Journal of Chromatography B, 885–886 (2012) 66–72

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Elucidating the selectivity of recombinant forms of *Aleuria aurantia* lectin using weak affinity chromatography

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ARTICLE INFO

Article history:
Received 27 September 2011
Accepted 14 December 2011
Available online 21 December 2011

Keywords:
Affinity
Aleuria aurantia lectin
Glycan interaction
Recombinant protein
Weak affinity chromatography

ABSTRACT

Aberrant glycosylation is connected to several pathological conditions and lectins are useful tools to characterize glycosylated biomarkers. The *Aleuria aurantia* lectin (AAL) is of special interest since it interacts with all types of fucosylated saccharides. AAL has been expressed in *Escherichia coli* as a fully functional recombinant protein. Engineered variants of AAL have been developed with the aim of creating monovalent lectins with more homogenous binding characteristics. Four different forms of AAL were studied in the present work: native AAL purified from *A. aurantia* mushrooms, recombinant AAL dimer, recombinant AAL monomer and recombinant AAL site 2 (S2-AAL). The affinities of these AAL forms toward a number of saccharides were determined with weak affinity chromatography (WAC). Disaccharides with fucose linked α1–3 to GlcNAc interacted with higher affinity compared to fucose linked α1–6 or α1–4 and the obtained dissociation constants (K_d) were in the range of 10 μM for all AAL forms. Tetra- and pentasaccharides with fucose in α1–2, α1–3 or α1–4 had K_d values ranging from 0.1 to 7 mM while a large α1–6 fucosylated oligosaccharide had a K_d of about 20 μM. The recombinant multivalent AAL forms and native AAL exhibited similar affinities toward all saccharides, but S2-AAL had a lower affinity especially regarding a sialic acid containing fucosylated saccharide. It was demonstrated that WAC is a valuable technique in determining the detailed binding profile of the lectins. Specific advantages with WAC include a low consumption of non-labeled saccharides, possibility to analyze mixtures and a simple procedure using standard HPLC equipment.

1. Introduction

The significance of protein glycosylation in protein folding, stability and function has gradually evolved in recent years. The high complexity and variability in glycosylation are severe challenges in the characterization of the "glycome", but techniques to perform such studies have been developed in recent years [1–4]. A number of structures have been identified of which fucosylated glycans have gained specific attention, as they are involved in a number of events related to cellular communication [5–8]. Abnormally fucosylated glycans have been recognized as biomarkers for diagnosis and prognosis of severe diseases such as chronic inflammation and cancer. Lectins have proven to be useful tools in order to characterize such glycosylated biomarkers [1,3,9] and the characterization and development of new lectins are of high priority.

One lectin of special interest in studying aberrant fucosylation is the *Aleuria aurantia* lectin (AAL) from the orange peel mushroom [10]. AAL interacts selectively with L-fucose and the dissociation constant (K_d) has been determined with surface plasmon resonance (SPR) and equilibrium dialysis to be 15–33 μM [10–13]. AAL also interacts with fucosylated oligosaccharides and glycopeptides, with fucose in either α1–2, α1–3, α1–4 or α1–6 position [14–16]. Nuclear magnetic resonance (NMR) and X-ray crystallography analysis have shown that the interaction site of AAL is restricted to the recognition of fucose and the linkage to the next saccharide unit [13].

AAL has a molecular weight of 72 kD and is composed of two identical subunits [10]. The AAL protein subunits are associated to form a homodimer through hydrophobic interaction "back to back" with its binding sites exposed to the environment. Each subunit has six tandem repeats organized as a six-bladed β-propeller with five fucose binding sites located between a pair of adjoining blades (the sixth site is non-functional) [11,17]. The five binding sites in the AAL monomer have a similar architecture but are not identical and site 2 has been proposed to have a higher affinity than the

Abbreviations: AAL, *Aleuria aurantia* lectin; ELLA, enzyme-linked lectin assay; K_d, dissociation constant; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; PA, pyridylaminated; Pmp, p-methoxy-phenyl; Pnp, p-nitrophenyl; SPR, surface plasmon resonance; WAC, weak affinity chromatography.

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doi:10.1016/j.jchromb.2011.12.015

other sites [11]. The lectin has been cloned and a functional protein with an identical structure and binding properties as the native lectin has been produced in *Escherichia coli* [18]. A recombinant His-tagged form of AAL was recently shown to exhibit two different K_d values toward fucose; 22 μM and 4 nM, respectively [12]. This form of recombinant AAL was not exposed to fucose in the cultivation or purification process and it was suggested that the high affinity site (possibly site 2) becomes permanently occupied by fucose in the normal production of AAL, which would explain why it has not been detected previously. Recombinant variants of AAL were further studied by producing two engineered variants of AAL; a monomeric form of AAL (free subunit with five binding sites, 36 kD) and an AAL-form containing only site 2 (S2-AAL, 14 kD) [19]. The binding profile of the proteins was studied with SPR, enzyme-linked lectin assay (ELLA) and hemagglutination. All proteins were found to bind fucosylated saccharides but S2-AAL differed from the other proteins as the affinity generally was lower and it was reported to bind sialylated structures less efficiently [19].

Engineered variants of AAL or other lectins could be useful in order to create "improved lectins" having altered selectivity, affinity or other properties. In developing these new affinity binders it is important to have reliable techniques to study the effect of introduced changes. SPR is a well-established method in such studies but the expensive equipment, high consumption of saccharides and the dependence of trained personnel are serious limitations [20]. NMR applications aiming at determining affinity constants [21] have similar problems. Different techniques based on affinity chromatography have shown to be valuable alternatives in studying protein–carbohydrate interactions due to specific merits such as robustness, high through-put, low consumption of analyte and the use of standard HPLC instrumentation [1,22–25]. Weak affinity chromatography (WAC) is a technique that combines high-performance liquid chromatography (HPLC) with affinity chromatography [22–24]. Since WAC utilizes high-resolution silica, small differences in peak retention are detectable and affinities in the mM range can be studied. From chromatographic theory the retention volume of an analyte (injected substance) is directly related to the affinity and the number of binding sites, under isocratic conditions. The K_d value of the interaction can therefore easily be extracted from the retention in combination with the number of binding sites, or by comparing the retention in relation to an analyte of known affinity [1,22,25]. Because of the separation step that is part of the analysis, it is possible to do accurate K_d determinations also when samples are impure or a mixture of several compounds.

In this study four different variants of AAL were evaluated using the WAC technique: native AAL purified from *A. aurantia* mushrooms (n-AAL), recombinant AAL dimer (r-AAL), recombinant AAL monomer (m-AAL) and AAL site 2 protein (S2-AAL). The affinity of a number of saccharides was determined and it was found that disaccharides with fucose linked α1–3, α1–4 or α1–6 to GlcNAc interacted with higher affinity compared to larger saccharides with identical fucosylation. When comparing the disaccharides, α1–3 linked fucose interacted with higher affinity compared to α1–4 or α1–6 linked fucose A large α1–6 fucosylated oligosaccharide did however bind with almost the same affinity as the disaccharides. It was also shown that S2-AAL had a slightly different binding profile compared to the other AAL forms.

2. Material and methods

2.1. Materials

Kromasil spherical silica with 5 μm particle size and 300 Å pores were kindly provided by EKA chemicals, Bohus, Sweden. The oligosaccharides LNT, LNFI, LNFII, LNFIII, A-tetra and A-tri were from BioCarb AB (Lund, Sweden); pnp-α-L-Fuc (p-nitrophenyl-α-L-fucose), pnp-β-D-Fuc, H-tri and Fucα1–3GlcNAc were from Sigma Aldrich (St. Louis, MO, USA); Fucα1–4GlcNAc and Fucα1–6GlcNAc were from Carbosynth (Compton United Kingdom); pmp-β-LeX (p-methoxy-phenyl-β-LeX), and pmp-β-sLeX were kindly provided by Ulf Ellervik (Lund University, Sweden). NA2 and NA2F were obtained from Dextra laboratories Ltd. (Berkshire, United Kingdom). Oligosaccharide structures are listed in Table 1. Native AAL (n-AAL) was obtained from Vector Laboratories (Burlingame, CA, USA).

Table 1
Oligosaccharide structures.

pmp-β-LeX	Galβ1-4GlcNAcβ1-pmp* | Fucα1-3
pmp-β-sLeX	Neu5Acα2-3Galβ1-4GlcNAcβ1-pmp* | Fucα1-3
H-tri	Fucα1-2Galβ1-3GalNAc
A-tri	GalNAcα1-3Gal | Fucα1-2
A-tetra	GalNAcα1-3Galβ1-4Glc | Fucα1-2
LNFI	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc
LNFII	Fucα1-4 | Galβ1-3GlcNAcβ1-3Galβ1-4Glc
LNFIII	Galβ1-4GlcNAcβ1-3Galβ1-4Glc | Fucα1-3
NA2F	R=Galβ1-4GlcNAcβ1- R-2Manα1-6 Fucα1-6 | | Manβ1-4GlcNAcβ1-4GlcNAc | R-2Manα1-3
LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc

*pmp = p-methoxy-phenyl

2.2. Expression and purification of the His-tagged recombinant forms of AAL

The construction of plasmids containing the recombinant full length dimeric AAL (r-AAL,), the monomeric Ser283Asp AAL mutant (m-AAL), and the AAL site 2 protein corresponding to amino acids Ser50 to Gly160 (S2-AAL), has previously been described [12,19]. Bacteria (BL21/DE3) harboring these plasmids were grown in Luria Broth medium containing 30 μg/mL kanamycin (Duchefa, Haarlem, the Netherlands) at 37 °C with shaking until OD_{600} was between 0.6 and 0.9. Isopropyl-beta-D-thiogalactopyranoside (IPTG, Fermentas, St. Leon-Rot, Germany), at a final concentration of 0.5 mM, was

added to induce synthesis of the recombinant AAL forms and the bacteria were further incubated by shaking at room temperature overnight. Cells were collected by centrifugation and sonicated for 4 × 30 s in 10 mM phosphate pH 7.4, 150 mM sodium chloride. The sonicate was centrifuged first at 3200 × *g* for 20 min then at 19000 × *g* for 15 min at 4 °C to remove debris. Recombinant AAL proteins were purified by affinity chromatography using a 1 mL Ni-column (HiTrap™ Chelating HP column, GE Healthcare Uppsala, Sweden). The binding buffer used was a 10 mM phosphate buffer with 0.5 M NaCl and 40 mM imidazole, whereas a 10 mM phosphate buffer with 0.5 M NaCl and 1.2 M imidazole was used as elution buffer. The purified proteins were desalted on a PD-10 column (GE Healthcare, Uppsala, Sweden) and then dialyzed against 10 mM phosphate pH 7.4, 150 mM sodium chloride.

2.3. Isothermal titration microcalorimetry measurements

Isothermal titration calorimetry (ITC) experiments were performed at 20 °C using a VP-ITC microcalorimeter (MicroCal, Northampton, MA). Carbohydrate and protein were dissolved in 10 mM phosphate pH 7.4, 150 mM sodium chloride. S2-AAL (1.8 mL, 12 μM) was added into the calorimeter cell, while the carbohydrate solution (360 μM pnp-α-L-Fuc) was loaded into the syringe injector. Titration was performed with 5 μL injections of the carbohydrate solution into the protein solution, resulting in an increase of the carbohydrate concentration with 1 μM for every injection. In total, 50 injections were done applying a 3.5 min delay between each injection. A dilution control was performed by titrating pnp-α-L-Fuc into 10 mM phosphate pH 7.4, 150 mM sodium chloride, using the same procedure as described above. The dissociation constant (K_d) of the interaction was calculated using the Origin 5.0 software supplied with the VP-ITC instrument.

2.4. Immobilization procedure

The Kromasil silica was derivatized into diol-silica and oxidized to aldehyde-silica, which was used for covalent coupling of the AAL proteins essentially as described before [22,24]. In short, n-AAL, r-AAL, m-AAL and S2-AAL were dissolved in 0.1 M sodium phosphate, pH 7.0, and coupled to aldehyde silica through reductive amination with sodium cyanoborohydride as reductive agent. The yield in the coupling procedure was measured as the difference in absorption at 280 nm of the protein solution before and after immobilization and in total 2.0 mg of r-AAL, 1.8 mg of mono-AAL, 1.7 mg of n-AAL and 1.4 mg of S2-AAL were immobilized to batches of 300 mg silica. About 200 mg of each AAL-silica preparation was packed in stainless steel columns, 50 × 3.2 mm (400 μL) equipped with 1 μm frits (Hichrom, Berkshire, UK), using an air driven Haskel pump (Haskel, Burbank, CA, USA). Packing pressure was 300 bars and 0.1 M sodium phosphate, pH 7.0, was used as packing buffer.

2.5. Chromatography

All chromatography operations were performed at room temperature (23 ± 1 °C) with 10 mM sodium phosphate pH 7.2, 140 mM sodium chloride as mobile phase at a flow rate of 0.25 mL/min, if not otherwise stated. The mobile phase was filtered through a 0.45 μm filter before use. All separations were performed under isocratic conditions.

Frontal chromatography was performed essentially as described previously [22,26,27]. A Varian HPLC system (Varian Inc., Walnut Creek, CA, USA) equipped with a 5012 pump, a manual injector with a 10 mL injection loop and a Varian 9050 single wavelength detector was used. A large volume (5–10 mL) of different concentrations of pnp-α-L-Fuc was injected and the signal at 300 nm was recorded. Data were evaluated with EZChrom software version 6.8 (Scientific Software, San Ramon, CA, USA) and the midpoints of the break-through curves were determined from the apex of the first derivative of the front. The break-through curve from GlcNAc (20 μg/mL) detected at 210 nm was used to measure the void volume of the columns. About ten individual measurements were carried out on each column with concentrations of pnp-α-L-Fuc ranging from 0.2 μM to 33 μM. A value of $\Delta\nu$ (retention volume subtracted with void volume) was calculated for each measurement, and the number of moles that saturated the column at each concentration ($\Delta\nu$ [pnp-α-L-Fuc]) was used to construct a one-site Langmuir binding hyperbola according to Eq. (1) using GraphPad Prism 5 (SanDiego, CA, USA). The number of binding sites (B_{max}) on each column was obtained via non-linear regression analysis.

$$\Delta\nu \times [\text{pnp-}\alpha\text{-L-Fuc}] = \frac{B_{max} \times [\text{pnp-}\alpha\text{-L-Fuc}]}{K_d + [\text{pnp-}\alpha\text{-L-Fuc}]} \quad (1)$$

Zonal chromatography was performed with an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a degasser, autosampler and multi-wavelength detector. Injection volume was 5 μL and the concentration of the saccharides was 20 μM if not otherwise stated. The signal from the detector at 220 nm was collected and data were evaluated with ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The concentrations of the injected saccharides in the zonal analysis were assumed to be much less than the K_d value which implicates that the concentration of the analyte in the denominator of Eq. (1) can be neglected, and the simplified version of Eq. (1) ($K_d = B_{max}/\Delta\nu$) can be used in the calculation of the affinity (K_d). Since B_{max} had been determined in the frontal chromatography evaluation it was possible to obtain the K_d value toward each saccharide directly from retention volume ($\Delta\nu$). The non-fucosylated oligosaccharide LNT was used as void marker. The retention volume was calculated from the mobile phase flow rate and the difference in elution time between LNT and the different saccharides. An average from three separate injections was used in the calculations. The small saccharides pnp-α-L-Fuc, Fucα1–3GlcNAc, Fucα1–4GlcNAc and Fucα1–6GlcNAc were evaluated with an increased flow rate of 0.5 mL/min in order to have a reasonable elution time. The affinity of these saccharides was relatively high and the simplified version of Eq. (1) was therefore not valid in determining the K_d value. The disaccharides were instead injected at different concentrations ranging from 20 to 1400 μM under non-linear conditions, and the apparent K_d values were plotted versus the injected concentrations. The actual K_d value of each saccharide was extrapolated from the intersection with the ordinate, which represent the K_d value at infinite dilution. The α1–6 fucosylated decasaccharide NA2F and the control NA2 were injected only once per column. A concentration of 100 μM was used in the evaluation and an apparent K_d value was estimated from the retention.

Non-labeled L-fucose does not absorb UV light and it is therefore impossible to determine the affinity of L-fucose by zonal chromatography with UV detection. Instead, competitive/inhibition chromatography was performed, in principal as described previously [22]. In the present study LNF III (5 μL, 20 μM) was used as reporter and fucose was dissolved in the mobile phase at various concentrations. The mobile phase was prepared at least 2 h before use to let fucose reach anomeric equilibrium, since the α and β anomers might differ in affinity and both has been reported to bind to AAL [11,17]. The concentration of fucose in the mobile phase was varied from 5.7 to 57 μM and the retention of a zonal injection of LNF III (t_{inhib}) was determined for each fucose concentration. The shift in retention of LNF III due to the addition of fucose in mobile phase was determined as $\Delta t = t_{max} - t_{inhib}$, where t_{max} was determined as the retention of LNF III without fucose in the mobile phase on each column. The column with S2-AAL was evaluated with a maximum concentration of 450 μM of fucose in the mobile phase

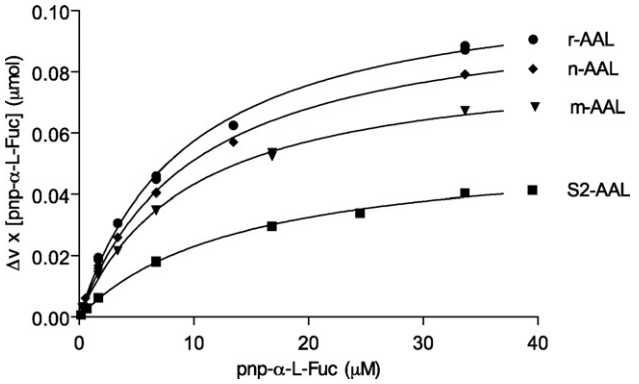

Fig. 1. Frontal chromatography; one-site binding hyperbolas from columns with immobilized r-AAL, n-AAL, m-AAL and S2-AAL, as indicated in figure. See Section 2 for chromatography conditions. The number of binding sites were determined to: r-AAL; 111 ± 3 nmol, n-AAL; 102 ± 2 nmol, m-AAL; 85 ± 2 nmol and S2-AAL; 55 ± 2 nmol. Dissociation constants (K_d) for pnp-α-L-Fuc were determined to: r-AAL; 9.4 ± 0.7 μM, n-AAL; 10.0 ± 0.5 μM, m-AAL; 9.6 ± 0.5 μM and S2-AAL; 14.1 ± 1.2 μM.

to approximately reach the same relative shift in retention as was obtained with the n-AAL, r-AAL and m-AAL columns. The relative shift in retention of LNF III ($\Delta t/\Delta t_{max}$) was used to construct a binding hyperbola according to Eq. (2); where $\Delta t_{max} = t_{max} - t_{LNT}$ and t_{LNT} is the retention time of the void marker LNT.

$$\frac{\Delta t}{\Delta t_{max}} = \frac{[\text{L-fucose}]}{K_d + [\text{L-fucose}]} \quad (2)$$

The resulting hyperbolas were fitted with a one-site binding model using GraphPad Prism 5 (San Diego, CA, USA) and the affinity was extracted from a non-linear regression analysis. A control experiment with glucose instead of fucose dissolved in the mobile phase was performed. The addition of glucose had no influence on the LNF III retention on any column (data not shown).

3. Results and discussion

3.1. Assessment of number of binding sites on AAL-immobilized columns by frontal chromatography

Frontal affinity chromatography is a reliable method to determine the affinity and the number of binding sites on a chromatography column [26,27]. It is however less suitable for screening purposes since the column has to be saturated with several concentrations of the analyte, which is time consuming and problematic when the analyte is scarce. Frontal affinity chromatography was applied in our study to assess the number of binding sites on the individual columns using pnp-α-L-Fuc as analyte. It was selected for the frontal study since it has a high UV response that made it possible to detect fronts at a wide concentration range. The number of active binding sites (B_{max}) on the immobilized columns was predicted from frontal chromatography to be 111 ± 3 nmol for r-AAL, 102 ± 2 nmol for n-AAL, 85 ± 2 nmol for m-AAL and 55 ± 2 nmol for S2-AAL (Fig. 1). It is presumed that other fucosylated saccharides bind to AAL in close analogy to pnp-α-L-Fuc and the obtained B_{max} values were used to determine K_d values of other saccharides using the zonal chromatography technique.

The calculated B_{max} values can also be used to estimate the fraction of active sites on the AAL columns by relating the obtained B_{max} to the immobilized amount of each column. The number of active sites per monomer was determined to be 2.8 for r-AAL, 3.2 for n-AAL and 2.5 for m-AAL. If each AAL monomer is assumed to have 5 sites, the result indicates that 50–64% of the sites were active in these columns. The S2-AAL column was estimated to have 0.8 binding sites per protein molecule, resulting in an activity of 80%, if one site per protein is assumed. It is known from previous WAC studies that the immobilization procedure induces some loss of activity, probably due to steric hindrance from a covalent coupling close to the binding site [22,24]. The obtained activity of the columns indicates that the proteins have been successfully coupled to the silica support.

The intact AAL proteins (n-AAL, r-AAL, and m-AAL) are predicted from crystallography studies on n-AAL to be heterogeneous regarding the affinity of the individual sites. Therefore different binding models were examined in the evaluation of AAL columns and it was found that both the one-site and two-site binding models produced good fits with R-values above 0.998. The two obtained K_d values from the two-site binding model were similar for n-AAL, r-AAL and m-AAL; about 4 μM and 40 μM, respectively, which probably reflect the approximate distribution in affinity of the five sites. The previous finding [12] of a high-affinity site in the nM-range was however not detected since it is outside the measuring range of the technique. To be able to measure such high affinities with frontal chromatography it has to be performed with an analyte concentration in the same range (nM), and this is far beyond the detection limit for non-labeled saccharides using UV detection. The confidence interval in the two-site binding model was however wide and a one-site binding approximation was therefore preferred in the evaluation. The obtained K_d values toward pnp-α-L-Fuc with a one-site binding model regarding n-AAL, r-AAL, and m-AAL were not statistically different and varied from 9.4 ± 0.7 to 10.0 ± 0.5 μM (see Fig. 1), while the affinity of S2-AAL was found to be significantly lower with a K_d value of 14.1 ± 1.2 μM. A previous frontal affinity study on a sepharose column with immobilized n-AAL reported a K_d value toward pnp-α-L-Fuc of 8.9 μM [28].

3.2. Affinity screening of fucosylated saccharides with zonal chromatography

Zonal chromatography is identical to the standard chromatographic procedure where a small volume of a diluted analyte is injected onto a column, and the retention of the corresponding peak is recorded. Under the prerequisite that the concentration of the injected analyte is much less than the K_d of the interaction (i.e. under linear conditions) the retention volume (Δv) can be used to calculate the K_d ($K_d = B_{max}/\Delta v$). The zonal chromatography technique is therefore a straightforward and efficient method to screen the affinity of a number of compounds. The affinities of a number of non-labeled fucosylated saccharides were determined with zonal chromatography by injecting about 0.1 μg of each saccharide and recording the retention on the different AAL columns. The result of the screening is presented in Table 2. The LNT pentasaccharide, which does not contain any fucose, was used to determine the void volume of each column. The columns were also examined by the injection of pnp-β-D-Fuc that was eluted close to the void (data not shown). The non-binding characteristics of these control saccharides demonstrate that the non-specific binding of the AAL columns is negligible. Saccharides were injected individually in this study but as seen in Fig. 2, mixtures could also be applied.

The affinities of the pentasacchrides LNF I, II and III have previously been determined with SPR, with reported K_d values for rAAL of 128, 92 and 77 μM, respectively [19], and the WAC results agree well with the affinity ranking of the saccharides. The K_d values determined with the two methods do however differ quite substantially, even though the binding was studied under almost identical conditions. The K_d values of LNF I, II and III determined with SPR are generally lower (i.e. of higher affinity) compared with WAC and the most pronounced difference was exhibited by the weakest binder LNF I. It had a SPR K_d value of almost a magnitude lower compared with WAC (K_d values for rAAL were for example 128 and 960 μM, respectively). The tighter binder LNF III, had a SPR K_d value of 77 μM

Table 2
Obtained K_d values.

Saccharide	K_d (μM)			
	r-AAL	m-AAL	n-AAL	S2-AAL
NA2F[a]	18	22	20	27
	25	27	25	43
LNFI	960	847	898	2250
LNFII	300	229	295	748
LNFIII	154	118	155	396
A-tetra	4850	3850	3760	6790
A-tri	110	95	113	232
H-tri	57.5	53.1	56.5	80.6
pmp-β-LeX	95.9	88.6	99.0	143
pmp-β-sLeX	56.3	36.9	46.3	264
pnp-α-L-Fuc	9.2	9.8	9.5	16.4
	9.4[b]	9.8[b]	10.0[b]	14.1[b]
				17[c]
L-Fuc-α-1-6GlcNAc	7.8	7.8	7.8	8.2
L-Fuc-α-1-4GlcNAc	9.6	9.3	9.7	15.4
L-Fuc-α-1-3GlcNAc	4.9	5.3	4.9	5.2
L-Fucose	15.5[d]	17.9[d]	17.7[d]	37[d]
LNT	–	–	–	–

[a] K_d values of the two major peaks in the chromatogram.
[b] K_d values obtained with non-linear chromatography.
[c] K_d value obtained with ITC.
[d] K_d values obtained with competitive/inhibition chromatography.
All other K_d values were obtained by WAC (zonal chrom.).

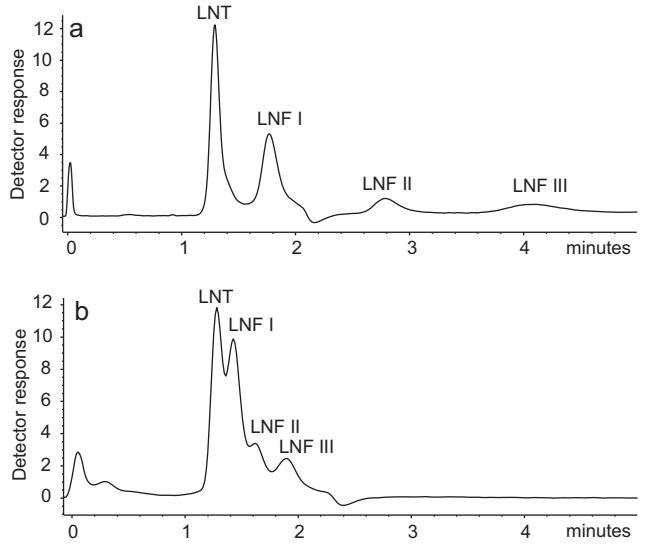

Fig. 2. Zonal chromatography of a mixture of LNF I, II and III on columns with immobilized n-AAL (a) and S2-AAL (b). The retention of each saccharide was evaluated from individual injections (not shown). Injection volume was 5 μL and sample concentration was 50 μg/mL of each analyte. Mobile phase was 0.01 mM sodium phosphate pH 7.2 with 140 mM sodium chloride, flow rate was 0.25 mL/min and temperature 23 ± 1 °C.

which is closer to the value obtained by WAC (154 μM). The most likely explanation for the discrepancy with SPR is that this study was performed with oligosaccharide concentrations below the K_d of the interaction, varying from 0.01 to 100 μM. The obtained K_d values in the SPR analysis will therefore have a high uncertainty for weakly interacting saccharides. This explains why the weakest binder (LNF I) demonstrated the largest deviation when measured with WAC and SPR. To receive reliable K_d values of low affinity analytes (K_d > 100 μM) in SPR, higher concentrations of the analyte have to be used, which in most cases are impossible to achieve due to technical as well as economical reasons. The accuracy of WAC in determining high K_d values, without the need of using high concentrations of analyte, is a distinguishing feature of WAC in contrast to SPR and other techniques such as fluorescence spectroscopy, NMR and ITC.

The result of the WAC oligosaccharide screening can be compared to a recent work where an n-AAL sepharose column was evaluated by frontal injections of pyridylaminated (PA) oligosaccharides [28]. The concentrations of the PA saccharides were in the nM range and the K_d values were calculated using the simplified equilibrium equation ($K_d = B_{max}/\Delta v$). The PA forms of the LNF I, II and III and A-tetra saccharides were reported not to have any affinity toward n-AAL in the frontal study, while the affinities of these oligosaccharides toward all forms of AAL were readily determined using WAC. The reason for the difference in results is most likely the low amount of n-AAL immobilized on the sepharose column in combination with a lower chromatographic resolution, resulting in an upper detection limit of about 50 μM in K_d regarding the frontal affinity study [28]. Derivatization of oligosaccharides might also influence the result making direct comparisons between different studies difficult. An example of this is that PA forms of LeX trisaccharide and sLeX tetrasaccharide was reported to have K_d values of 5 and 3 μM, respectively [28], while the affinity of p-methoxyphenyl (pmp) derivatized LeX and sLeX included in our study had K_d values of 99 and 46 μM, respectively, on the n-AAL column. Previously reported values from SPR and NMR studies of non-labeled LeX and sLeX oligosaccharides are in the range of 100–400 μM [11,13].

The fucosylated disaccharides turned out to be highly retained on the AAL column indicating a K_d value in the low μM range. As a consequence, the injected concentration should be less than 1 μM in order to be in the linear range of zonal chromatography. Overriding this concentration will shorten the retention of the injected saccharides and the simplified version of Eq. (1) ($K_d = B_{max}/\Delta v$) will not be valid. As the desired concentration was far beyond the detection limit of non-labeled saccharides, the retention of the fucosylated disaccharides were determined from injecting the disaccharides under non-linear conditions. The retention from injecting the saccharides at different concentrations was determined and a graph of the calculated apparent K_d values versus the injected concentrations was constructed for each saccharide. The relation in the graph was empirically found to follow an exponential equation and the "true" K_d value was extrapolated from the intersection with the ordinate (Fig. 3). In order to validate the non-linear WAC technique, the affinity of S2-AAL toward pnp-α-L-Fuc was evaluated with non-linear WAC, frontal chromatography and isothermal calorimetry (ITC). The obtained K_d values (Table 2); 16 μM from non-linear WAC (Fig. 3), 14 μM from frontal chromatography (Fig. 1) and 17 μM from ITC (data not shown) are in close agreement. The identical K_d values of ITC and the two different chromatography methods (non-linear WAC and frontal chromatography) confirm that the produced values are reliable K_d estimations.

The determined K_d values of the disaccharides (Fucα1–3GlcNAc, Fucα1–4GlcNAc and Fucα1–6GlcNAc) were all in the 10 μM range (Fig. 3 and Table 2). It has been shown with STD NMR experiments of LeX saccharides that fucose and the first glycosidic bond interact with the AAL binding pocket, while the rest of the saccharide seems to have a minimal contact with the protein [13]. The almost equivalent affinities of the disaccharides seem to confirm this view of AAL binding. Comparing the affinities in detail, it was found that the disaccharide with fucose in α1–3 position showed the highest affinity with all AAL forms, followed by the saccharides with fucose in α1–6 and α1–4 position. It has been postulated that fucose in position 6 generally should be the most advantageous for AAL binding because of the higher accessibility of fucose in this position [14,15]. Later work does however indicate that fucosylated disaccharides bind to AAL with approximately the same affinity regardless of the fucose position [11,13], a view that is supported by our results. We could also confirm the previous observation that small fucosylated disaccharides generally have a higher affinity compared to larger

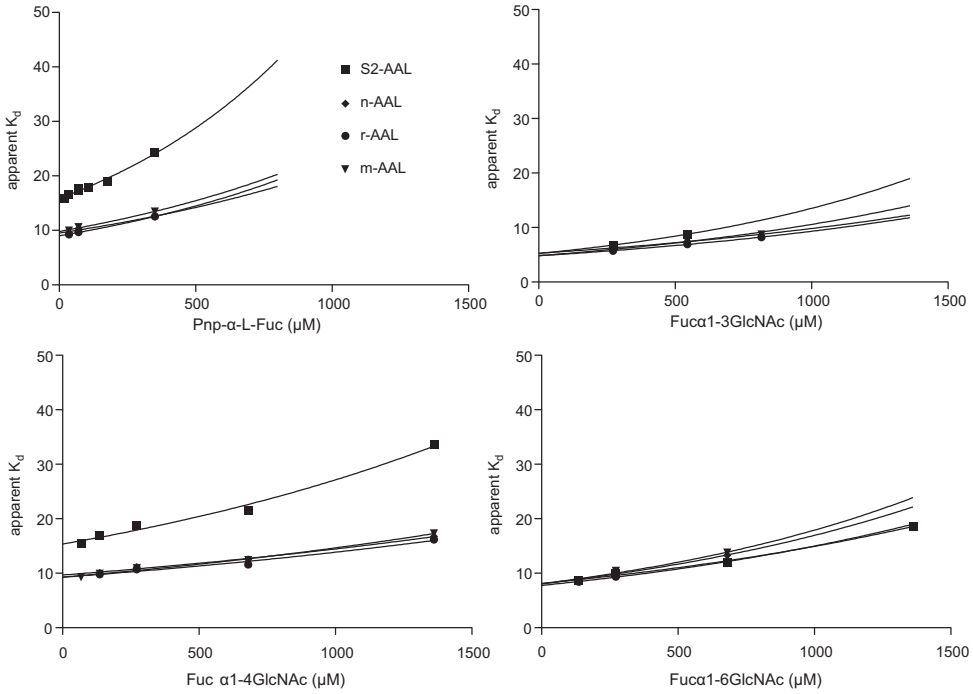

Fig. 3. Non-linear zonal chromatography; different concentrations (exceeding the linear isotherm) of pnp-α-L-Fuc, Fucα1–3GlcNAc, Fucα1–4GlcNAc and Fucα1–6GlcNAc were injected on the columns with immobilized S2-AAL, n-AAL, r-AAL and m-AAL, as indicated in figure. The K_d value of each saccharide was extrapolated at an infinite dilution as the intersection with the ordinate. Chromatography conditions were identical to Fig. 2, except for the flow rate that was 0.5 mL/min.

saccharides, even though they contain the same fucosylated unit (Table 2).

The reason for the weaker affinity of fucosylated trisaccharides and larger oligosaccharides compared to the fucosylated disaccharides is probably the intramolecular stacking of fucose in larger oligosaccharide structures, which has been shown for LeX trisaccharides [13]. The fucose stacking has to be distorted in order to bind to AAL, which means that the conformation of the bound saccharide is of higher energy and different from the most populated conformation found in solution. Disaccharides are too small to exhibit any stacking and the resulting affinity is therefore similar for all disaccharides and of higher affinity compared to larger saccharides (Table 2). In conclusion we can confirm that the AAL interaction is highly dependent on the availability of fucose in a saccharide, while the linkage between fucose and the next unit seems to be of minor importance. The greater flexibility of the Fucα1–6 bond is probably advantageous in binding to AAL but only when it is part of a large oligosaccharide [15,28]. The tetra- and pentasaccharides included in our study have fucose bound α1–2, α1–3 or α1–4 and they all interact with a low affinity, having a $K_d > 0.1$ mM (Table 2). The analysis of NA2F, a decasaccharide with fucose bound α1–6 to GlcNAc (see Table 1), produced two major peaks that were considerably more retained compared to the tetra-and pentasaccharides. The origin of the two peaks in the NA2F sample is unclear but could possibly represent two NA2F conformers. The non-fucosylated control NA2 did not produce any retained peaks. The analysis was performed under non-linear conditions and the apparent K_d values of the peaks were about 20 μM regarding r-AAL, m-AAL and n-AAL while the K_d value found with S2-AAL was about 30 μM (Table 2).

3.3. Determination of fucose affinity with competitive/inhibition chromatography

Competitive/inhibition chromatography was used to determine the affinity of non-labeled L-fucose, since it was impossible to determine K_d with zonal chromatography due to the poor UV absorbance of L-fucose. The principle of competitive/inhibition chromatography is to use a reporter compound (that has good detection properties) in order to determine the affinity of another analyte (having poor detection properties), which is dissolved in the mobile phase at various concentrations. The reporter and the analyte in the mobile phase should bind to the same site on the immobilized protein. By changing the concentration of the analyte in the mobile phase, a saturation-binding curve is obtained based on the change in retention of a zonal injection of the reporter. In this study LNF III was used as reporter and L-fucose was dissolved in the mobile phase. The retention of LNF III without any L-fucose in the mobile phase (Δt_{max}) was about 3 min on the n-AAL, r-AAL and m-AAL columns while it was less retained on the S2-AAL column, exhibiting a Δt_{max} of 0.6 min (Fig. 2). The shorter retention of the reporter on the S2-AAL column made the K_d determination less accurate compared to the other AAL columns. The obtained K_d values were 17.7 ± 0.3 μM for n-AAL, 15.5 ± 0.4 μM for r-AAL, 17.9 ± 0.1 μM for m-AAL and 37 ± 3 μM for S2-AAL (Fig. 4). These values are in close agreement with previously reported K_d values regarding n-AAL and r-AAL [10–13,16].

3.4. Comparison of the binding profile of the different AAL forms

When comparing the AAL proteins it appears that n-AAL, r-AAL and m-AAL have almost identical affinities toward fucosylated saccharides while S2-AAL generally has a lower affinity. Another difference is that S2-AAL is sensitive to sialic acid in combination with fucose, which is seen in the decrease in affinity of pmp-β-sLeX compared with pmp-β-LeX, while the other AAL forms have a higher affinity of the sialylated saccharide (Table 2). Similar results have previously been observed in ELLA (enzyme linked lectin assay) and SPR where other sialylated and fucosylated saccharides were analyzed [19]. The observed shifts in selectivity of S2-AAL

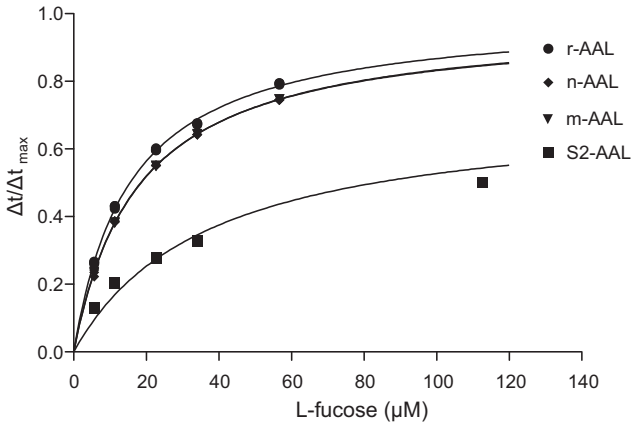

Fig. 4. Competitive/inhibition chromatography; binding hyperbolas from columns with immobilized r-AAL, n-AAL, m-AAL and S2-AAL, as indicated in figure. See Section 2 for chromatography conditions. The affinities toward fucose were determined to: r-AAL; 15.5 ± 0.4 μM, n-AAL; 17.7 ± 0.3 μM, m-AAL; 17.9 ± 0.1 μM and S2-AAL; 37 ± 3 μM. S2-AAL was evaluated with a maximum concentration of 450 μM L-fucose (data not shown).

compared to native AAL are the first step in the development of a linkage specific fucose recognizing lectin.

4. Conclusions

This study demonstrates the use of weak affinity chromatography (WAC) as a powerful technique in the evaluation of the binding characteristics of engineered lectins, such as AAL. The different forms of AAL showed various weak affinities to fucose-containing saccharides in the mM to μM range and by applying the WAC technique structure–affinity relationships could be elucidated. WAC benefits from the possibility to determine K_d values with high precision, without the need of using high concentrations or labeled saccharides. Another distinguishing feature of WAC is that racemates, sample mixtures and impure samples can be analyzed as the analysis is based on the principle of separation. If the constituents of the mixture have different affinities toward the immobilized protein, affinity of each component can be determined concomitantly. Finally, standard HPLC instrumentation is used in the analysis and a selection of operating parameters such as pH, buffer composition and temperature could easily be varied.

Funding

This study was supported by grants from Linnæus University, Linköping University and the Medical council of Southeast Sweden.

Acknowledgments

We thank Prof. Ulf Ellervik for generously providing us with pmp-LeX saccharides and Johan Olausson for preparing the AAL proteins.

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