Usefulness of specific antibodies to immobilize pyridylaminated *N*-glycans for solid-phase interaction analyses

Na Le[*], Mari Kato[*], Haruko Ogawa[*†]

[*]Course of Chemistry and Biochemistry, Faculty of Advanced Sciences, Graduate School of Humanities and Sciences and [†]the Glycoscience Institute, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610

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ABSTRACT

Pyridylamination is a method of fluorescence-tagging oligosaccharides that enables efficient purification of each glycan from mixtures of biological materials and structural analyses of glycans by HPLC mapping techniques. A method to utilize purified pyridylamino (PA)-oligosaccharides immediately in interaction studies with specific binding proteins would be useful to study the biological functions of glycans. To achieve this, we prepared a neoglycoprotein by complexing periodate-oxidated PA-$GlcNAc_2$ and rabbit serum albumin (RSA) by reductive amination. Immunizing a rabbit with the neoglycoprotein, specific polyclonal antibodies, generated anti-PA-$GlcNAc_2$-IgGs that were purified from the rabbit antiserum by using a protein A column and subsequently an RSA-Sepharose column to remove anti-RSA-IgGs. When the wells of a plastic plate were coated with anti-PA-$GlcNAc_2$-IgGs and PA-$GlcNAc_2$ was added to the wells, PA-$GlcNAc_2$ was immobilized and concentration-dependently bound with biotinylated concanavalin A or *Psathyrella velutina* lectin (PVL) by ELISA. The IgGs were capable of immobilizing PA-*N*-glycans in the solid phase. Using $F(ab')_2$ fragments prepared from the IgGs as an immobilizing reagent for PA-$GlcNAc_2$ decreased the background absorbance in the ELISA, suggesting that *N*-glycans in the Fc region of the IgGs interfered with lectin binding. The effects of de-*N*-glycosylation of $F(ab')_2$ suggested that the *N*-glycans which are reactive to Con A but not involved in the antigen recognition are present in the fragments. Quantitative studies using surface plasmon resonance indicated that the dissociation constant of the interaction between the $F(ab')_2$ and PA-$GlcNAc_2$ was 6×10^{-6} M. The results demonstrate the utility of the IgGs and $F(ab')_2$ as immobilizing reagents for PA-*N*-glycans. Our method provides a new approach to interaction analyses of PA-oligosaccharides.

INTRODUCTION

Due to the microheterogeneity of glycans in natural glycoconjugates, elucidation of the structures and biological roles of glycans was difficult until a method for fluorescence labeling of

glycans was developed [1]. As a fluorescent reagent, 2-aminopyridine labels oligosaccharides with high efficiency and achieves highly sensitive detection of oligosaccharides at sub-pmol levels, enabling purification and structural analysis of oligosaccharides by using 2- or 3-dimensional HPLC [2]. The use of mass spectrometry for analyzing glycoconjugate structures has expanded over the years [3]. However, fluorescent tagging is still useful because it is non-destructive and especially efficient in purification of glycans with novel structures, which is required prior to structural analyses by NMR; further, pyridylamination makes it possible to separate highly hydrophilic oligosaccharides by reverse-phase liquid chromatography prior to mass spectrometry of glycan mixtures due to the hydrophobicity of pyridylamino (PA) groups [4].

In addition to the ability of pyridylamination to purify and analyze glycans, its usefulness in biological studies will expand if we can analyze the interactions with binding proteins or receptors after structural characterization of PA-oligosaccharides. Although several methods of interaction analysis of PA-oligosaccharides have been reported, including affinity chromatography [5, 6], frontal affinity chromatography (FAC) [7], and surface plasmon resonance (SPR) [8], these methods require immobilization of proteins on the chromatography adsorbent or sensor chip, which sometimes alters the protein activity and/or these assays often require a large amount of PA-oligosaccharides. A method to convert a PA group chemically to an amino group was developed [9], but it is not still an ideal solution because preparation of probes by glycobiologists needs to be technically practical and it decreases the yield of the amino-oligosaccharides from the starting minute amounts of PA-oligosaccharides. A suitable method to immobilize PA-oligaccharides immediately onto the solid phase will solve these problems and allow application of PA-oligosaccharides to various interaction analyses. In this study, we report the preparation of specific antibodies against *N*-linked PA-oligosaccharides for the first time and application of the PA-oligosaccharides to binding studies with lectins by ELISA.

MATERIALS AND METHODS

Materials

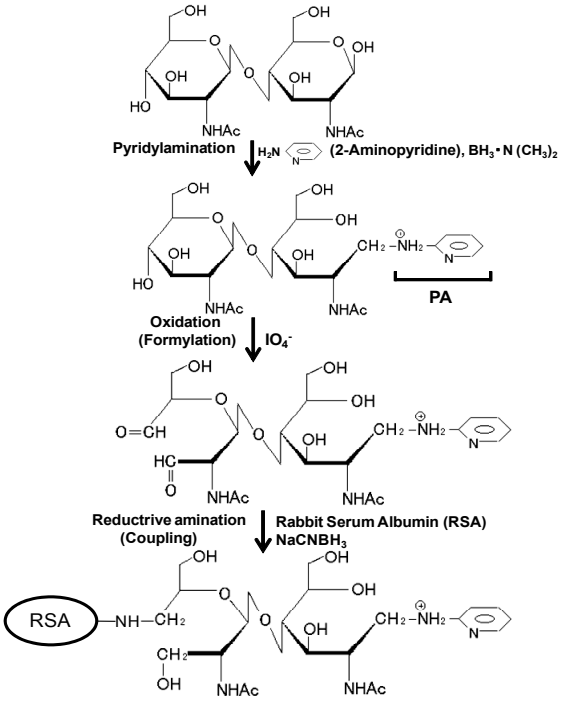
2-Aminopyridine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and recrystallized in hexan. Tri-*N*-acetylchitotriose was prepared from chitin according to the procedure reported by Rupley [10]. (Xylβ1-2)(Manα1-3)(Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc ($XylMan_3FucGlcNAc_2$) was prepared in our laboratory [11]. Rabbit serum albumin, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, anti-$F(ab')_2$, and anti-Fc antibodies were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH). For production of antibodies, a New Zealand white rabbit aged 3-4 months was used. Pepsin was purchased from Sigma. *N*-glycosidase F was obtained from Roche Diagnostics (Mannheim, Germany). Concanavalin A (Con A) [12] and *Psathyrella*

velutina lectin (PVL) [5, 6] were prepared in our laboratory as described previously. The products related to SPR were supplied by GE Healthcare (Uppsala, Sweden). The running buffer was HBS-EP+ 10× (pH 7.4, 0.1 M HEPES, 1.5 M NaCl, 30 mM EDTA, and 0.5% v/v surfactant P20). A CM5 sensor chip and an amine-coupling kit containing 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 1 M ethanolamine-HCl, pH 8.5, were used to immobilize the ligands [13]. Unless otherwise stated, reagents of special grade were used which were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of PA-oligosaccharides

Pyridylamination of oligosaccharides was performed according to the method of Kondo et al. [14]. A coupling reagent was prepared by dissolving 2-aminopyridine (1.5 g) in 500 µl acetic acid and adjusting the pH to pH 7.0. *N,N'*-di-*N*-acetyl-chitobiose or $XylMan_3FucGlcNAc_2$ (250 nmol) and 500 µl of coupling reagent were added to a PAL tube. In the improved method, pyridylamination of oligosaccharides was performed by using a PALSTATION (Takara, Kyoto, Japan). After pyridylamination, the sample was dissolved in 200 µl of distilled water and applied to a Sephadex G-10 column (1 × 30 cm) or G-25 column (2.5 x 100 cm, for large scale preparation) for desalting. The PA-oligosaccharides were eluted with 10 mM ammonium bicarbonate (pH 7.0) and detected by absorbance at 280 nm.

Preparation of PA-$GlcNAc_2$-RSA The preparation procedures are summarized in Scheme 1. PA-$GlcNAc_2$ (500 nmol) was dissolved in 100 µl of distilled water. Sodium periodate (15 µl/0.037 M) was then added and the mixture was incubated for 18 h at 4°C. The reaction was stopped by adding 50 nmol of ethylene glycol. Then, 1 mg rabbit serum albumin (RSA) was applied, and 1/5 volume of the reaction solution of 1 M bicarbonate buffer (pH 8.3) was added to the final concentration of 0.2 M. The reduction of the imine was then performed at 4°C by adding 1 mg $NaCNBH_3$ for 7 d. About 4 mol of PA-$GlcNAc_2$ was coupled to 1 mol of RSA by this method, which was quantified as PA-GlcNAc by sugar analysis using HPLC after acid hydrolysis of PA-$GlcNAc_2$-



Scheme 1. Preparation of the antigen, PA-$GlcNAc_2$-RSA

RSA, according to the method as described previously [15].

Immunization of rabbit and purification of IgGs

RSA-$GlcNAc_2$-PA (RSA-$GlcNAc_2$-PA=8.3:1, v/v) in saline (120 μl) was mixed with 120 μl adjuvant (TiterMax Gold, CytRx, Norcross, GA) according to the manufacturer's protocol. The rabbit was immunized by injecting 100 μl of the antigen solution into the right and left thigh muscles every three weeks for four months until the titer of the antibody was more than 1000 on ELISA.

The IgGs were purified from antiserum and preimmune serum using a HiTrap protein A-column (1 ml, GE Healthcare), and eluted with 0.1 M citrate-phosphate buffer (pH 3.5), followed by the same buffer (pH 4.5) according to the manufacturer's instructions.

ELISA

To measure the activity of the IgG antibodies produced, ELISAs of IgG in the serum were performed. Immulon 1B plates (Thermo Labsystems, Franklin, MA) were coated with RSA-$GlcNAc_2$-PA or RSA (0.5 μg/100 μl) in PBS at 4°C overnight. The plates were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature (r.t.) in order to prevent nonspecific binding. Rabbit antiserum, anti-RSA-$GlcNAc_2$-PA IgGs, or pre-immune IgGs were added to each well in 100-μl aliquots. The mixture was incubated for 1 h at r.t., followed by washing three times with PBS. After that, the plates were blocked with 200 μl 3% BSA in PBS again. HRP-goat anti-rabbit IgGs (100 μl) were added and incubated at r.t. for another hour. The plates were then washed three times, and the substrate, *o*-phenylenediamine (OPD) containing H_2O_2, was added. The enzyme-substrate reaction was terminated by addition of 50 μl 2 M H_2SO_4 to each well, and the absorbance was read at 490 nm.

Western blotting

RSA-$GlcNAc_2$-PA or RSA (0.1 μg each) was loaded onto a 9.5 % gradient polyacryamide gel. After SDS-polyacrylamide gel electrophoresis (SDS-PAGE)[16], proteins in the gel were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) at 100 mA at r.t. for 150 min. The Western blots were blocked with 5% skim milk in 10 mM TBS for 1 h at r.t., washed three times with 10 mM PBS containing 0.1% Tween 20, and then incubated for 1 h with 5 μg/ml anti RSA-$GlcNAc_2$-PA IgGs in TBS. The membrane was washed five times for 5 min each with PBS containing 0.1% Tween 20, and then incubated with horseradish peroxidase (HRP)-conjugated anti-IgGs (ICN Biochmicals, Costa Mesa, CA) in PBS containing 1% skim milk (1:2000 dilution) for 1 h. For color development, the membrane was treated with DAB and H_2O_2.

Reactivity of PA-oligosaccharides with lectins by ELISA using anti-PA-GlcNAc$_2$ antibodies as immobilizing reagent

The wells of Immulon 2HB plates were coated with IgG, F(ab')$_2$, or de-*N*-glycosylated F(ab')$_2$ (1 μg/100 μl in TBS) overnight at 4°C. The wells were washed lightly with PBS, followed by blocking with 3% BSA/PBS. Various concentrations of PA-oligosaccharides were added and incubated for 1 hour at r.t. The wells were washed and blocked with 3% BSA/PBS for 1 h at r.t., and biotinylated Con A or PVL (1 μg/100 μl) was then added. After 1 h incubation, the wells were washed and blocked again. For quantifying the bound lectins, ABC-HRP (0.1 μg/100 μl PBS) was added and incubated for 1 hour. OPD detection was performed as described above.

Preparation and de-N-glycosylation of F(ab')$_2$ fragments of rabbit IgG antibodies

Prior to pepsin digestion, purified IgGs in 10 mM PBS (pH 7.3) were dialyzed against 0.2 M acetate buffer (pH 4.5). The IgGs (7.02 mg/ml) were then incubated with 5 μl pepsin solution (2.5 mg/0.25 ml 0.01 M HCl) for 20 hours at 37°C. To stop the reaction, the pH of the mixture was adjusted to 8.0 with 1 M NaOH, followed by ultrafiltration with a membrane with a cut-off size of 10 kDa (YM-10, Millipore). For de-*N*-glycosylation, the digested pepsin fragments were treated with 5 μl N-glycosidase F (100 U/0.1 ml) at 37°C for 3 days. For a control, the procedures were performed without *N*-glycosidase F. De-*N*-glycosylation was assessed by SDS-PAGE and by the reactivity on the membrane with biotinylated Con A.

Quantification of interaction between antibodies and PA-GlcNAc$_2$ by SPR

The binding kinetics of the interaction between antibodies and PA-oligosaccharides were determined by SPR using a BIAcore T-100 (GE Healthcare). Rabbit anti-serum IgGs (50 μg/ml) in 10 mM acetate buffer (pH 5.0), F(ab')$_2$ fragments (150 μg/ml) in 10 mM acetate buffer (pH 4.0), or rabbit pre-immune serum IgGs (50 μg/ml) in 10 mM acetate buffer (pH 5.0) were immobilized on the CM5 sensor chip by amine coupling. An underivatized flow cell on the same sensor chip was used as a control. As analytes, GlcNAc$_3$-PA instead of GlcNAc$_2$-PA, was used because an analyte with a higher molecular weight has a better response. Various concentrations of PA-GlcNAc$_3$ between 2 μM and 50 μM in 10 mM PBS (pH 7.2) were prepared, and 15 μl of each was injected onto the antibody-immobilized sensor chip. Because analyte sugars dissociated quickly, the sensor chip was not regenerated when each injection time was over. All biosensor analyses were carried out at a constant flow rate of 10 μl/min at 25°C. Kinetic parameters were calculated by affinity analysis using the BIA evaluation software version 2.0.2.

RESULTS

Reactivities of rabbit IgGs with PA-GlcNAc$_2$-RSA or RSA

As shown in Figure 1A, the purified rabbit IgGs eluted from the protein A column showed concentration-dependent binding to RSA-GlcNAc$_2$-PA in ELISA, and to a lesser extent to underivatized RSA, while the preimmune IgGs did not bind to either RSA or neoglycoproteins. The antiserum IgGs bound much better to RSA-GlcNAc$_2$-PA than to RSA, indicating that the rabbit antiserum contained IgG subpopulations that specifically recognized the PA-GlcNAc$_2$ moiety linked with RSA. Western blotting revealed that specific IgGs bound to RSA-GlcNAc$_2$-PA, whereas only weak binding to oligomerized RSA was observed (Figure 1B). The results indicate that anti-RSA-GlcNAc$_2$-PA IgGs were produced, but some IgGs that recognize underivatized RSA were suggested to be present. We tried to remove the RSA-binding IgGs from the anti-RSA-GlcNAc$_2$-PA IgG antibodies by passing through an RSA-Sepharose column that was prepared by coupling RSA to formyl-Sepharose with or without cross-linking by glutaraldehyde according to the method previously described [17], but the IgGs did not bind to the column. Therefore, the anti-RSA-GlcNAc$_2$-PA IgGs were used for further analyses without more purification than a protein A column.

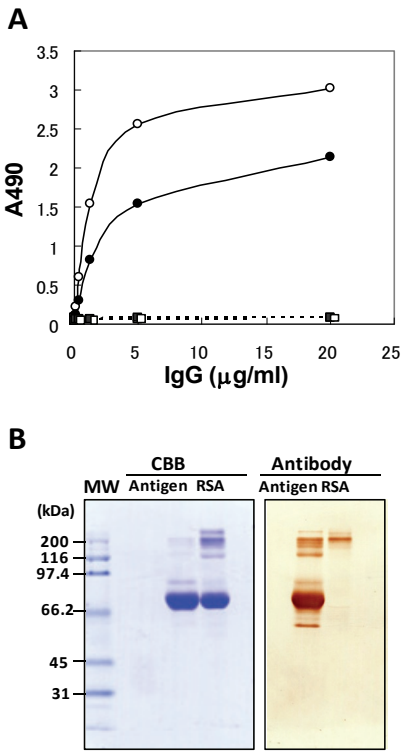


Figure 1. Reactivities of rabbit IgGs with PA-GlcNAc$_2$-RSA or underivatized RSA

(A) PA-GlcNAc$_2$-RSA (neoglycoprotein used as antigen) or underivatized RSA (each 0.5 μg) was coated onto the wells of plastic plates, blocked with 3% BSA, and reacted with serially 4-fold diluted IgGs purified from antiserum or preimmune serum. The bound IgGs were detected with HRP-goat anti-rabbit IgGs and OPD/H_2O_2. Symbols indicate the binding activity between PA-GlcNAc$_2$-RSA-antiserum IgGs (○), RSA-anriserum IgGs (●), PA-GlcNAc$_2$-RSA-preimmune serum IgGs (□), and RSA-preimmune serum IgGs (■).

(B) PA-GlcNAc$_2$-RSA or RSA (each 5 μg) was applied to 9.5% acrylamid gel, electrophoresed, and transferred to a PVDF membrane. The blots were stained with Coomassie brilliant blue (CBB), or probed with rabbit anti-PA-GlcNAc$_2$-RSA IgGs and HRP-goat anti-rabbit IgGs, then developed with DAB/H_2O_2, as described in the text. 'MW' stands for molecular weight markers.

Binding between lectins and PA-oligosaccharides using IgG antibodies as capturing reagents by ELISA

In order to determine whether IgGs are able to immobilize $GlcNAc_2$-PA to the well of a plastic plate, a GlcNAc-specific lectin, PVL, was used to detect the $GlcNAc_2$-PA by ELISA. As shown in

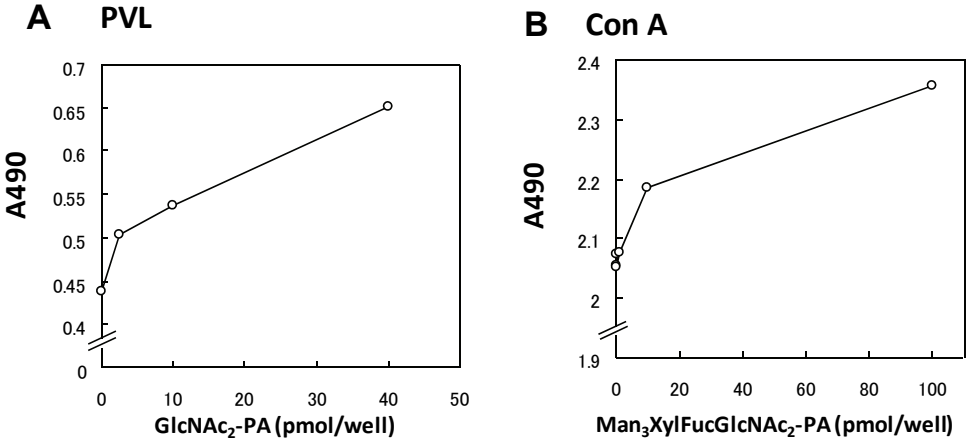


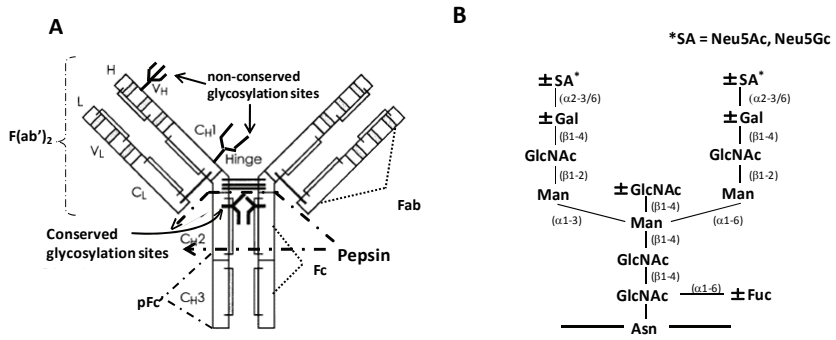
Figure 2. Reactivities of PA-oligosaccharides immobilized on anti-RSA-$GlcNAc_2$-PA IgGs with PVL (A) and Con A (B) by ELISA

In order to immobilize PA-oligosaccharides, 1 μg/100 μl of anti-PA-$GlcNAc_2$-IgGs was coated onto a microtiter plate, then various concentrations of $GlcNAc_2$-PA (A) or $XylMan_3FucGlcNAc_2$-PA (B) were added to the wells and captured by the antibodies. The immobilized PA-oligosaccharides were incubated with biotin-PVL (A) or -Con A (B), respectively. The bound lectins were detected with ABC-HRP and OPD/H_2O_2 as described in the text.

Figure 2A, the bound PVL increased as the concentrations of $GlcNAc_2$-PA added to the IgG-immobilized wells were increased. On the other hand, Con A was used to demonstrate whether the IgGs can capture $XylMan_3FucGlcNAc_2$-PA, a major *N*-glycan of sophoragrin, a Man/Glc-specific legume lectin from *Sophora japonica* [11]. Con A showed concentration-dependent binding to the plate (Figure 2B). The results clearly demonstrate that PVL and Con A bind to the PA-oligosaccharides and that the IgGs on the wells successfully immobilized the two PA-oligosaccharides. However, the background absorbance in the absence of the PA-oligosaccharides was extremely high, especially in ELISA with Con A. This was considered to be caused by ConA and PVL binding to the *N*-glycans of IgGs.

Preparation of $F(ab')_2$ and de-N-glycosylated $F(ab')_2$ fragments from the IgGs

Because Fc region of mammalian IgGs have been known to contain complex-type *N*-glycans at the hinge region and rabbit IgGs were shown to possess heterogeneous biantennary complex-type *N*-glycans at Asn-297 in both the Fc regions (see Scheme 2) [18], we treated the IgGs with pepsin to remove Fc region. As shown in Figure 3, lane P, SDS-PAGE under the reducing condition indicated



Scheme 2. Structure of rabbit IgG

(A) The domain structure, intra- and inter-chain disulfide bonds, glycosylation sites (⟶) and the cleavage sites by pepsin (– · ➔) of rabbit IgG. The conserved *N*-linked glycans are attached to Asn-297 in the Fc. The *N*-glycans are often attached to non-conserved glycosylation sites in the Fab.

(B) Composite structure of the *N*-glycans most typical of rabbit IgG.

that IgGs were cleaved into F(ab')$_2$ and other fragments with pepsin treatment; intact H-chain (50 kDa) disappeared, concomitantly with the appearance of 27 kDa band which bound with anti-F(ab')$_2$ antibodies, with a low molecular weight fragment of 14 kDa. Intact IgGs and F(ab')$_2$ exhibited clear reactivities with Con A, suggesting that *N*-glycans are present not only at Fc' peptides (pFc') but Fab' fragments. Rabbit IgGs has been often reported to possess *N*-glycans in the Fab and Fd regions in addition to Fc region [19] (Scheme 2A). Therefore, we subsequently treated the pepsin-digested IgGs with *N*-glycosidase F to avoid interference of remaining *N*-glycans in the rabbit IgGs. As shown in Fig. 3, lane PN, the reactivity with anti-F(ab')$_2$ antibodies was increased by the *N*-glycosidase F treatment, suggesting that the F(ab')$_2$ region was originally *N*-glycosylated.

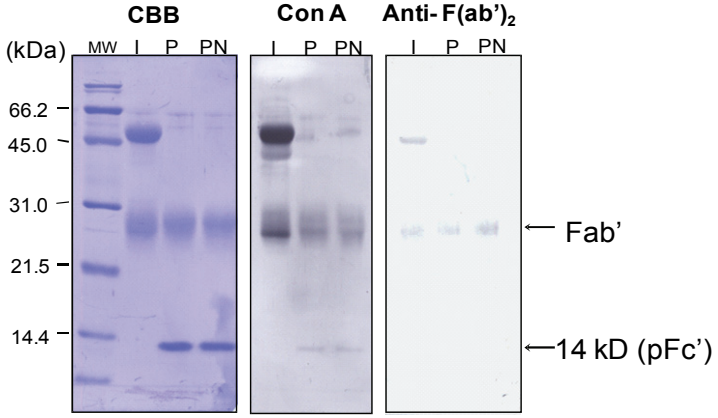


Figure 3. Preparation and de-*N*-glycosylation of F(ab')$_2$ fragment

IgGs purified from the antiserum were treated with pepsin (80:1, w/w) at 37℃ for 20 hours and stopped. Pepsin-treated IgGs were de-*N*-glycosylated with *N*-glycosidase F. I: intact IgGs, P: pepsin-treated IgGs, and PN: pepsin-treated and de-*N*-glycosylated IgGs. Each sample (2.5 μg) was subjected to SDS-PAGE on a 12.5% polyacrylamide gel and electrotransferred onto a PVDF membrane. The membrane was probed with CBB (left), biotin-Con A (middle), or anti-(Fab')$_2$ antibodies (right), and the color was developed with 4-chloro-1-naphthol/H_2O_2, as described in the text.

Binding between lectins and PA-GlcNAc$_2$ using F(ab')$_2$ or de-N-glycosylated F(ab')$_2$ as immobilizing reagents in ELISA

As shown in Fig. 4A, ELISA with PVL showed lower background absorbance when F(ab')$_2$ was used as the capturing reagent for PA-GlcNAc$_2$ rather than IgGs. This indicates that the glycans located in the Fc region bound to PVL as well as PA-GlcNAc$_2$ when IgGs were used as the capture reagent, and that removal of the pFc' region by pepsin digestion abolished the interference. Because we previously reported that PVL can bind to the *N*-acetylneuraminic acids of the trisialylated *N*-glycans, as well as to GlcNAc residues to a lesser extent [6], PVL may have bound to the sialylated *N*-glycans in the Fc region. De-*N*-glycosylation of F(ab')$_2$ produced capturing activity for PA-GlcNAc$_2$ equal to that of F(ab')$_2$ in detection with PVL (Fig. 4A), suggesting that de-*N*-glycosylation does not decrease the PA-GlcNAc$_2$-binding activity of the antibodies.

On the other hand, de-*N*-glycosylation of F(ab')$_2$ affected the binding of Con A, as shown in Fig. 4B; the background absorbance markedly decreased, and moreover, the concentration-dependency of detection was attenuated compared to the results using untreated F(ab')$_2$. The decreased absorbance coincided with that of Con A bound to the Fab' fragment of the rabbit IgGs in Western blotting (Fig. 3), and because de-*N*-glycosylation did not decrease the reactivity with PVL (Fig. 4B), the Fab' region might contain high-mannose type oligosaccharides, as has been reported for mouse variable IgG regions [20]. The background absorbance of Con A was much lower in the case of F(ab')$_2$ (Fig. 4B) from that of IgGs (Fig. 2B), indicating the *N*-glycans in the Fc region interfered with the Con A binding.

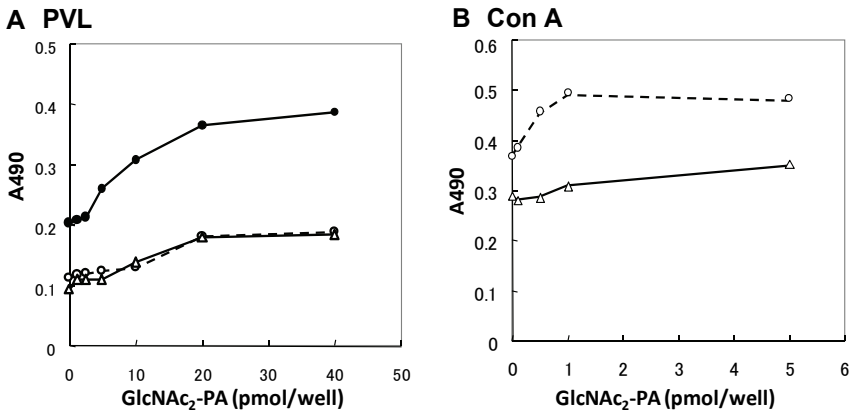


Figure 4. Reactivities of PA-GlcNAc$_2$ captured on the IgGs, F(ab')$_2$, or de-*N*-glycosylated F(ab')$_2$ with PVL (A) and Con A (B) by ELISA.

To capture PA-GlcNAc$_2$, intact IgGs (●), F(ab')$_2$ (○), or de-*N*-glycosyated F(ab')$_2$ (D) (10 μg/ml each) was coated onto microtiter plates. Different concentrations of PA-GlcNAc$_2$ were then added and incubated with antibodies. The immobilized PA-GlcNAc$_2$ was then probed with biotin-PVL (A) or -Con A (B). The bound lectins were detected by ELISA with ABC-HRP and OPD/H_2O_2.

Quantitative analysis of interaction between anti-PA-oligosaccharide IgGs-F(ab')$_2$ and PA-oligosaccharides by SPR

Immobilized ligands showed 15000-20000 resonance units (RU, 1000 RU=1 ng/mm^3) on the sensor chip. As shown in Fig. 5A, PA-GlcNAc$_3$ clearly bound to a sensor chip that was immobilized with F(ab')$_2$ of rabbit anti-PA- oligosaccharide IgGs, but GlcNAc$_3$ or 2-aminopyridine did not, indicating that both the PA group and GlcNAc$_3$ are necessary for binding with the F(ab')$_2$ fragment. When various concentrations of PA-GlcNAc$_3$ were injected onto the surface of the sensor chip, concentration-dependent binding was shown (Fig. 5B). Because the anti-PA- GlcNAc$_2$ antibodies are polyclonal antibodies, the affinity between the F(ab')$_2$ and the PA-oligosaccharides are heterogeneous, and therefore the obtained dissociation constant (K_d) is apparent, representing those of the total polyclonal F(ab')$_2$ fragments. The apparent K_d was calculated to be 6.2×10^{-6} M by affinity analysis.

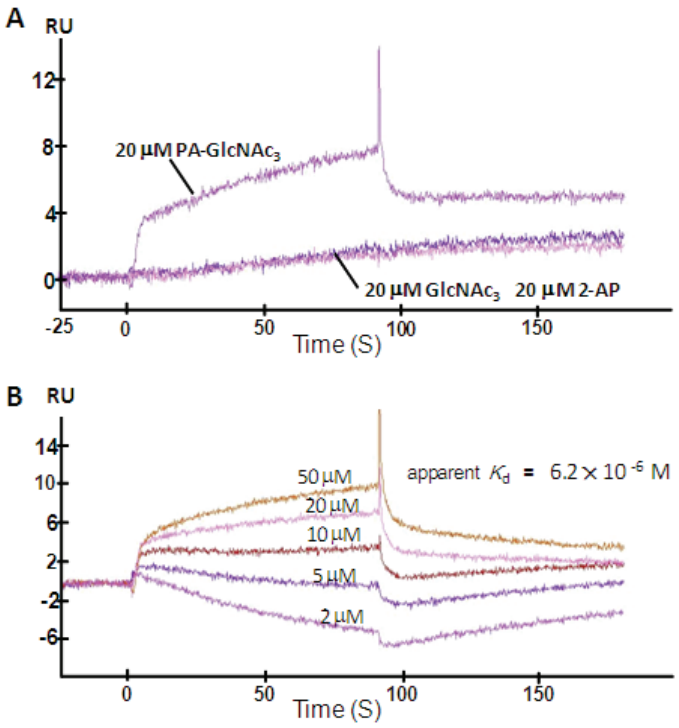


Figure 5. Quantitative analysis of anti-PA-GlcNAc$_2$-IgGs-F(ab')$_2$ and PA-oligosaccharides by SPR

Anti-PA-GlcNAc$_2$-IgGs-F(ab')$_2$ was immobilized on a CM5 sensor chip. Various analytes, 2-aminopyridine, GlcNAc$_3$, or PA-GlcNAc$_3$ (A), or five concentrations of PA-GlcNAc$_3$ (B) in 10 mM PBS (pH 7.2), were separately injected onto the surface of sensor chip at a flow rate of 10 µl/min at 25ºC. The response was expressed as the change in the number of resonance units (RU) induced by analyte binding to immobilized F(ab')$_2$, which was corrected for the bulk effect by subtracting the change on the reference cell.

Taken together, these results suggest that the antibody against PA-oligosaccharides prepared in this study is a potential binding reagent for the specific oligosaccharide. On the other hand, the interaction between PA-GlcNAc$_3$ and anti-PA-oligosaccharide IgGs was also analyzed (data not shown), but the RU of PA-GlcNAc$_3$ binding to the IgG-immobilized flow cell became negative after subtracting the RU of the reference flow cell. This might be due to immobilization of large amounts of IgGs on the sensor chip, which may limit mass transport of analytes on the IgG-immobilized sensor chip and inhibit the interaction analyses.

DISCUSSION

In this study, we prepared an original neoglycoprotein by complexing periodate-oxidized PA-$GlcNAc_2$ and RSA by reductive amination, and generated anti-PA-oligosaccharide IgG antibodies by immunizing a rabbit with the neoglycoprotein. The antibodies made possible the study of interactions between the lectins and PA-*N*-glycans possessing $GlcNAc_2$-PA as a common determinant. To achieve this, we considered that free PA-oligosaccharides would generate a weak IgM response, while the PA-$GlcNAc_2$ covalently conjugated to RSA would become effective immunogens for naive B cells. The B cells recognizing the PA-$GlcNAc_2$ via IgM receptors on the cell surface should internalize and degrade the neoglycoprotein, which must be recognized by T cells as an extracellularly presented foreign PA-$GlcNAc_2$ fragment to be activated. As expected, the neoglycoprotein induced specific IgG responses. We then demonstrated in this study that the antibody is useful to immobilize a variety of *N*-glycans. This protocol is easily applicable to various types of glycans by choosing the appropriate PA-oligosaccharide in designing the antigen. This would contribute to the study of the biological functions of a wide variety of glycans that are present in biological systems.

Although the anti-PA-oligosaccharide IgGs was demonstrated to be capable of capturing PA-*N*-glycans for binding study using ELISA, interference by the *N*-glycans of IgGs was one problem in this study. It was considerably ameliorated by using $F(ab')_2$ instead of IgGs. The other problem was the affinity of the antibody for the antigen: $F(ab')_2$ fragments exhibited the apparent K_d of 6.2×10^{-6} M for PA-$GlcNAc_3$ by SPR (Fig. 5), which is not high considering that the average K_d for antibody-antigen interactions has been reported to be 10^{-6}–10^{-8} M [21]. However, compared to the K_d values of most lectin-monovalent sugar interaction, which has been reported to be in the10^{-3}-10^{-4} M range [22], the $F(ab')_2$ exhibited considerably high affinity to PA-$GlcNAc_3$. The $F(ab')_2$ antibodies will be applicable to most cases for which lectins are used, not only for interaction analyses but detection and isolation of PA-*N*-glycans.

Every molecule of IgGs has 2–3 glycan chains in which *N*-linked glycans are located at Asn237 in both Fc regions [23, 24]. The glycans of IgGs were shown to bind to ConA as well as PVL (Fig. 2) to increase the absorbance from the interaction between anti-RSA-$GlcNAc_2$-PA IgGs and $GlcNAc_2$-PA. In order to solve this problem, we prepared $F(ab')_2$ fragments and de-*N*-glycosylated $F(ab')_2$ fragments by treatment with pepsin and subsequently *N*-glycosidase F. With $F(ab')_2$, the detection of $GlcNAc_2$-PA was significantly improved (Fig. 4). However, de-*N*-glycosylation was less effective than the removal of the Fc region for $GlcNAc_2$-PA detection by Con A, attenuating the concentration-dependent binding. The function of *N*-linked glycans of $F(ab')_2$ fragments has been reported; the glycosylation in this supervariable region significantly affects the affinity for the antigen [25-27]. In this study, the capturing activity for $GlcNAc_2$-PA was unchanged by de-*N*-glycosylation as shown by reactivity with PVL (Fig. 4A) indicating that the *N*-glycans were not involved in the antigen recognition. Therefore the reason why de-*N*-glycosylation of $F(ab')_2$ did not improve the detection for

$GlcNAc_2$-PA by Con A (Fig. 4) might be related to the decrease in the avidity effect that the high Man-type *N*-glycans of $F(ab')_2$ contributed to the positive cooperative binding of the $GlcNAc_2$-PA with multivalent Con A, because the binding affinity of Con A to β-GlcNAc is originally low compared to that of high Man-type glycans [28, 29]. To increase the antigen-binding affinity of the antibodies, we tried to polymerize the IgGs by various cross-linking reactions, including one that utilizes the formyl groups generated by periodate-oxidation of glycans of IgGs. However, every trial examined to date has failed to increase the affinity. The influence of *N*-glycans on antigen recognition may be one reason for the difficulty. The preparation of monoclonal antibodies using genetically modified B-cells to change or delete *N*-glycosylation site(s) in the $F(ab')_2$ region may be effective in producing antibodies with high affinity.

Previously, synthesis of a oligosaccharide adduct with biotinylated diaminopyridine (BAP) was reported [30]. The oligosaccharide-BAP adduct was then attached to streptavidin, and specific polyclonal antibodies against the oligosaccharide moieties were prepared. The generated IgGs recognized the oligosaccharides and were utilized for detection and interaction analyses of the glycans. However, the concept of our study is completely different. The antibodies in our study enable PA-oligosaccharides to be applicable to various interaction analyses by immobilizing them onto the solid phase to search for specific binding proteins/receptors by using the PA-glycan-antibody complex as probes. Thus, our simple method provides a versatile method to immobilize or trace various PA-glycans. Moreover, our procedures for the preparation of anti-PA-oligosaccharides are technically feasible with short steps compared with the preparation of complexes of oligosaccharides-BAP. Combined with analytical techniques and databases such as GALAXY to analyze PA-oligosaccharide structures [31], our concept and protocol to prepare antibodies against PA-oligosaccharides would be a useful and important tool to study the functions of glycans.

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著者名：楽　娜、加藤真利、小川温子

住所：東京都文京区大塚２－１－１　お茶の水女子大学大学院人間文化創成科学研究科

E-mail:　ogawa.haruko@ocha.ac.jp