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Evidence that *Agaricus bisporus* agglutinin (ABA) has dual sugar-binding specificity ☆

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Abstract

Agaricus bisporus agglutinin (ABA) is known as a useful lectin to detect T-antigen (Core1) disaccharide (Galβ1–3GalNAcα) and related O-linked glycans. However, a recent X-ray crystallographic study revealed the presence of another intrinsic sugar-binding site, i.e., for GlcNAc. To confirm this possibility, detailed analysis was performed using two advanced methods: lectin microarray and frontal affinity chromatography (FAC). In the lectin microarray, intense signals were observed on ABA spots for both *N*-glycanase-treated and *O*-glycanase/β1–4galactosidase-treated Cy3-labeled asialofetuin. This indicates substantial affinity for both O-linked and agalactosylated (GlcNAc-exposed) N-linked glycans. A further approach by FAC using 20 pNP and 130 PA-oligosaccharides demonstrated that ABA bound to Core1 ($K_d = 3.4 \times 10^{-6}$ M) and Core2 (1.9×10^{-5} M) but not to Core3 and Core6 O-linked glycans. It also showed substantial affinity to mono-, bi-, and tri-antennary agalactosylated complex-type N-linked glycans ($K_d > 1.8 \times 10^{-5}$ M). These results establish ABA as a lectin having dual sugar-binding sites with distinct specificity, i.e., for Gal-exposed O-linked glycans and GlcNAc-exposed N-linked glycans.

Keywords: Lectin; GlcNAc; T-antigen disaccharide; Carbohydrate binding specificity; Frontal affinity chromatography; *Agaricus bisporus* agglutinin

Lectins are valuable tools used to differentiate between complex carbohydrates expressed on various types of cells and in glycoconjugates. This is especially the case for glycomics, which involve the comprehensive analysis of glycans from both structural and functional viewpoints [1]. However, previous studies on lectin specificity lack the quantitative aspects required for systematic analysis. In this context, there is still much room for re-investigation of valuable lectins for profiling complex features of glycans (i.e., to extend the uses of known lectins into new areas).

Agaricus bisporus agglutinin (ABA) is a conventional lectin used to detect T-antigen (also designated Core1) disaccharide (Galβ1–3GalNAcα), a major type of O-linked glycan [2]. Although ABA is often compared with Jacalin, another T-antigen binder [3], ABA barely recognizes Tn-antigen (GalNAcα) [4], whereas Jacalin does so strongly. Moreover, 6′-sialylation or sulfation of the reducing-end GalNAc still allows recognition by ABA [5], whereas this modification abolishes the affinity to Jacalin [6]. ABA is also known to have anti-neoplastic activity [7], though its molecular mechanism remains to be elucidated.

As described, ABA binds to O-linked glycans, though there are several reports, contradictory to this idea, which suggest the lectin can also recognize N-linked glycans contained in human IgG, IgA2, and α2-macroglobulin [8,9]. Moreover, recent X-ray crystallographic studies revealed that ABA possesses two intrinsic sugar-binding sites in one polypeptide; one for T-antigen and the other for GlcNAc [10]. Although these observations strongly suggest that ABA recognizes different types of glycans, it remains

☆ *Abbreviations:* ABA, *Agaricus bisporus* agglutinin; FAC, frontal affinity chromatography; PA, pyridylaminated; pNP, *p*-nitrophenyl; ASF, asialofetuin.

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unclear whether the latter GlcNAc-binding site is adopted for agalactosylated (GlcNAc-exposed) N-linked glycans, or for GlcNAc-containing O-linked glycans, such as Core2 and Core3. We decided to address this confusion by re-investigating the sugar-binding specificity of ABA from a more systematic and quantitative view point. In this study, we employed two advanced techniques recently developed in our laboratory, evanescent-field fluorescence-assisted lectin microarray [11] and frontal affinity chromatography [1,12–15]. The results clearly demonstrate the dual specificity of ABA for O-linked and N-linked glycans, and that each is recognized by a different binding site. This conclusion suggests that the results of glycan profiling previously performed with this lectin should be re-evaluated.

Materials and methods

Materials. *p*-Nitrophenyl (pNP) glycosides were commercially obtained as described previously [6]. Pyridylaminated (PA) N-linked glycans used are listed in Fig. 1. N-linked glycans were purchased from Takara Bio Inc. (Kyoto, Japan) and Seikagaku Co. (Tokyo, Japan) [12].

Agaricus bisporus agglutinin (ABA) was purchased from Seikagaku Co. ACA, GSL-II, ECA, and ConA were from Vector or Seikagaku Co.

Lectin-microarray analysis. For lectin microarray analysis, glass slides with multiple lectin spots were prepared as described previously [11]. For interaction analysis, 100 μl of Cy3-labeled glycoprotein solution in the probing buffer (TBS containing 1% Triton X-100) was applied to wells on the glass slide, and the slide incubated at 20 °C until the binding reaction reached equilibrium. For the inhibition assay, 100 mM (final concentration) GlcNAc was added to the probing solution. After binding of the Cy3-labeled glycoprotein with immobilized lectins, the reaction solution was discarded and the glass slide was rinsed once with the probing buffer. A fluorescence image of the microarray was acquired by using an evanescent-field fluorescence scanner, GTMASScan III (Nippon Laser & Electronics Lab.), as described previously [11]. All data were analyzed with the Array Pro analyzer ver. 4.5 (Media Cybernetics, Inc., MD). The net intensity value for each spot was calculated as the signal intensity minus a background value. Three spots of the signal net intensity values were averaged.

Preparation of glycosidase-treated asialofetuin. Cy3-labeled bovine asialofetuin (Cy3-ASF) having only O-linked glycans was prepared by enzymatic removal of N-linked glycans from Cy3-ASF. Ten micrograms of Cy3-ASF was dissolved in 40 mM $NaHCO_3$, pH 8.3, containing 200 mM NaCl, and was treated with 4 mU of PNGase F (Takara Bio Inc., Shiga, Japan) at 37 °C for 16 h. A series of Cy3-ASFs having galactosylated glycans, agalactosylated glycans, and tri-mannose type glycans were prepared as follows by serial digestion with glycosidases. Thirty micrograms of Cy3-ASF was dissolved in 50 mM sodium phosphate buffer, pH 7.0, and was treated with 3.75 mU of *O*-glycanase (Prozyme, CA, USA) at 37 °C overnight (as in ASF having galactosylated glycans). A 20 μg aliquot of the *O*-glycanase-treated Cy3-ASF was further digested with 2 mU of β-galactosidase from *Streptococcus* 664K (Seikagaku Co., Tokyo, Japan) at 37 °C for 4 h (as in ASF having agalactosylated glycans). ASF

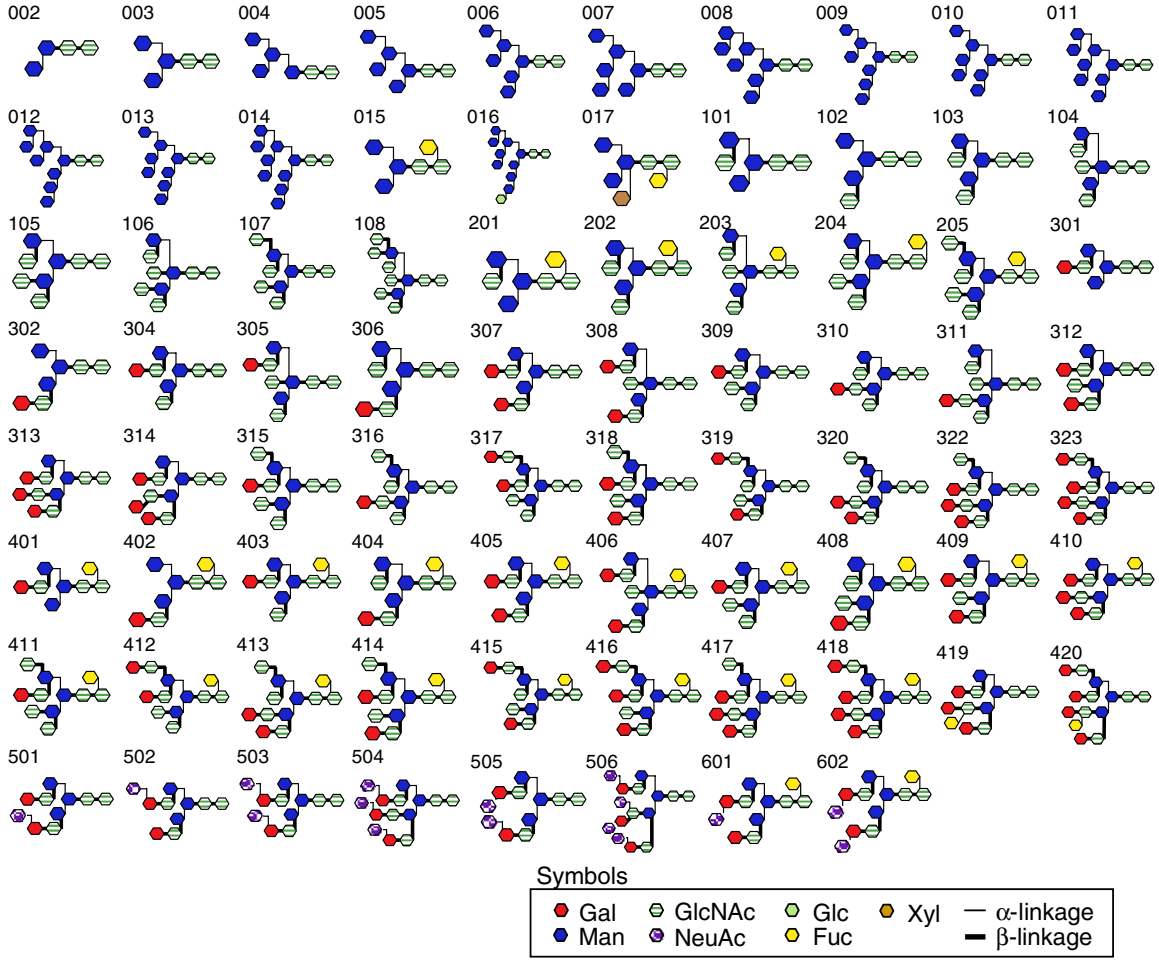

Fig. 1. Schematic representation of PA-oligosaccharides used in this study. The reducing terminal is pyridylaminated. Symbols used to represent pyranose rings of monosaccharides are shown in the box at the bottom of the figure [12].

having exposed tri-mannose type glycans was prepared by further digestion of 10 μg of the *O*-glycanase/galactosidase-treated Cy3-ASF with 0.4 U of β-*N*-acetylhexosaminidase (Prozyme) at 37 °C for 4 h.

Preparation of lectin columns. ABA dissolved in 10 mM $NaHCO_3$ was coupled to NHS-activated Sepharose 4FF (Amersham). The resultant lectin–Sepharose was suspended in 10 mM Tris–HCl buffer, pH 7.4, containing 0.8% NaCl (TBS), and the derived slurry was packed into a miniature column (ϕ 2 × 10 mm, 31.4 μl) specially designed for FAC as previously described [12].

Frontal affinity chromatography (FAC). Frontal affinity chromatography was performed using an automated system for FAC (FAC-1) as described previously [1,12]. The flow rate and the column temperature were kept at 0.125 ml/min and 25 °C, respectively. The concentrations of pNP- and PA-glycans were adjusted to 5 μM (pNP) and either 2.5 or 5.0 nM (PA), respectively, and an excess volume (0.5–0.8 ml) of each glycan solution was successively injected into the column by an autosampling system. Elution of pNP- and PA-glycans was monitored by measuring UV (280 nm) and fluorescence (ex/em = 310/380 nm), respectively. The elution front relative to that of an appropriate standard oligosaccharide (i.e., PA-lactose), i.e., $V - V_0$, was determined [13]. The dissociation constant (K_d) was obtained from $V - V_0$ and B_t (effective ligand content of the column), according to the basic equation of FAC, $K_d = B_t/(V - V_0) - [A]_0$ [12–14].

The FAC inhibition assay was carried out using 125 nM of agalactosylated, bi-antennary N-linked glycan (103), 20 μM of Galβ1–3GalNAcα(Core1)-pNP or Galβ1–3(GlcNAcβ1–6)GalNAc-α(Core2)-pNP in the presence of non-labeled GlcNAc at concentrations between 0 and 125 mM. Residual binding (%) values compared with $V - V_0$ values without GlcNAc were plotted.

Concentration-dependence analysis. To determine the B_t value, concentration-dependence analysis was carried out as described previously [13]. Various concentrations ($[A]_0$) of Core1-pNP and GlcNAcα-pNP were successively injected into the column, and $V - V_0$ values were calculated according to the method originally developed by Arata et al. [15]. Woolf–Hofstee-type plots, $(V - V_0)$ vs. $(V - V_0)[A]_0$, were made to determine B_t and K_d values from the intercept of the axis and the slope of the fitted curves, respectively.

Results and discussion

Analysis by lectin microarray

Although a recent X-ray crystallographic study suggests that ABA can bind GlcNAc-containing glycans [10], it is not known whether they are O-linked glycans or N-linked glycans, of which the non-reducing ends are GlcNAc. In this context, there is no systematic data available on the sugar-binding specificity of ABA. As a first approach to solving this problem, evanescent-field fluorescence-assisted lectin microarray was adopted. As probes, Cy3-labeled asialofetuin (Cy3-ASF) [16,17], and its glycosidase-treated derivatives were prepared, and their binding was assessed using the array equipped with 43 lectins including ABA (see supplemental Figure). The system was validated by the behavior of other standard lectins, e.g., ACA specific for Core1 [18], ECA (LacNAc [19]), GSL-II (agalactosylated tri-/tetra antennary glycans, unpublished data), and ConA (high-mannose type glycans [19]).

When the microarray was probed with Cy3-ASF, which has bi- and tri-antennary N-linked glycans (NA2 and NA3) and Galβ1–3GalNAcα O-linked glycans (Core1) [16], intense signals were observed on ABA, ACA, and ECA (Fig. 2A). After removal of N-linked glycans with PNGase F

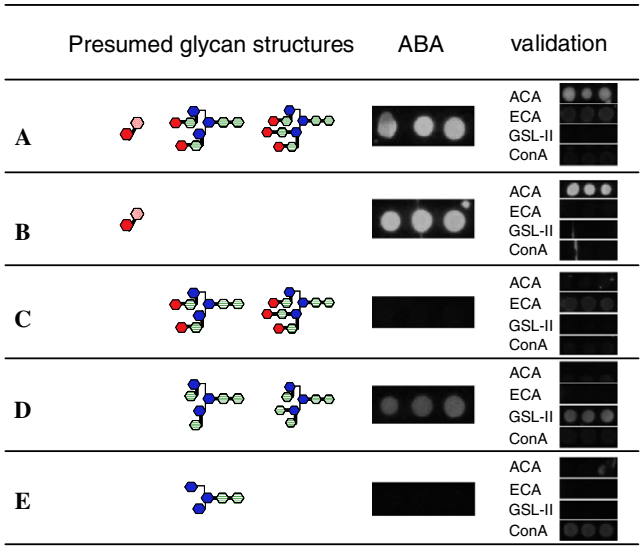

Fig. 2. Evanescent-field fluorescence-assisted lectin microarray using Cy3-labeled asialofetuins (Cy3-ASFs) with/without glycosidase treatment. For validation of glycosidase treatment, spots of ACA (having affinity for Galβ1–3GalNAc), ECA (Galβ1–4GlcNAc), GSL-II (agalactosylated glycans), and ConA (high-mannose-type glycans) are shown together. (A) non-treated Cy3-ASF, (B) PNGase F-treated, (C) *O*-glycanase-treated, (D) *O*-glycanase, and β-galactosidase-treated, and (E) *O*-glycanase, β-galactosidase, and *N*-acetyl-hexosaminidase-treated Cy3-ASF. Symbols used to describe glycan structures are shown in Fig. 1.

(Fig. 2B), signals on ECA disappeared as expected, whereas those on ABA and ACA remained. The latter observation clearly indicates that ABA has affinity for O-linked glycans. On the other hand, when Cy3-ASF was treated with *O*-glycanase to remove Core1 (T-antigen) disaccharide, no signal was detected for ABA as well as ACA, while high signal intensity was retained on ECA (Fig. 2C). This confirms that *O*-glycanase-treated ASF preserves galactose-exposed complex-type N-linked glycans. However, subsequent treatment with β-galactosidase regenerated binding to ABA as well as to GSL-II, while it canceled that to ECA (Fig. 2D). This observation strongly suggests that ABA and GSL-II recognized GlcNAc-exposed N-linked glycans of Cy3-ASF. In fact, additional treatment with *N*-acetylhexosaminidase resulted in no binding to these GlcNAc-binding lectins, while it enhanced the binding to ConA, a mannose-binding lectin (Fig. 2E). These results confirm that ABA has distinctive sugar-binding abilities, which are sensitive to either PNGase F (N-linked glycan) or *O*-glycanase (O-linked glycan) treatment. The fact that ABA binding was regenerated by β-galactosidase and cancelled by subsequent *N*-acetylhexosaminidase treatments implies that ABA specifically binds to agalacto-type N-linked glycans, as well as Core1-type O-linked glycans.

To test the above prediction, an inhibition assay using non-labeled GlcNAc was carried out for the above series of Cy3-ASF. When 100 mM GlcNAc was added, ABA interaction with PNGase F-treated Cy3-ASF was retained, whereas that with *O*-glycanase and β-galactosidase-treated

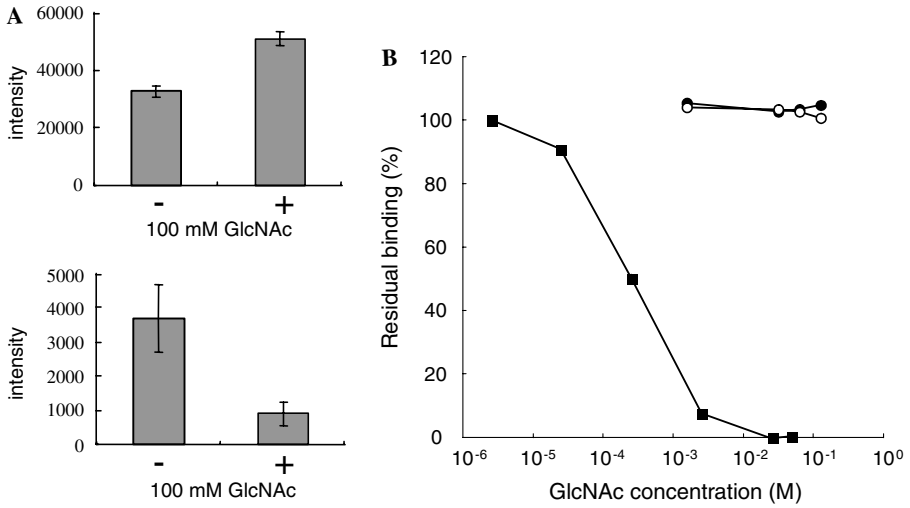

Fig. 3. (A) Inhibition assay using non-labeled GlcNAc by means of lectin microarray. Cy3-labeled ligands (top; PNGase F-treated ASF, bottom; *O*-glycanase- and β-galactosidase-treated ASF) were probed on to the lectin microarray with/without 100 mM GlcNAc. Average intensities of three spots are shown. (B) Inhibition assay by means of FAC. In the presence of various concentrations of non-labeled GlcNAc, $V - V_0$ values of 103 (bi-antennary, agalactosylated N-linked glycan), Core1-pNP (closed circle), and Core2-pNP (open circle), were determined as described in Materials and methods. Residual binding (%) relative to $V - V_0$ values without non-labeled GlcNAc is plotted.

Cy3-ASF was completely inhibited (Fig. 3A). These lines of observation suggest that O-linked and N-linked glycans of ASF bind to different sites of ABA in an independent manner.

Analysis by FAC

Evaluation of T-antigen-binding site

Though the above result for the lectin microarray showed that ABA could bind not only to O-linked but also to N-linked glycans, the detailed oligosaccharide specificity remained to be elucidated. To determine dissociation constants (K_d) by FAC, a panel of pNP-derivatized sugars and PA-oligosaccharides was prepared, and used for analysis of O-linked glycans and N-linked glycans, respectively.

As regards pNP-derivatives, Core1 (T-antigen disaccharide; Galβ1–3GalNAcα) and Core2 (Galβ1–3(GlcNAcβ1–6)GalNAcα) O-linked glycans were found to have significant affinity for ABA, while none of the other pNP-sugars, GalNAcα, Core3, and Core6 showed detectable retardation in terms of $V - V_0$ (<3 μl; Table 1). As a result of concentration-dependence analysis using Core1- and Core2-pNP (Fig. 4), the column capacity (B_t) and their K_d values were determined to be 1.11 nmol and 3.4 μM, respectively, for Core1-pNP, whereas they were 1.29 nmol and 19 μM, respectively, for Core2-pNP (Table 1). The K_d value obtained for Core1-pNP agreed well with that determined by a different method [20]. Apparent lack of affinity for GalNAcα-pNP implies that the whole structure of Core1 disaccharide (Galβ1–3GalNAc) is required for ABA-recognition. Considering the greatly diminished (i.e., 5.6 times) affinity for Core2-pNP in comparison with Core1-pNP, the 6-OH group of the reducing-end GalNAc is an important, but not absolute, requirement for the binding.

Table 1
Dissociation constants for binding of pNP-sugars to ABA by FAC

pNP-sugar	K_d (M)
T-antigen (Core1) (Galβ1–3GalNAcα)	3.4×10^{-6}
Core2 (Galβ1–3(GlcNAcβ1–6)GalNAcα)	1.9×10^{-5}
Core3 (GlcNAcβ1–3GalNAcα)	N.D.
Core6 (GlcNAcβ1–6GalNAcα)	N.D.
GalNAcα	N.D.
GlcNAcα	2.1×10^{-5}

N.D., not detected. Galα/β, GalNAcβ, Manα/β, Glcα/β, GlcNAcβ, Lacβ, LacNAcβ, Malα, maltopentaose, $(GlcNAc)_2$, $(GlcNAc)_3$ showed no detectable affinity.

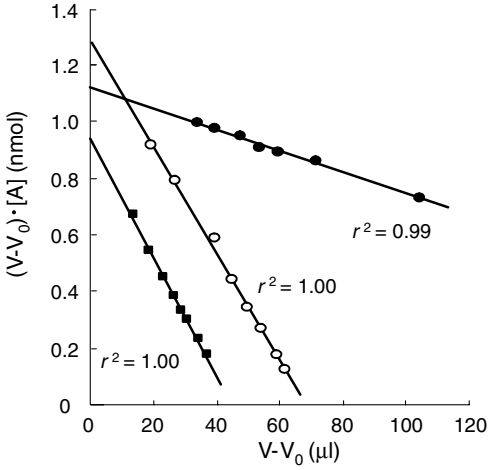

Fig. 4. Woolf–Hofstee-type plots for an ABA-immobilized column. Various concentrations of appropriate pNP-sugars were applied to the ABA-immobilized column, and K_d and B_t values were obtained from the intercept and the slope of Woolf–Hofstee-type plots, respectively. Closed circles: Galβ1–3GalNAcα-pNP (Core1, 5–30 μM); open circles: Galβ1–3(GlcNAcβ1–6)GalNAcα-pNP (Core2, 2–50 μM); closed squares: GlcNAcα-pNP (5–50 μM).

A similar observation has been reported for 6′-sialylated and sulfated Core1 structures [5].

Evaluation of the GlcNAc-binding site

To evaluate the GlcNAc-binding site in ABA, the B_t value was determined using pNP-derivatives of α- and β-GlcNAc. Only GlcNAcα-pNP showed substantial affinity for ABA. Concentration-dependence analysis was then performed with GlcNAcα-pNP (Fig. 4). As a result, B_t and K_d values were determined to be 0.93 nmol and 21 μM, respectively. The B_t value (0.93 nmol) was similar to those for the GalNAc-binding site determined for Core1-pNP (1.11 nmol) and Core2-pNP (1.29 nmol), as described above. We therefore conclude that ABA possesses an equivalent number of Core1- and GlcNAc- binding sites.

Fine specificity to N-linked glycans

FAC analysis using 130 PA-oligosaccharides revealed that ABA recognizes N-linked glycans, whereas it showed no affinity for glycolipid-type glycans (Fig. 1; for detailed glycan structures of glycolipid-type glycans and others (701–911), see Fig. 1 in [12]). No chito-oligosaccharide was a good ligand for ABA (data not shown). For better comparison of affinity strength, a bar graph was constructed using K_a (=1/K_d) values for O-linked, pNP- (Fig. 5A) and N-linked, PA-glycans (Fig. 5B), though K_d continues to be used in the text.

Evidently, ABA has substantial affinity for a series of partially and completely agalactosylated, complex-type glycans (>18 μM; Fig. 5B). However, the affinity for these N-linked glycans was >5.3 times weaker than that for O-linked glycans (Fig. 5A). No detectable affinity was observed for high-mannose type (002–017) and completely galactosylated glycans (301, 302, 307, 308, 313, 314, 323, 401, 402, 405, 406, 410, 418–420, 501–602). These results, together with the observation by lectin microarray, clearly indicate that ABA recognizes the non-reducing-end GlcNAc of N-linked glycans, but not reducing-end chitobiose or GlcNAc of O-linked glycans (Core2, 3, and 6).

Another novel feature investigated in this work is that of N-linked glycans. ABA distinguishes branching patterns. Its affinity declines in the following order; bi-antennary glycans having two intact GlcNAc residues (103 and 202; K_d = 18 and 19 μM, respectively) > mono- or bi-antennary glycans having a single intact GlcNAc residue transferred by GnT-I (102, 304, and 403; K_d = 37, 35, and 35 μM, respectively) > tri-antennary glycans having two intact GlcNAc residues transferred by GnT-I and II (105, 204, and 310; K_d = 43, 44, and 38 μM, respectively) > mono- or bi-antennary glycans having a single intact GlcNAc residue transferred by GnT-II (101, 201, 306, and 404; K_d = 54, 49, 57, and 53 μM, respectively) > tri-antennary glycans having two intact GlcNAc residues transferred by GnT-I and IV (309 and 407; K_d = 167 and 307 μM, respectively) > tetra-antennary glycans (no detectable affinity; 107, 205, 315–320, 322, and 411–417). In addition, the affinity was reduced to 30–50% by the presence of bisecting GlcNAc (103 > 104, 105 > 106, 202 > 203 and 310 > 311).

To understand the recognition mechanism of O-linked and N-linked glycans, FAC inhibition analysis was carried out using 125 nM of PA-oligosaccharide 103 (agalactosylated, bi-antennary N-linked glycan; note that the K_d value for 103 is 18 μM) or 20 μM of pNP-oligosaccharides (Core1- and Core2-pNP) with the copresence of non-labeled GlcNAc (25 μM–250 mM). Binding to the former saccharide was inhibited by GlcNAc in a dose-dependent manner (Fig. 3B). This confirms that 103 binds to the GlcNAc-recognition site of ABA. On the other hand, binding to Core1-pNP (K_d = 3.4 μM) was not inhibited at all, even at the highest concentration used for inhibition (250 mM). Therefore, the Core1-binding event is essentially independent from GlcNAc recognition. In this context, ABA-binding to Core2 (Galβ1–3(GlcNAcβ1–6)GalNAcα) was also not inhibited by GlcNAc (Fig. 3B). Thus, binding to

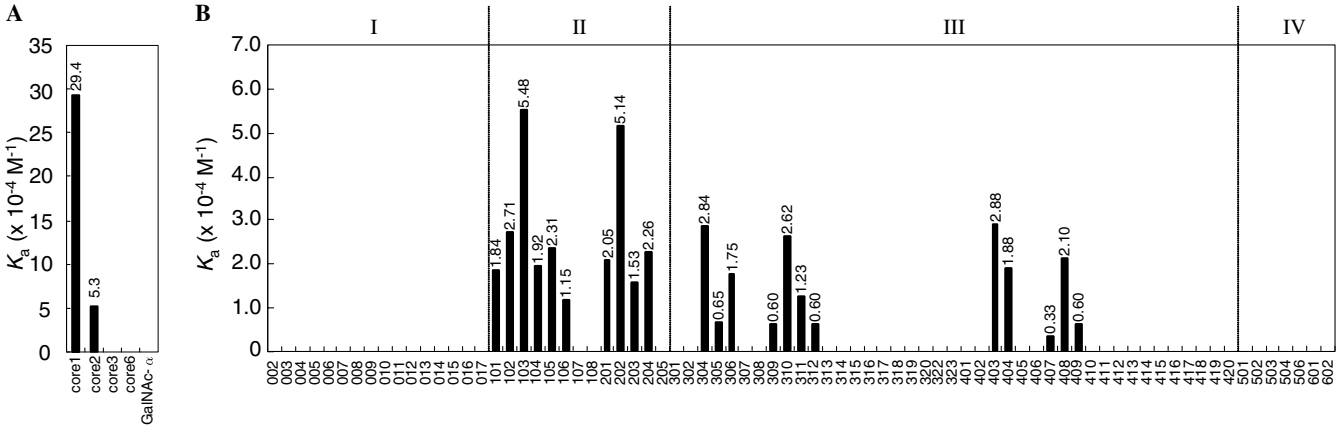

Fig. 5. Bar graph representation of affinity constants (K_a) of pNP- (A) and PA-sugars (B) for ABA. The small Arabic numerals at the bottom of graphs correspond to sugar numbers indicated in Fig. 1; small Arabic numerals at the top of the bars indicate K_a values ($K_a \times 10^{-4}$ M^{-1}); large Roman numerals at the top of graphs represent types of glycans; high-mannose-type (I), agalacto-type (II), galactosylated-type (III), and sialylated-type (IV) N-linked glycans.

O-linked glycans (Galβ1–3GalNAc) and N-linked glycans (agalactosylated, complex-type) proved to be independent from one another.

ABA has been widely used as a tool to detect and capture glycoconjugates targeting O-linked glycans. However, the dual specificity revealed in this study shows that there is a risk of misunderstanding glycan analysis using this lectin.

We conclude that sugar-binding specificity elucidated by FAC is capable of contributing greatly to various aspects of structural and functional glycomics.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.06.073.

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