltered, and concentrated.

Deglycosylation of the Anti-human AFP Antibody — Anti-human AFP antibody NB-011 (150 μg) was incubated with 25 U of *N*-glycosidase F (Roche Applied Science) in a total volume of 1 mL of glycosidase reaction buffer (20 mM sodium phosphate buffer (pH 7.4)) containing 0.5% *n*-octyl glucoside (Dojindo Laboratories, Kumamoto, Japan) for 24 h at 37°C. The deglycosylated antibody was stored in aliquots at 4°C with 0.1% sodium azide (31).

Biotinylation of Lectin — PhoSL was incubated with biotin amidocaproate *N*-hydroxysuccinimide ester (Sigma-Aldrich Co. LLC.) in 0.1 M $NaHCO_3$ with haptenic sugar for 12 h at 4 ºC and then desalted with Sephadex G-25 columns (GE Healthcare UK Ltd.)

ELISA — Interaction between glycosylated proteins and L-Fuc-specific lectins was detected by ELISA. Ninety-six-well ELISA plates (Greiner Bio-One, Frickenhausen, Germany) were coated by adding 25 μl of each diluted protein or glycoprotein (100 ng/ml) in 0.1 M carbonate buffer (pH 9.5) per well, and the plates were then incubated overnight at 4°C. Subsequently, the plates were blocked with

PBS containing 1% bovine serum albumin (BSA) for 1.5 h at room temperature, and then rinsed with wash buffer (PBS containing 0.05% Tween 20, pH 7.4) 3 times before the addition of each biotinylated lectin in blocking buffer (1 μg/ml). After incubation for 1 h at room temperature, the plates were washed 3 times before the addition of horseradish peroxidase-streptavidin (Vector, Laboratories Inc., Burlingame, CA). After the plates were washed, TMB microwell peroxidase substrate system (KPL) was used for colorimetric analysis, and the absorbance was measured at 450 nm.

Human Serum Samples Information — Human serum samples for the study were prepared by KAC Co., Ltd., with informed consent from the patients. The normal volunteers were NV-1 (sample ID S018282, female, age 41), NV-2 (sample ID S01828, male, age 46), and NV-3 (sample ID S018290, male, age 29). The HCC patients were HCC-1 (sample ID S09119, male, age 71, Grade 00, TNM T3NxM0, Stage III, CEA 2.3, AFP 6.9), HCC-2 (sample ID S09069, male, age 44, Grade G3, TNM T1N0M0, Stage N/A, CEA N/A, AFP 956.31), and HCC-3 (sample ID S09227, male, age 50, Grade G2, TNM T3N0M0, Stage III, CEA 0.606, AFP 10.66). Human serum samples were pre-treated with Proteome Purify™ 12 (R&D Systems, Inc. Minneapolis, MN).

Antibody–Lectin Sandwich Assay — Ninety-six-well ELISA plates were coated by adding 25 μl of diluted deglycosylated antibody in 0.1 M carbonate buffer (pH 9.5) per well, and the plates were then incubated overnight at 4°C. The next day, the plates were blocked for 1.5 h at room temperature with 150 μl blocking buffer (PBS containing 1% BSA) and then rinsed with wash buffer (PBS containing 0.05% Tween 20, pH 7.4) 3 times. Each sample in the blocking buffer or the human serum samples was allowed to react for 1.5 h at 37°C, and the plates were washed 3 times before the addition of each lectin in the blocking buffer (1 μg/ml). After incubation for 1 h at room temperature, the plates were washed 3 times before the addition of horseradish peroxidase-streptavidin. After the plates were washed, TMB microwell peroxidase substrate system was used for colorimetric analysis, and the absorbance was measured at 450 nm.

Human colon cancer array analysis — Human colon cancer array slides carrying primary or metastatic colon cancers were obtained from KURABO Industries Ltd. (Osaka, Japan). In total, 124 colon cancer tissues, including 79 primary and 45 metastatic cancer tissues and 11 normal colon tissues, were subjected to immunohistochemical analysis. Tissue microarray slides were deparaffinized with xylene and ethanol. The slides were pretreated with avidin/biotin solution and then with Peroxidase Blocking Reagent (DAKO, Carpinteria, CA). After washing twice with PBS, the slides were incubated in TBST containing 5% BSA at 4°C overnight. Next, the slides were incubated with biotinylated AAL (5.0 μg/ml) or PhoSL (50 μg/ml) for 1 h at room temperature. The slides were then washed 3 times with PBS and incubated with ABC kit (Vector, Laboratories Inc.) for 30 min at room temperature. After washing 3 times with PBS, positive staining was visualized using diaminobenzidine (DAKO). Statistical analysis was performed using the $\chi 2$ test.

RESULTS

Isolation and Molecular Properties of PhoSL — A lectin was purified from extracts of fruiting bodies of *Pholiota squarrosa* using DEAE-Sepharose and affinity chromatography on thyroglobulin-agarose. Since very little of the lectin activity was recovered from the affinity support (the thyroglobulin-agarose) by elution with the haptenic sugar, L-fucose, even at a concentration of 0.2 M, the lectin was eluted with 0.2 M ammonia. The purified lectin, designated as PhoSL, gave a band with a mass of 4.5 kDa on SDS-PAGE in the presence (Figs. 1A, lane 1 and 1B, lane 1) and two bands with masses of 4.5 kDa and 14 kDa in the absence of 2-mercaptoethanol(Fig 2, lane 2). Isoelectric focusing gave a band near *p*I 4.0 (Fig. 1C). The use of assay kits for the detection of glycoproteins and phosphoproteins showed no significant bands on the membrane, suggesting that very low or undetectable levels of glycosylation and phosphorylation were present in the protein (data not shown). MALDI-TOF mass analysis of PhoSL yielded molecular ions from *m/z* 4229 to 4455 and small peaks at *m/z* 8932 and 13373 (data not shown). HPLC gel filtration of the intact lectin gave a peak at an elution volume corresponding to a molecular mass of 14 kDa (Supplementary data, Fig, S2).

N-*Terminal Amino Acid Sequence Analysis* — *N*-Terminal amino acid sequence analysis of PhoSL gave the forty-amino-acid sequence NH_2-APVPVTKLVCDGDTYKCTAYLDFGD GRWVAQWDTNVFHTG-OH (Fig. 2). The amino acid sequence of PhoSL was analyzed by the BLAST program, and the sequence showed homology to a lectin from *Rhizopus stolonifer* (RSL) (85%) (Fig. 2).

Stability of PhoSL — The lectin activity of PhoSL was extraordinarily stable over a wide range of temperatures between 4ºC and 100ºC at an incubation time of 30 min (Fig. 3A). The activity was also retained at 60ºC for 12 h and at 80ºC for 6 h (Fig. 3B). Half of the lectin activity was maintained even at 100ºC for 3 h (Fig. 3B). Similarly, the lectin activity was very stable over the wide range of tested pH values (pH 2.0–11.0) (Fig. 3C). Treatment with EDTA or the addition of the metal cations $CaCl_2$, $FeCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$ did not produce any changes in the lectin activity. This result indicates that the lectin does not require metal ions for binding (date not shown). PhoSL was soluble in all the buffers used; however, LCA was soluble in 10 mM Tris-HCl buffer (pH 7.4), 10 mM sodium phosphate buffer (pH 7.4), and 50 mM veronal buffer (pH 8.6) but not soluble in PBS, 10 mM potassium phosphate buffer (pH 7.4), or 10 mM sodium citrate buffer (pH 7.4) (data not shown).

Hemagglutination and Inhibition Assays — As shown in Table 1, PhoSL agglutinated intact erythrocytes from rabbit, horse, pig, goose, and guinea pig. As shown in Table 2, various monosaccharides, oligosaccharides, and glycopeptides were able to inhibit the hemagglutination activity of PhoSL. None of the mono- and oligosaccharides used bound to PhoSL. Among the tested glycoproteins, only IgG and thyroglobulin inhibited the hemagglutination activity of PhoSL. A peptide possessing the determined amino acid sequence was synthesized chemically. The synthetic peptide did not agglutinate intact rabbit erythrocytes (data not shown).

FAC Analysis of PhoSL — The detailed sugar-binding specificity of PhoSL was also elucidated by FAC analysis. Among 114 kinds of PA-glycans used (Supplementary data, Fig. S1), only the 21 glycans possessing the core α1-6 fucose bound to the lectin (Fig. 4B). The B_t and K_d values were determined to be 0.09 nmol and 3.3×10^{-6} M, respectively, for the immobilized PhoSL (1 mg/ml) using PA-403 (Fig. 4A). The strength of affinity of each PA-glycan for the immobilized lectin is shown as a K_a value (M^{-1}) in Figs. 4B and 5B. Manα1-3(Manα1-6 Manβ1-4GlcNAcβ(Fucα1-6)1-4GlcNAc-PA (PA-15, $K_a = 5.0 \times 10^{-5}$ M) showed the strongest affinity to the immobilized lectin (Fig. 4B). The sialylated *N*-glycans ±Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 (Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-PA (PA-601, $K_a = 2.4 \times 10^{-5}$ M and 602, $K_a = 1.2 \times 10^{-5}$ M) also bound to the lectin. In contrast, *O*-glycans having L-Fuc (PA-718 to PA-723, PA-726 to PA-731, PA-739, PA-909) or Fucα1-3 linkages (PA-419 and 420) did not show any significant affinity to the immobilized PhoSL.

The detailed oligosaccharide-binding specificity of PhoSL was compared to that of LCA, which has been reported previously (Fig. 5) (3). For an easier understanding of the structural elements required for the recognition of PhoSL and LCA, the K_a values for a series of core-fucosylated glycans have been arranged in the order of affinity strength, and the core-fucosylated glycans have been represented using the "GRYP" code proposed in a previous report (3,32). In this system, the branch positions of *N*-glycans (GlcNAc) are numbered from I to VI according to the corresponding mammalian GlcNAc-transferases, and the nonreducing end sugars are shown in different colors: GlcNAc (blue), Gal (yellow), and NeuAc (purple). The presence of the core fucose (α1-6Fuc) is emphasized with another box colored in red. Both PhoSL and LCA showed high specificity for mannose-type (PA-015), mono- (PA-201, 401, 402), and bi-antennary (PA-202, 203, 403, 404, 405, 406, 601, 602) *N*-glycans containing core fucose. PhoSL recognized not only mono- or bi-antennary oligosaccharides but also tri- or tetra-antennary ones (PA-015, 201-204, 401–411, 413, 601, 602) (Fig. 5B). However, LCA bound to only mono- and biantennary oligosaccharides (PA-015, 202, 203, 205, 401, 402, 403, 406, 601, 602). No binding of LCA to core-fucosylated tri- (PA-407, 408, 409, 410) and tetra-antennary (PA-411, 413, 415, 418) *N*-glycans was observed (Fig. 5B).

The sugar-binding specificity of the synthetic peptide was almost the same as that of PhoSL (Supplementary data, Fig. S3).

ELISA of PhoSL — To compare the detailed carbohydrate binding specificity of PhoSL with that of LCA, we examined the binding of biotinylated lectins to immobilized proteins or glycoproteins using ELISA (Fig. 6). Each protein or glycoprotein was immobilized on the plates, and biotinylated PhoSL or LCA was used as the analyte. Among the major human serum glycoproteins (HSA, IgG, TF, Fib, IgA, a2M, IgM, A1AT, C3, HP, and AGP) tested, the most potent binding glycoproteins for PhoSL were IgA and IgG (Fig. 6A). Among the serum tumor markers (PSA, AFP, AFP-L3) tested, PhoSL bound to fucosylated AFP (AFP-L3). TG (bovine) and LF (human milk) were also bound to PhoSL. All the glycoproteins that bound to PhoSL in the assay possessed the core fucose. Although the profile of LCA was similar to that of PhoSL, the binding to the glycoproteins was much weaker than that of PhoSL (Fig. 6B).

Antibody-Lectin Sandwich ELISA — The sugar-binding specificity of PhoSL to fucosylated AFP (AFP-L3) obtained from the sera of 3 patients with HCC (HCC-1 to -3) was further investigated by antibody-lectin sandwich ELISA (Fig. 7). Prior to the ELISA, the existence of AFP-L3 in the serum samples was confirmed by the conventional method, lectin affinity electrophoresis (LAE), using LCA. All the samples contained AFP-L3 (Supplementary data, Fig. S4). In the antibody-lectin sandwich ELISA, *N*-glycosidase F-treated anti-AFP antibody was immobilized on the plate, since IgG has fucosylated oligosaccharides in its Fc site. AFP from the 3 patients (HCC-1 to -3) and 3 volunteers (NV-1 to NV-3) was detected by anti-AFP (Fig. 7A). Both PhoSL and LCA bound to the AFP in a dose-dependent manner (Fig. 7B and 7C).

Application of PhoSL to Histochemistry — To demonstrate the utility of PhoSL in the immunohistochemical analysis of human cancer tissues, 124 colon cancer tissues, including primary and metastatic colon cancers, on tissue arrays were stained with biotinylated–PhoSL and –AAL. AAL is a lectin from *Aleuria aurantia* that binds to all types of fucosyl linkage. Staining intensities were classified into 4 groups: negative staining (0), low staining intensity (1), medial staining intensity (2), and high staining intensity (3). A representative tissue from each group is shown in Supplementary data, Fig. S5. Representative images of normal colon, primary cancer, and metastatic cancer are shown in Fig. 8A. Normal colon mucosa was stained with AAL, but not PhoSL, because of abundant mucin, which carries α1-3/4 fucosylated glycans on *O*-glycans, suggesting that PhoSL does not bind to α1-3/4 fucosylated glycans (Fig. 8A and B). As shown in Fig. 8C and D, both AAL and PhoSL bound to primary colon cancer tissue at a similar intensity. In contrast, only AAL, but not PhoSL, bound to metastatic colon cancer tissue (Fig. 8E and F). The staining intensities of all tissues examined in this study are summarized in Table 3. Approximately 70% of the primary and metastatic cancer tissues were classified into a high-intensity group after AAL staining (Fig. 8G, H and Table 3). No difference in AAL-staining intensity was observed between primary and metastatic cancer tissues. In contrast, PhoSL exhibited a significantly lower binding capacity to the metastatic cancer tissues than the primary tissues (Fig. 8G, H and Table 3). Only 25% of the metastatic tissues showed medial and strong intensities (group 2 and 3) following PhoSL staining, despite the fact that 84% of these tissues were stained with AAL.

DISCUSSION

PhoSL was purified from the edible mushroom *Pholiota squarrosa*. The lectin gave a band with a mass of 4.5 kDa on SDS-PAGE in the presence (Figs. 1A, lane 1 and 1B, lane 1) and two bands with masses of 4.5 kDa and 14 kDa in the absence of 2-mercaptoethanol (Fig 2, lane 2). Its primary structure consisted of 40 amino acids, as determined by *N*-terminal amino acid sequence analysis. The synthetic PhoSL peptide corresponding to the determined sequence exhibited identical binding specificity to native PhoSL in FAC analysis (Supplementary data, Fig. 2), but did not exhibit hemagglutination activity. HPLC gel filtration of the intact lectin gave a peak at an elution volume corresponding to a molecular mass of 14 kDa. PhoSL possessed no sugar chains or phosphate groups. All the results mentioned above indicated that PhoSL is composed of three or four 4.5 kDa subunits with S-S linkage and exhibits true polyvalent binding during agglutination of erythrocytes and/or precipitation of appropriate cell surface polysaccharides, but the oligomeric form is not necessarily in direct binding assays (FAC analysis, ELISA, etc.).

The BLAST search revealed that PhoSL has 85% sequence homology (22/26 amino acids) with the α1-6 linked fucose-specific lectin from *Rhizopus stolonifer*. RSL has also been isolated as a core fucose specific lectin. RSL has high affinity toward saccharides with α1-6Fuc, and weak affinity toward saccharides with α1-2Fuc, α1-3Fuc, and α1-4Fuc (33).

The unique property of PhoSL is its strict sugar-binding specificity to α1-6Fuc (Fig. 4B). α1-6 Fucosylation is one of the most important oligosaccharide modifications in carcinogenesis; however, although many studies related to fucosylation have been conducted, they have not completely clarified the difference between α1-2, α1-3, or α1-4 fucosylation and α1-6 fucosylation. A hindrance to this clarification has been the lack of a tool for the specific detection of α1-6 fucosyl linkage; AAL, which is used in many studies, recognizes all types of fucosyl linkages (13,17).

The FAC results indicate that PhoSL recognizes α1-6 fucosyl linkages exclusively, that all the α1-6 oligosaccharides were bound to the lectin, and that LCA could not bind to some α1-6 oligosaccharides (Figs. 4 and 5). In addition, the K_a value of PhoSL was 3.2×10^5 M^{-1} for the fully galactosylated, biantennary *N*-glycan with a core fucose (PA-405), which is the major *N*-glycan in AFP-L3 from Huh7 cells and HCC patients (Fig. 4B) (34). On the other hand, the K_a value of the binding between LCA and PA-405 was 4.7×10^4 M^{-1} (3). The affinity of PhoSL towards the oligosaccharide was higher than that of LCA. Furthermore, LCA also bound to non-fucosylated, high mannose-type *N*-glycans. For example, LCA showed affinity for a larger high mannose-type *N*-glycans, Man8 [Manα1-2Manα1-2Manα1-3 (Manα1-2Manα1-6 (Manα1-3) Manα1-6) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-PA, PA-012], with a K_a of 2.5×10^4 M^{-1} (3). LCA is now the only commercially available diagnostic agent that can detect α1-6 fucosyl linked sugar chains specifically.

The superiority of PhoSL over LCA was confirmed by ELISA using biotin-labeled PhoSL and LCA and immobilized glycoproteins. Although the specificity of both lectins showed similar tendencies, the binding strength of PLT to the α1-6 fucosylated glycoproteins was greater than that of LCA (Fig. 6). The promising potential of PhoSL as a diagnostic agent was also shown by antibody-lectin sandwich ELISA using AFP-L3 and the partially purified AFP from the sera of HCC patients and normal volunteers (NV) (Fig. 7). The sensitivity and selectivity of PhoSL to AFP-L3 in the sera of HCCs and NVs were much greater than those of LCA and even anti-AFP (Fig. 7). AFP is a biomarker that was discovered in 1963 by Abelev (35) and belongs to the albumin-like superfamily. This protein, whose molecular mass is 65 kDa, consists of 590 amino acids and has a bi-antennary sugar chain at Asp^{232} (36). A variety of sugar chains are on the protein, and AFP-L3 is one of them. The structure of AFP-L3 has been determined to be GlcNAcβ1-2Manα1-3 (Galβ1-4GlcNAcβ1-2Manα1-6) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-AFP by lentil lectin affinity electrophoresis. Since slightly increased serum concentrations of total AFP have been observed in patients with chronic hepatitis and liver cirrhosis, conditions that are known to be associated with premalignant HCC lesions, a wide overlap in total AFP has been observed between HCC and such benign liver diseases. The sera of patients with HCC are known to contain relatively large amounts of AFP-L3. Therefore, AFP-L3 has been recognized as a specific marker for HCC. Further, analysis using this marker could be useful for monitoring treatment responses and disease recurrence and could also be a tool for recognition of HCC earlier than that possible by using imaging modalities (34,37-42). In recent years, in addition to AFP, new biomarkers possessing fucosides have been discovered. For example, golgi protein 73 (GP73) content in the blood of patients with HCC was found to be elevated, and the protein was α1-6-hyperfucosylated (43-46). In addition, patients with liver cirrhosis and liver cancer had increased levels of triantennary glycancontaining outer arm (α1-3)-fucosylation in α-antitrypsin (A1AT) in the blood, but increases in core (α1-6)-fucosylation were observed only on A1AT from patients with liver cancer (47). Physiological functions of the core fucose has been investigated recently. The lack of core fucosylation of transforming growth factor-β1(TGF-β1) receptors induces severe growth retardation and death during postnatal development (48,49). Mutations of the GDP-mannose-4,6-dehydratase (GMDS) gene that plays a pivotal role in fucosylation in human colon cancer resulted in resistance to TRAIL-induced apoptosis followed by escape from immune surveillance. This pathway by GMDS mutation could be a novel type of cancer progression through cellular fucosylation and NK cell–mediated tumor surveillance. However the cellular fucosylation type has not determined yet (50,51).

Fig. 8 shows that AAL bound to both primary and metastatic colon cancer tissues with a similar intensity. However, PhoSL bound to the primary colon cancer tissues more strongly than it did to the metastatic tissues. These results suggest that, in some cases, α1-6 fucosylation is increased in the early phase of colon cancer development and subsequently decreased in the metastatic phase. The decreased expression of α1-6 fucosylation in metastatic cancer tissues may be responsible for the escape of cancer cells from NK cell-mediated tumor surveillance. The mechanism and meaning underlying the decreased expression of α1-6 fucosylation in metastatic cancer tissues should be revealed in a future study. PhoSL, the lectin characterized in this study, may be useful for the detection of AFP-L3 and other new biomarkers, and for determining the physiological functions of oligosaccharides (52).

In summary, PhoSL very strongly and specifically binds to Fucα-oligosaccharides. Moreover, it is highly stable over a wide range of pHs and temperatures and is highly soluble in various buffers. These advantages indicate that PhoSL can become a powerful tool to analyze biological functions of core fucoses and serve as a diagnostic agent in the near future.

Abbreviations used: PhoSL, *Pholiota squarrosa* lectin; LCA, *Lens culinaris* agglutinin; AAL, *Aleuria aurantia* lectin; AOL, *Aspergillus oryzae* lectin; RSL, *Rhizopus stolonifer* lectin; PBS, 10 mM phosphate-buffered saline, pH 7.4; DEAE, diethylaminoethyl; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue; pM, peptide marker; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; PA, pyridylaminated; NHS, *N*-hydroxysuccinimide; FAC, frontal affinity chromatography; TBS, 10 mM Tris-HCl buffer containing 0. 15 M NaCl, pH 7.4; PVDF, poly-vinylidene fluoride; TBS-T, 10 mM Tris-HCl buffer, pH 7.4, containing 0.8% NaCl and 0.05% Tween 20; Tris, tris(hydroxymethyl)aminomethane; ELISA, enzyme-linked immunosorbent assay; LAE, lectin affinity electrophoresis; BSA, bovine serum albumin; TG, thyroglobulin; LF, lactoferrin; OVA, ovalbumin; BSM, bovine submaxillary gland mucin; PSM, porcine stomach mucin; HAS, human serum albumin; IgG, Immunoglobulin G; TF, transferrin; Fib, fibrinogen; IgA, Immunoglobulin A; a2M, alpha-2-macroglobulin; IgM, Immunoglobulin M; A1AT, alpha-1-antitrypsin; HP, haptoglobin; AGP, alpha-1-acid glycoprotein; PSA, prostate-specific antigen; AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; NV, normal volunteers; Fc site, fragment, crystallizable site of antibody; all sugars are of D-configuration unless otherwise stated.

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FIGURE LEGENDS

FIGURE 1. Characterization of PhoSL.
A, SDS-PAGE linear gradient gel (10–15%). Lane M indicates marker proteins; lane 1, purified PhoSL under reducing conditions with 2-mercaptoethanol; lane 2, purified PhoSL non-reduced.
B, High-density SDS-PAGE gel. Lane pM indicates marker peptides; lane 1, purified PhoSL under reducing conditions with 2-mercaptoethanol; lane 2, purified PhoSL non-reduced; lane pM, marker proteins.
C, Isoelectric focusing of PhoSL Lane M indicates marker proteins; lane 1, PhoSL.

FIGURE 2. Multiple alignment of PhoSL and RSL.
The residues in the first row describe the amino acid sequence of PhoSL, and those in rows 2 describe the amino acid sequences of *Rhizopus stolonifer* lectin (RSL). Shown in gray shading are the amino acid residues that are identical between PhoSL and RSL.

FIGURE 3. The stability of PhoSL over a range of temperature and pHs.
The stability of PhoSL was investigated over a broad range of temperatures for 30 min (*A*), long incubation times for 4 different temperatures (*B*), and incubation in different pH buffers (*C*).

FIGURE 4. FAC analysis of PhoSL.
A, Elusion pattern of several types of PA-oligosaccharides on the PhoSL-immobilized column. *B*, Association constants (K_a) values of the purified PhoSL to various types of PA-glycans.

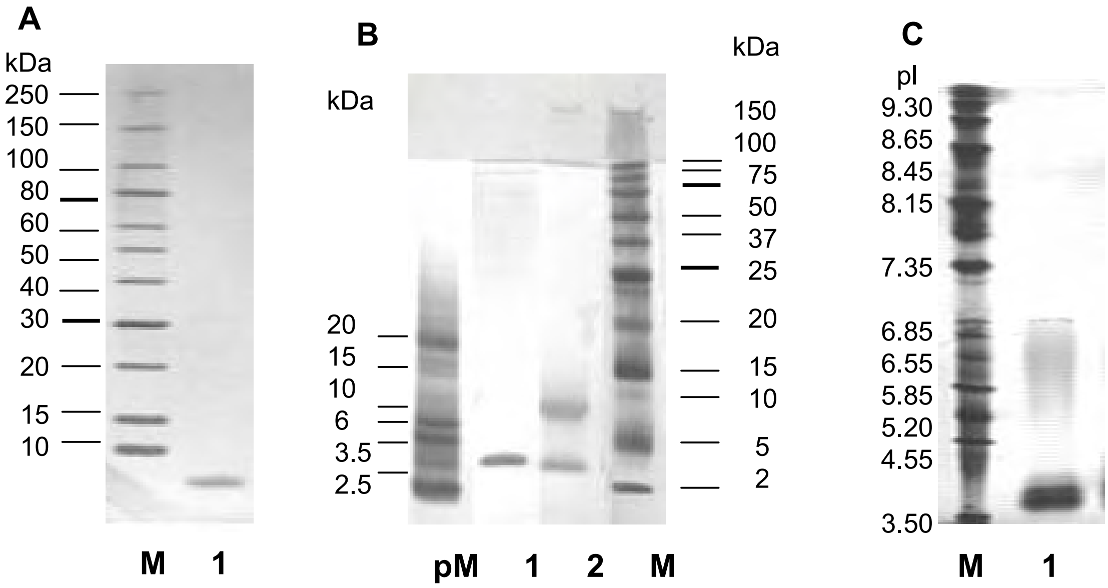
FIGURE 5. Comparative analysis of glycan-binding specificity of PhoSL and LCA by the

GRYP code.
A, Definitions of the GRYP code for representing the branch positions and non-reducing end residues. The non-reducing end sugars and the core fucose are shown in different colors in the left panel. Each branch is numbered from I to VI corresponding to GlcNAc transferases, as shown in the middle panel.
B, Bar graph representations of the association constants (K_a) of PhoSL toward core-fucosylated *N*-glycans. Numbers at the bottom of the bar graphs correspond to the sugar numbers indicated in Figure S2.
C, Bar graph representations of the association constants (K_a) of LCA.

FIGURE 6. Binding of PhoSL and LCA to immobilized glycoproteins by ELISA.
A, Binding activity of biotin-labeled PhoSL and various immobilized glycoproteins.
B, Binding activity of biotin-labeled LCA and various immobilized glycoproteins.
The immobilized glycoproteins are HAS, human serum albumin; IgG, Immunoglobulin G; TF, transferrin; Fib, fibrinogen IgA Immunoglobulin A; a2M, alpha-2-macroglobulin; IgM, Immunoglobulin M; alpha-1-antitrypsin; C3, third components of complement; HP, haptoglobin; AGP, alpha-1-acid glycoprotein; PSA, prostate-specific antigen; AFP, a-fetoprotein; AFP-L3, 1-6 fucosylated fetoprotein; TG, thyroglobulin; LF, lactoferrin; FT, fetuin; BSM, bovine submaxillary gland mucin; PSM, porcine stomach mucin; Inv, invertase; OVA, ovalbumin; BSA, bovine serum albumin.

FIGURE 7. Antibody-lectin sandwich ELISA using purified AFP-L3 and the sera of hepatocellular carcinoma (HCC) patients and normal volunteers (NV).
ELISA with Anti-AFP (*A*), PhoSL (*B*), and LCA (*C*).

FIGURE 8. Immunohistochemical analysis of human colon cancer tissues with PhoSL and **AAL**
Human colon cancer tissue arrays comprising normal colon tissues (*A* and *B*), primary colon cancers (*C* and *D*), and metastatic colon cancers (*E* and *F*) were subjected to immunohistochemical analysis with PhoSL and AAL. *G* and *H*, The ratio of the numbers in each staining-intensity group to the total number is shown.


A
kDa
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150
100
80
60
50
40
30
20
15
10
M
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B
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15
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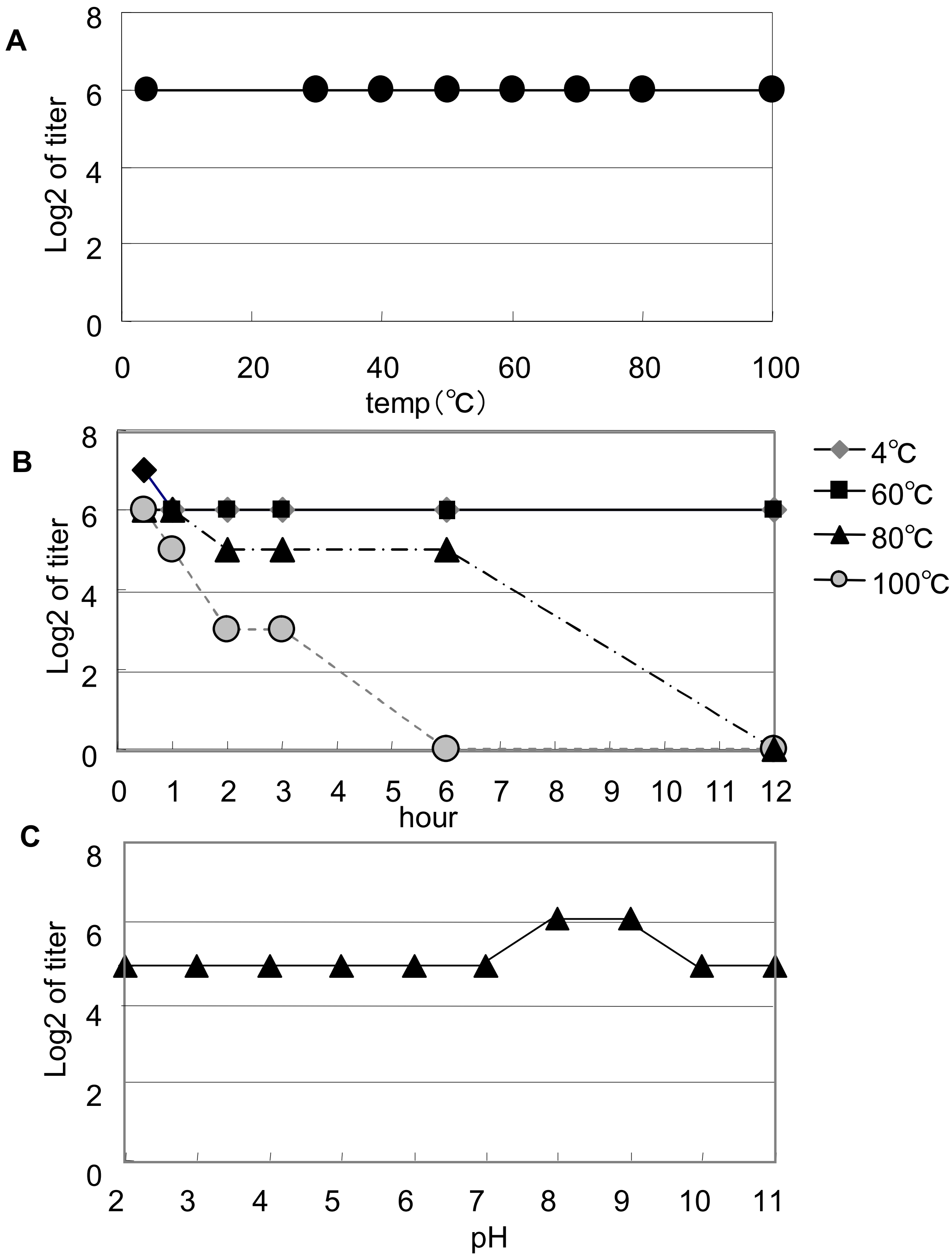
6
3.5
2.5
kDa
150
100
75
50
37
25
20
15
10
5
2
pM
1
2
M
C
pI
9.30
8.65
8.45
8.15
7.35
6.85
6.55
5.85
5.20
4.55
3.50
M
1

Fig. 1.

PhoSL	APVPVTKLVCDGDTYKCTAYLDFGDGRWVAQWDTNVFHTG	1-40aa
RSL	IDPVNVKKLQCDGDTYKCTADLDFGDGR	1-28aa

Fig. 2.


A
Log2 of titer
8
6
4
2
0
0
20
40
60
80
100
temp（℃）
B
Log2 of titer
8
6
4
2
0
0
1
2
3
4
5
6
7
8
9
10
11
12
hour
4℃
60℃
80℃
100℃
C
Log2 of titer
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6
4
2
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2
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10
11
pH

Fig. 3.

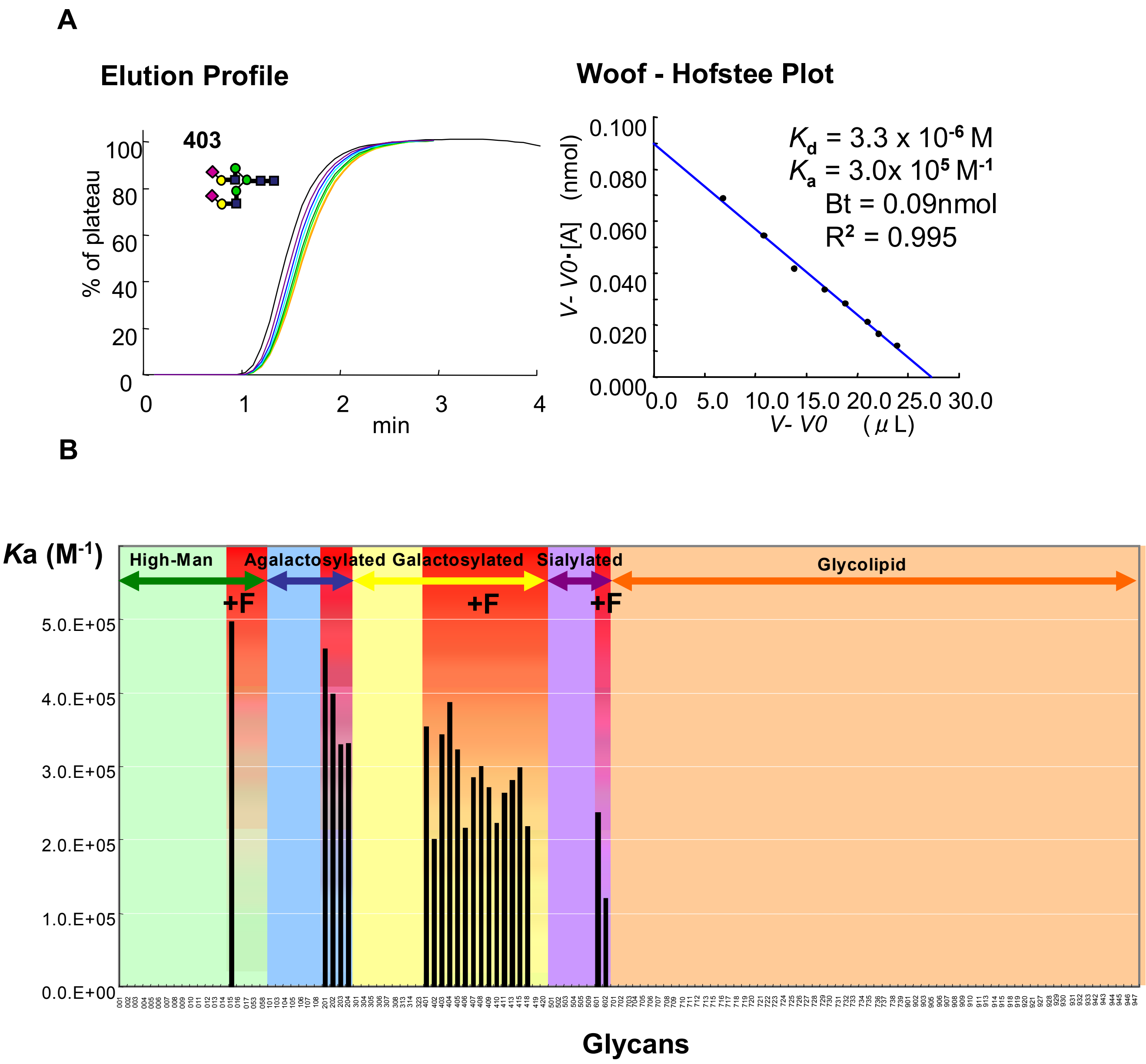

A
Elution Profile
403
% of plateau
100
80
60
40
20
0
0
1
2
3
4
min
Woof - Hofstee Plot
K_d = 3.3 x 10^{-6} M
K_a = 3.0x 10^5 M^{-1}
Bt = 0.09nmol
R^2 = 0.995
V- V0・[A] (nmol)
0.100
0.080
0.060
0.040
0.020
0.000
0.0 5.0 10.0 15.0 20.0 25.0 30.0
V- V0 (μL)
B
Ka (M^{-1})
High-Man
Agalactosylated
Galactosylated
Sialylated
Glycolipid
+F
+F
+F
5.0.E+05
4.0.E+05
3.0.E+05
2.0.E+05
1.0.E+05
0.0.E+00
Glycans

Fig. 4.

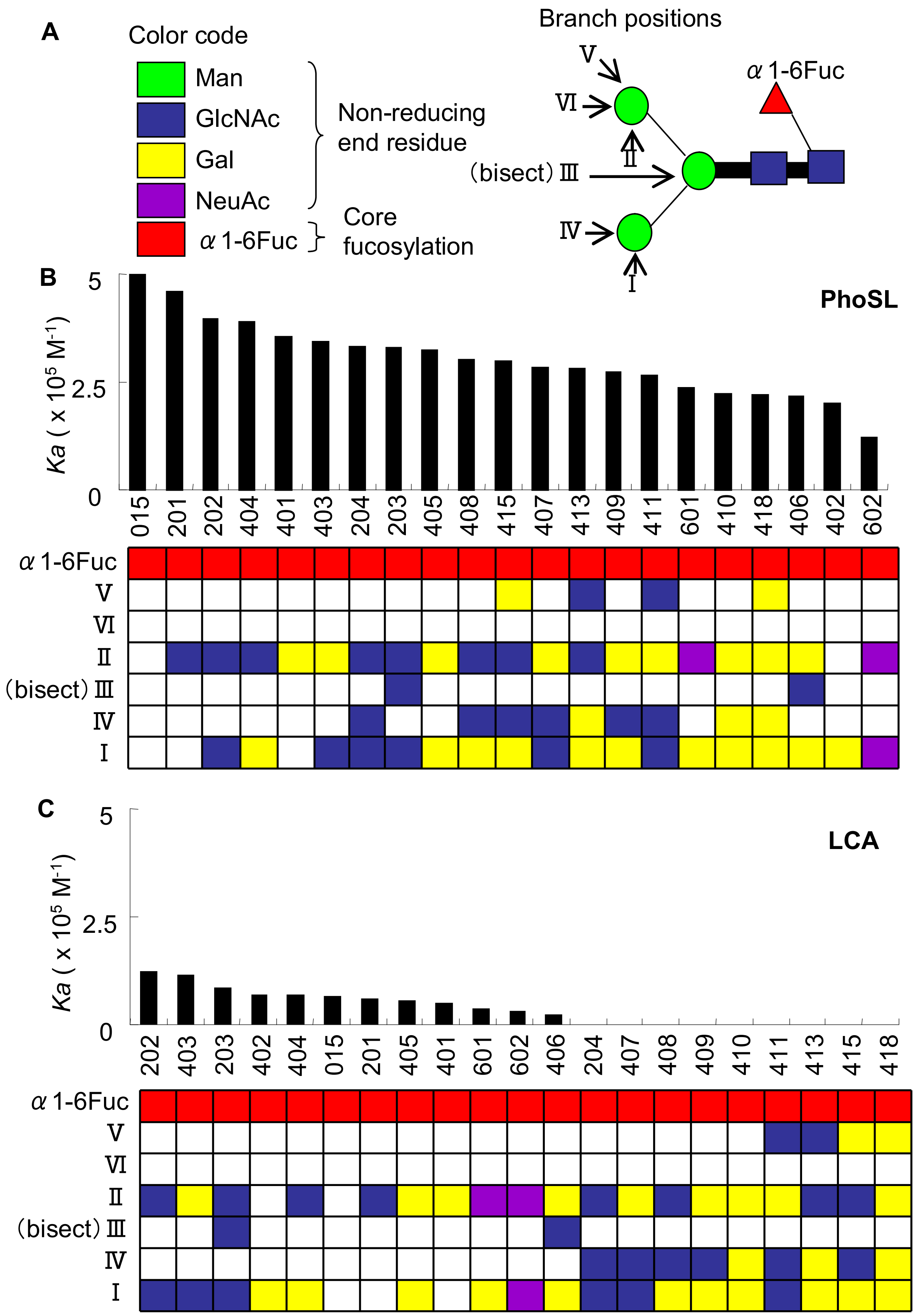

A
Color code
Man
GlcNAc
Gal
NeuAc
α1-6Fuc
Non-reducing end residue
Core fucosylation
Branch positions
V
VI
II
(bisect) III
IV
I
α1-6Fuc
B
PhoSL
Ka (x 10^5 M^-1)
5
2.5
0
015 201 202 404 401 403 204 203 405 408 415 407 413 409 411 601 410 418 406 402 602
α1-6Fuc
V
VI
II
(bisect) III
IV
I
C
LCA
Ka (x 10^5 M^-1)
5
2.5
0
202 403 203 402 404 015 201 405 401 601 602 406 204 407 408 409 410 411 413 415 418
α1-6Fuc
V
VI
II
(bisect) III
IV
I

Fig. 5.

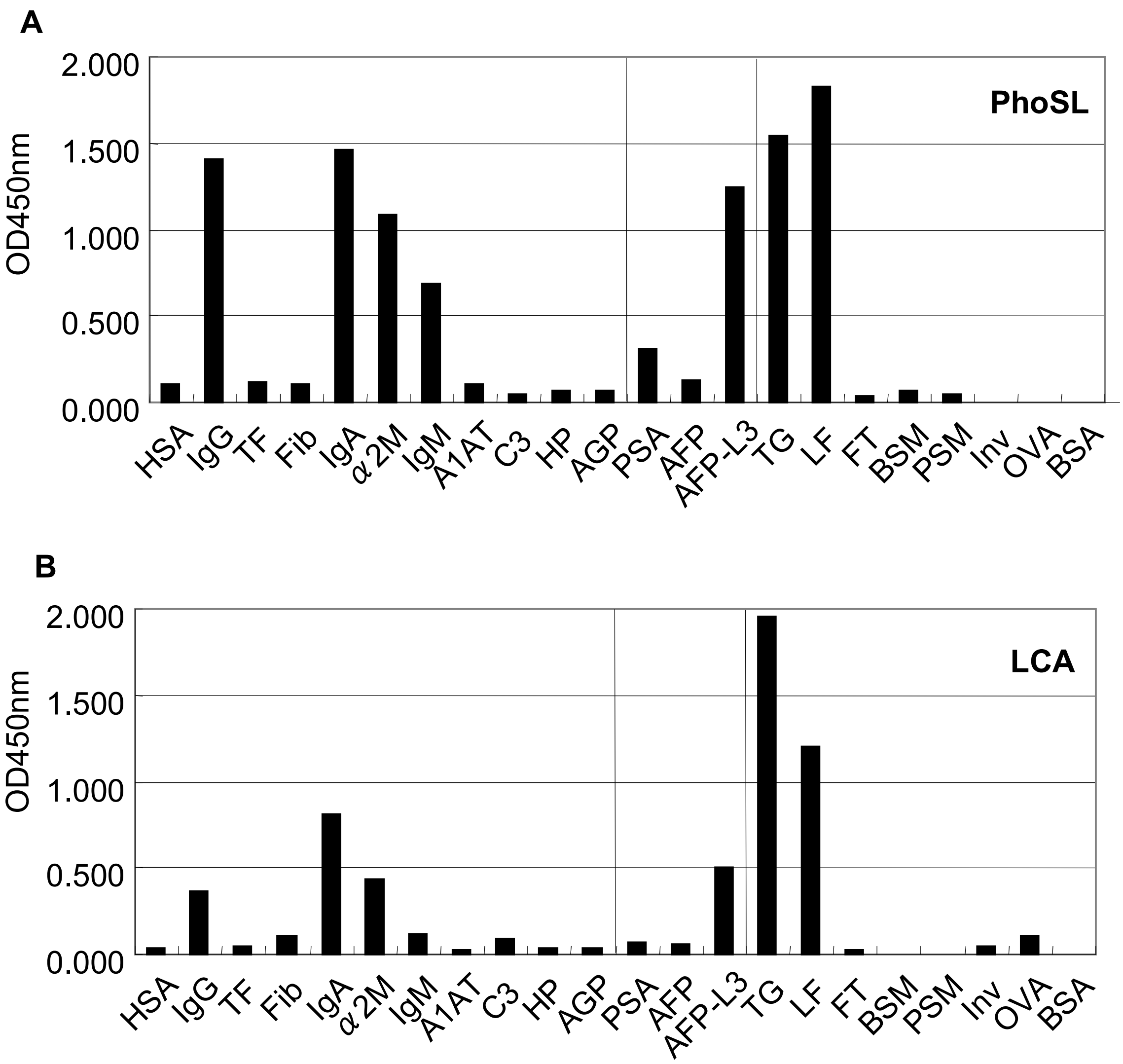
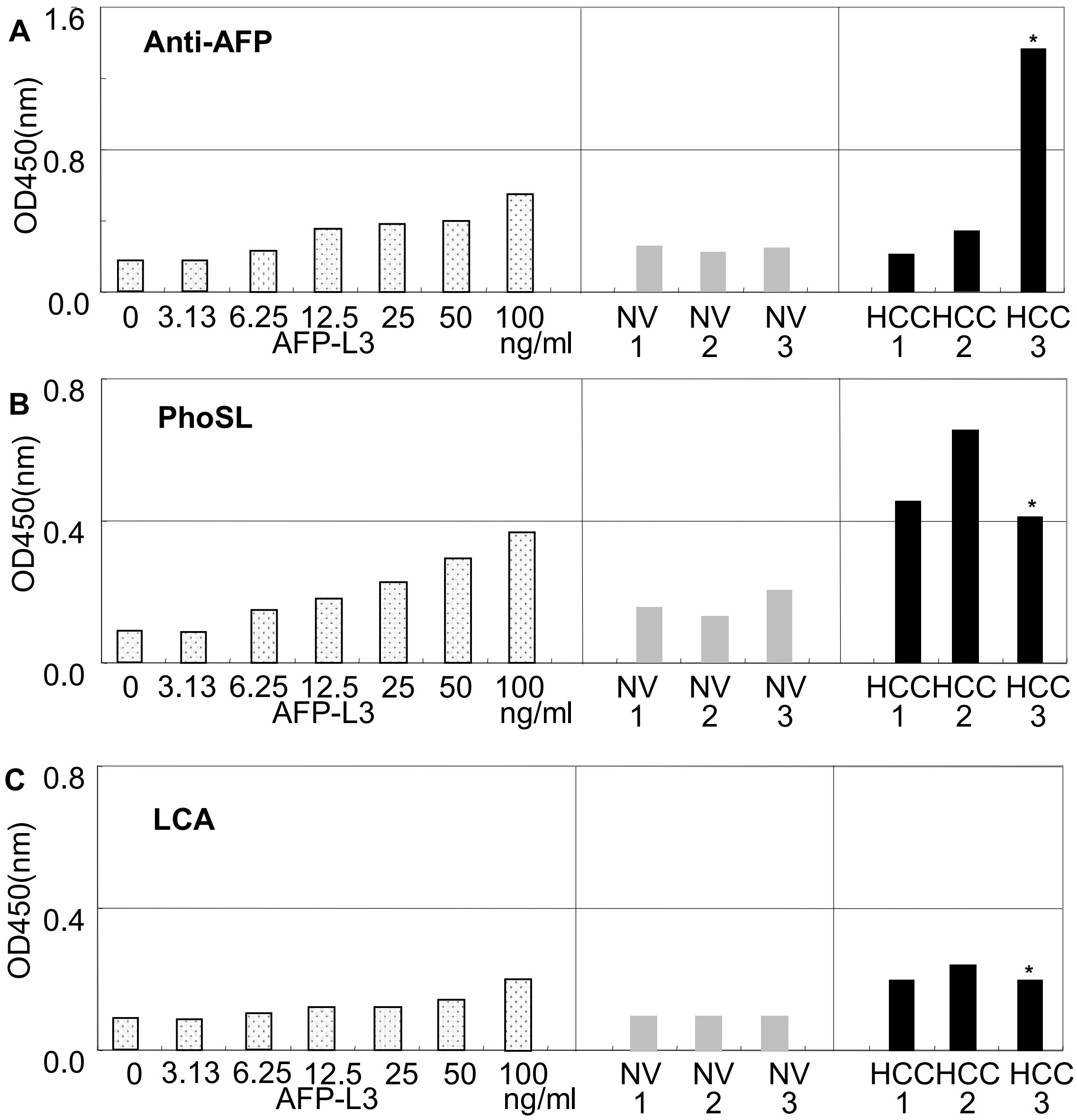

A
PhoSL
OD450nm
2.000
1.500
1.000
0.500
0.000
HSA IgG TF Fib IgA α2M IgM A1AT C3 HP AGP PSA AFP AFP-L3 TG LF FT BSM PSM Inv OVA BSA
B
LCA
OD450nm
2.000
1.500
1.000
0.500
0.000
HSA IgG TF Fib IgA α2M IgM A1AT C3 HP AGP PSA AFP AFP-L3 TG LF FT BSM PSM Inv OVA BSA

Fig. 6.



*10-fold diluted sample

Fig. 7.

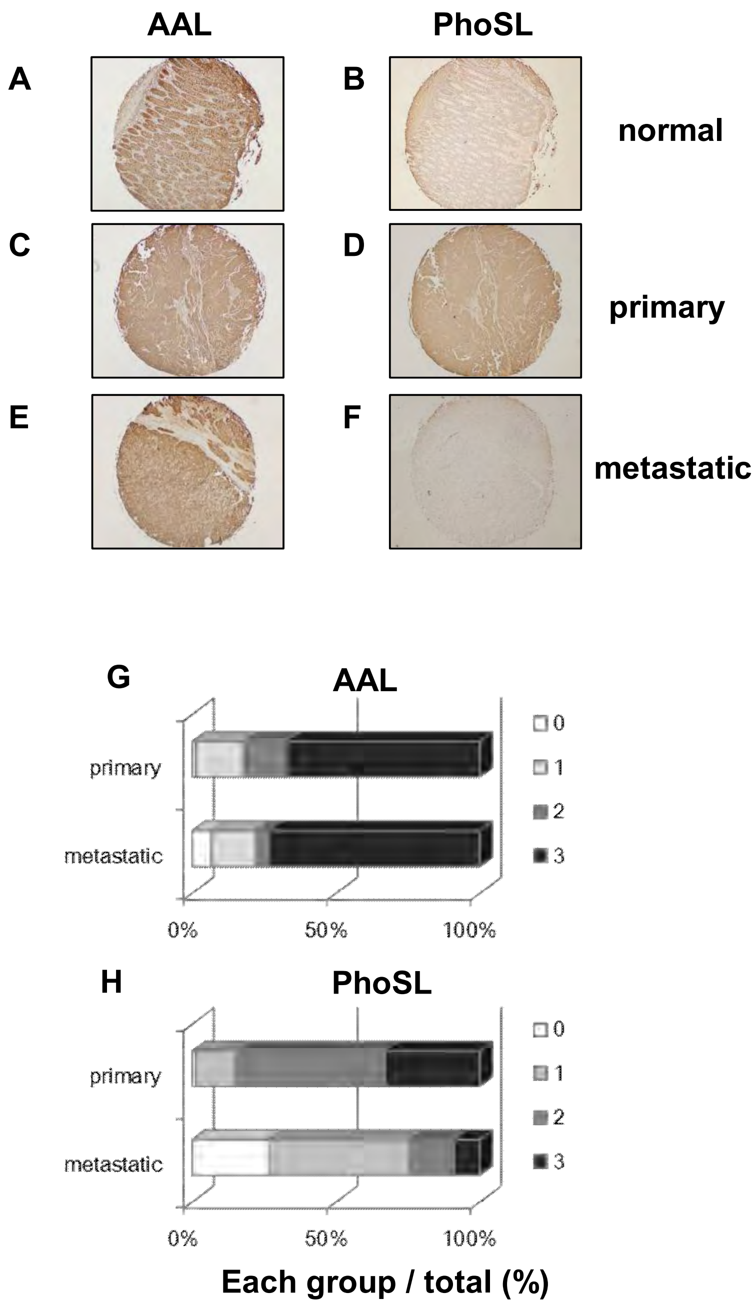

AAL
PhoSL
A
B
normal
C
D
primary
E
F
metastatic
G
AAL
primary
metastatic
0
1
2
3
0%
50%
100%
H
PhoSL
primary
metastatic
0
1
2
3
0%
50%
100%
Each group / total (%)

Fig. 8.

TABLE I

Agglutination profiles of PhoSL(0.5 mg/ml)

Group of erythrocytes	Titer[a]
Rabbit	2^{9}
Sheep	N.A.[b]
Bovine	N.A.
Horse	2^{8}
Pig	2^{8}
Chicken	N.A.
Goose	2^{10}
Guineapig	2^{8}
Human A	N.A.
Human B	N.A.
Human O	N.A.

[a] The hemagglutination titer was defined as the reciprocal of the highest dilution exhibiting hemagglutination.

[b] N.A., not agglutinin.

TABLE II

Inhibition of PhoSL-mediated hemagglutination by glycoproteins

Inhibitor[a]	MIC[b] (μg/ml)
Thyroglobulin	125
IgG	250

[a] Glucose, galactose, mannose, fucose, L-fucose, xylose, L-rhamnose, GlcNAc, GalNAc, ManNAc, LacNAc, lactose, maltose, fructose, saccharose, melibiose and raffinose did not inhibit at concentrations up to 0.2 M. *N*-Acetylneuraminic acid and *N*-glycolylneuramic acid did not inhibit at concentrations up to 20 mM. Fetuin, asialo-fetuin, α_1-acid glycoprotein, transferrin, BSM, asialo-BSM, PSM, asialo-PSM, and albumin did not inhibit at concentrations up to 500 μg/ml.

[b] Minimum inhibitor concentration required for inhibition of 4 hemagglutination dose of the lectin.

[c] BSM : bovine submaxillary gland mucin.

[d] PSM : porcine stomach mucin.

TABLE III

Staining intensities of AAL and PhoSL in human colon cancer tissue analyses

		AAL[a]			PhoSL[b]		
		Normal	Primary	Metastatic	Normal	Primary	Metastatic
staining intensity	0	0	1 (1.3%)	3 (6.7%)	7	1 (1.3%)	12 (26.7%)
	1	0	13 (16.5%)	7 (15.6%)	4	11 (13.9%)	22 (48.9%)
	2	5	12 (15.2%)	2 (4.4%)	0	41 (51.9%)	7 (15.6%)
	3	6	53 (67.1%)	33 (73.3%)	0	26 (32.9%)	4 (8.9%)
total		11	79	45	11	79	45

* [a]Not significant, [b]P<0.01, compared between primary and metastatic cancers (χ^2 test).

SUPPLEMENTARY DATA

FIGURE S1: Schematic representation of the PA-oligosaccharides used for FAC analysis

Note that the reducing terminal is pyridylaminated for FAC analysis.

FIGURE S2: HPLC elution profile of PhoSL.

Column, TSK-gel G3000SWXL(7.8 × 300 mm); temperature, 25°C; solvent, 20 mM phosphate buffer (pH 7.4) containing 20% acetonitrile; flow rate , 0.5 ml/min; detection, 280 nm.

FIGURE S3: Comparison of the glycan-binding specificity of PhoSL and a synthetic peptide based on the PhoSL sequence determined by FAC analysis

A, Bar graphs representing the elution profiles of PhoSL in the presence of core-fucosylated *N*-glycans. The numbers at the bottom of the bar graphs correspond to the sugar numbers indicated in Figure S2.

B, Bar graphs representing the elution profiles of the synthetic PhoSL peptide. The numbers indicated in the elution profiles are retardation volumes (V-V_0, in ml).

FIGURE S4: Comparison of serum samples by LAE using purified AFP-L1, AFP-L3, and sera from patients with hepatocellular carcinoma (HCC)

Conventional lectin affinity electrophoresis (LAE) is shown for the sera of HCC patients, AFP-L3 (cell culture), and AFP-L1 (human cord serum). AFPs in the patient sera were analyzed for isoforms (glycosylation type) by LAE, as described previously. Briefly, 2 µl of the serum samples (HCC1, 2, and 3), diluted to contain AFP at 100 ng/ml and AFP-L3 at 0, 20, or 100 ng/ml, were applied to an agarose gel containing LCA. Following electrophoresis, the AFP was transferred to a nitrocellulose membrane (ADVANTEC) pre-coated with monoclonal mouse antibody to human AFP (NB-011). The membrane was reacted first with rabbit immunoglobulins to AFP (DAKO A0008), and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (BIO-RAD).

FIGURE S5: Staining intensities of PhoSL and AAL

Representative staining intensities of both PhoSL and AAA are shown. 0: negative staining, 1: low-intensity staining, 2: medial-intensity staining, 3: high-intensity staining.

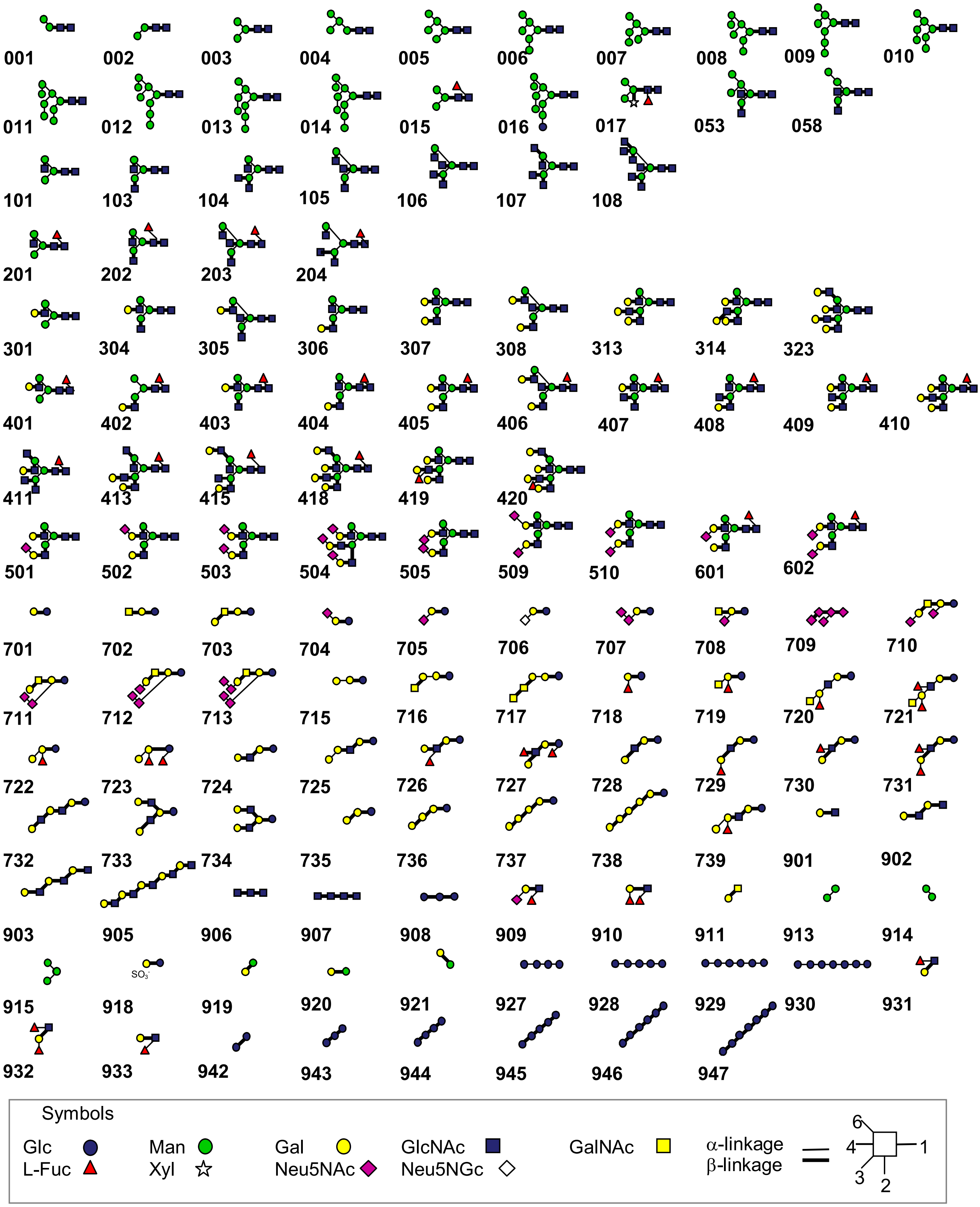

001 002 003 004 005 006 007 008 009 010
011 012 013 014 015 016 017 053 058
101 103 104 105 106 107 108
201 202 203 204
301 304 305 306 307 308 313 314 323
401 402 403 404 405 406 407 408 409 410
411 413 415 418 419 420
501 502 503 504 505 509 510 601 602
701 702 703 704 705 706 707 708 709 710
711 712 713 715 716 717 718 719 720 721
722 723 724 725 726 727 728 729 730 731
732 733 734 735 736 737 738 739 901 902
903 905 906 907 908 909 910 911 913 914
915 918 919 920 921 927 928 929 930 931
932 933 942 943 944 945 946 947
SO3-
Symbols
Glc
L-Fuc
Man
Xyl
Gal
Neu5NAc
GlcNAc
Neu5NGc
GalNAc
α-linkage
β-linkage
6
4
1
3
2

Fig. S1.

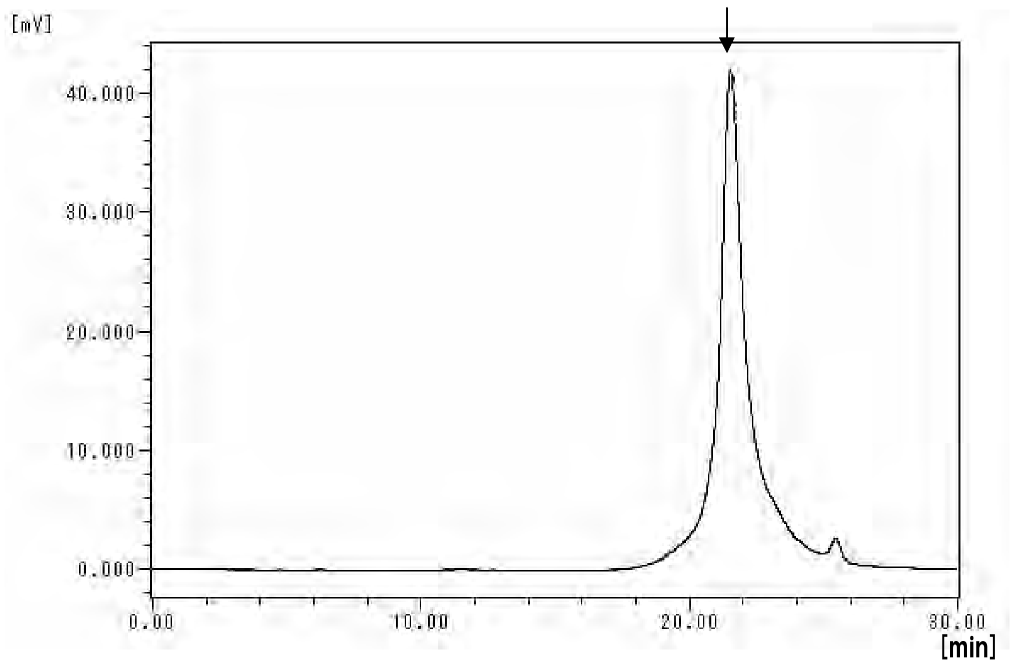
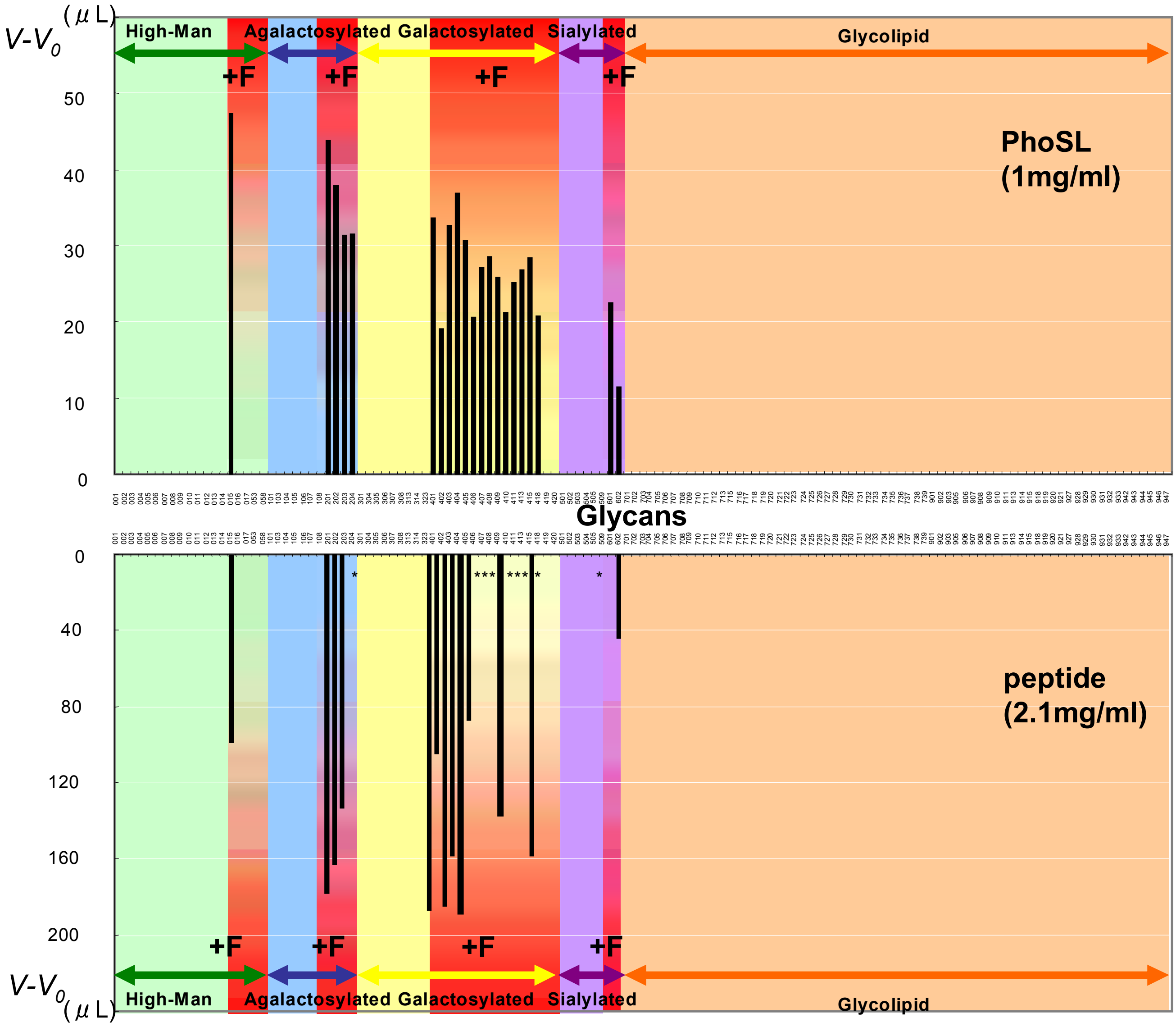
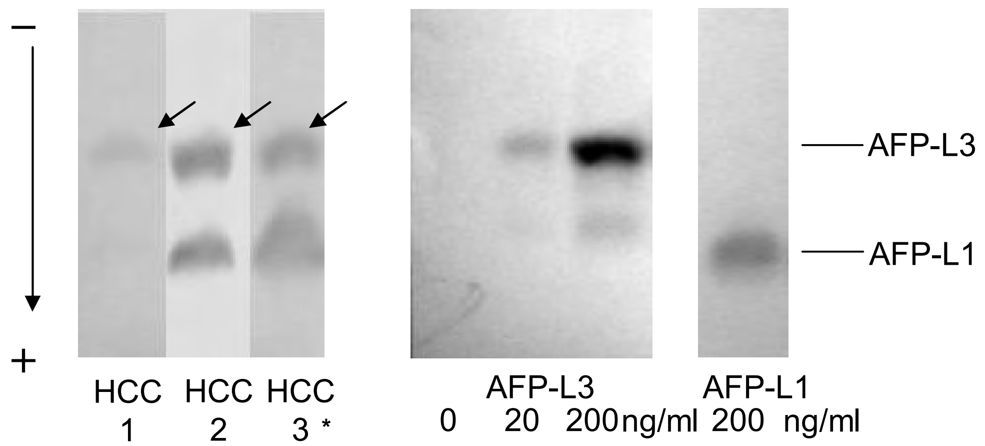


Fig. S2.



*not tested

Fig. S3.



*10-fold diluted sample

Fig. S4.

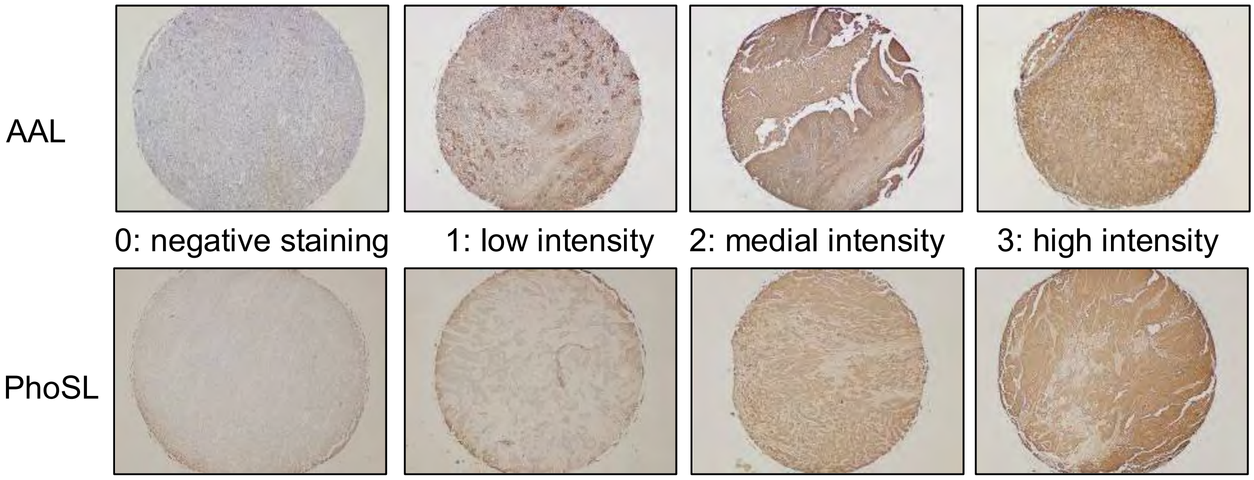

AAL
0: negative staining
1: low intensity
2: medial intensity
3: high intensity
PhoSL

Fig. S5.