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Detection of a high affinity binding site in recombinant *Aleuria aurantia* lectin

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Abstract Lectins are carbohydrate binding proteins that are involved in many recognition events at molecular and cellular levels. Lectin-oligosaccharide interactions are generally considered to be of weak affinity, however some mushroom lectins have unusually high binding affinity towards oligosaccharides with K_d values in the micromolar range. This would make mushroom lectins ideal candidates to study protein–carbohydrate interactions. In the present study we investigated the properties of a recombinant form of the mushroom lectin *Aleuria aurantia* (AAL). AAL is a fucose-binding lectin composed of two identical 312-amino acid subunits. Each subunit contains five binding sites for fucose. We found that one of the binding sites in rAAL had unusually high affinities towards fucose and fucose-containing oligosaccharides with K_d values in the nanomolar range. This site could bind to oligosaccharides with fucose linked α1-2, α1-3 or α1-4, but in contrast to the other binding sites in AAL it could not bind oligosaccharides with α1-6 linked fucose. This binding site is not detected in native AAL (nAAL) one possible explanation may be that this site is blocked with free fucose in nAAL. Recombinant AAL was produced in *E. coli* as a His-tagged protein, and purified in a one-step procedure. The resulting protein was analyzed by electrophoresis, enzyme-linked lectin assay and circular dichroism spectroscopy, and compared to nAAL. Binding properties were measured using tryptophan fluorescence and surface plasmon resonance. Removal of the His-tag did not alter the binding properties of recombinant AAL in the enzyme-linked lectin assay. Our study forms a basis for understanding the AAL-oligosaccharide interaction and for using molecular techniques to design lectins with novel specificities and high binding affinities towards oligosaccharides.

Keywords Aleuria aurantia lectin · Fucose · Surface plasmon resonance · Tryptophan fluorescence · Oligosaccharides

Abbreviations

AAL	*Aleuria aurantia* lectin
BSA	Bovine serum albumin
CD	Circular dichroism
ELLA	Enzyme-linked lectin assay
K_d	Dissociation constants
MIC	Minimum inhibiting concentration
nAAL	Native *aleuria aurantia* lectin
PBS	Phosphate buffer saline
rAAL	His-tagged recombinant aleuria aurantia lectin
SPR	Surface plasmon resonance

Introduction

Lectins are a class of proteins of non-immune origin that binds carbohydrates without modifying them. They are involved in many recognition events at molecular and cellular levels. Since lectins differ in the types of carbohydrate structures they recognize they are used to detect and separate cells, bacteria, and viruses with different carbohydrate content. Lectins are also useful tools for

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investigating the structure, distribution and function of different carbohydrate chains on glycoproteins and glycolipids [1–3]. The *Aleuria aurantia* lectin (AAL) from the fruit bodies of *Aleuria aurantia* mushroom has been extensively used in structural studies of oligosaccharides. AAL is specific for L-fucose and differs from other fucose-binding lectins by having a broad specificity towards fucosylated oligosaccharides [4–7]. AAL can bind to oligosaccharides with fucose in α1-2, α1-3, α1-4 and α1-6 linkages, with the strongest affinity towards fucose in α1-6 linkage, but is relatively insensitive to structural differences in the oligosaccharide backbone [5, 8]. Since AAL is one of the few fucose-binding lectins with a preferential binding to α1-6 linked fucose it has been widely used in fractionation of glycoproteins with core-fucosylated complex-type N-glycans [3, 9]. Since changes in fucosylation is often associated with inflammatory conditions and oncogenic transformation AAL has also been used for fractionation and analysis of disease-associated glycosylation [10–13]. However, production of carbohydrate binding reagents with more discriminating and specific recognition of specific carbohydrate epitopes would be valuable for diagnostic purposes.

AAL is a non-glycosylated protein that has a molecular weight of 72 kDa and is composed of two identical 312 amino acid subunits [4]. The lectin was recently crystallized and each monomer was shown to have a sixfold β-propeller structure with five binding sites for L-fucose [8, 14]. The slight structural differences at the five binding sites as well as the results from site specific mutagenesis studies indicated that the five possible binding sites for fucose differ in affinities towards fucose [8, 14, 15]. Site 2 and 4 have the highest affinities towards fucose, site 1 have medium affinity whereas site 3 and 5 bind fucose with the weaker affinities [8, 14]. Crystallization of a commercially available preparation of AAL revealed that free L-fucose was bound to three of the five binding sites [14]. Since the protein was extensively dialyzed before crystallization, this indicates that some sites in AAL may have very high affinity towards free fucose. Consequently, L-fucose remaining in high affinity binding sites of AAL preparations may affect the reported binding affinities for fucosylated oligosaccharides [14].

Lectin-oligosaccharide interactions are generally characterized by weak affinities (millimolar range) for monovalent binding. This low affinity is usually compensated by the fact that most lectins are multivalent. However, several bacterial and fungal lectins have been shown to display unusually high affinity towards carbohydrate ligands compared to plant or animal lectins, with K_d-values in the micromolar range [16–18]. A further understanding of the binding properties of these lectins will be important for designing high-affinity carbohydrate-binding proteins. The possibility of using molecular biology techniques to modify binding properties of these lectins may further increase these possibilities [19, 20].

AAL has previously been expressed and purified from both *E. coli* and *Pichia Pastoris*. The recombinant forms of AAL have been shown to retain their agglutinating properties [6, 15]. However, there have been no previous studies that specifically analyze the specificity and affinity of recombinant AAL (rAAL).

In this study we investigated the properties of recombinantly produced AAL. Since rAAL was purified without using any L-fucose it was devoid of fucose. The present study revealed a fucose binding site in rAAL that is blocked by free fucose in native AAL (nAAL). This site seems to have an unusually high affinity (nM) towards free fucose and fucosylated oligosaccharides.

Materials and methods

Oligosaccharides and glycoconjugates

Milk oligosaccharides and oligosaccharide-BSA (bovine serum albumin) conjugates were from BioCarb AB (Lund, Sweden). Complex-type biantennary oligosaccharide NA2F was from Dextra Laboratories Ltd (Berkshire, United Kingdom) and L-fucose was from Sigma-Aldrich (Stockholm, Sweden). Oligosaccharide structures are listed in Table 1.

Expression and purification of His-tagged recombinant AAL (rAAL)

A pGEM plasmid containing the *AAL* gene was a gift from Prof. Yoshiho Nagata (Chiba University, Japan). The *AAL* fragment was excised using restriction enzymes *Nde*I and *Xho*I (Invitrogen Carlsbad, CA) and then introduced into the *Nde*I/*Xho*I site of the expression vector pET-28b (Novagen, Darmstadt, Germany), which adds a His-tag to the C-terminal of AAL. The resulting plasmid was transformed into the *E. coli* strain BL21/DE3 (Invitrogen). BL21/DE3 harbouring the recombinant pET-28b-*AAL* plasmid was added to 500 ml of LB-medium containing 30 μg/ml kanamycin and incubated at 37°C with shaking until OD_{600} was between 0.6–0.9. To induce the synthesis of rAAL, isopropyl-beta-D-thiogalactopyranoside (IPGT) was added to a final concentration of 0.5 mM and the cells were incubated at room temperature over night with shaking. Cells were collected by centrifugation and sonicated for 4×30 s in 10 mM phosphate buffer saline, pH 7.2 (PBS). The sonicate was centrifuged first at 2000×*g* for 20 min then at 19,000×*g* for 15 min both at 4°C to remove debris. Recombinant AAL was purified from the supernatant by affinity chromatography using a 1 ml

Table 1 Oligosaccharide structures

Trivial name (abbreviated)	Oligosaccharide structure
LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
LNF I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc
LNnF I	Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glc
LNF II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 | Fucα1
LNF III	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 | Fucα1
NA2	Galβ1-4GlcNAcβ1-2Manα1 | 6 Manβ1-4GlcNAcβ1-4GlcNAc 3 | Galβ1-4GlcNAcβ1-2Manα1
NA2F	Galβ1-4GlcNAcβ1-2Manα1 Fucα1 | | 6 6 Manβ1-4GlcNAcβ1-4GlcNAc 3 | Galβ1-4GlcNAcβ1-2Manα1
LDFT	Fucα1-2Galβ1-4Glc 3 | Fucα1

Ni-column (HiTrapTM Chelating HP column, Amersham Biosciences, Uppsala, Sweden) at a flow rate of 1 ml/min.

Circular dichroism (CD)

Far-UV CD (180–260) and near-UV CD (240–320) spectra of both native AAL (nAAL) and rAAL (0.6 mg/ml in PBS) were recorded on a CD6 spectrodichrograph (Jobin-Yvon instruments SA, Longjumeau, France) under constant N_2 purging. For the far-UV measurements a 0.5 mm path length cell was used whereas a 5.0 mm path length cell was used for near-UV. Spectra were typically recorded as an average of three scans. All measurements were carried out in room temperature.

Enzyme-linked lectin assay (ELLA)

Purified rAAL and nAAL were biotinylated using IMMUNOPROBE™ Biotinylation Kit (Sigma-Aldrich, Stockholm, Sweden) according to the manufacturer's instruction. The biotin/protein ratio was determined to 1.1 biotin moieties per protein molecule for both rAAL and nAAL. The biotinylated nAAL and rAAL were used in an ELLA as previously described [10].

Briefly, microtiter plates (Nunc MaxiSorp™ eBioscience, San Diego, CA) were coated with 0.2 μg of LNnF I-BSA, LNF II-BSA, LNF III-BSA or LNT-BSA in 100 μl coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02 % NaN_3, pH 9.6). Then 100 μl of biotinylated nAAL or rAAL (0.002 μg/μl) was added to the wells. After addition of ExtrAvidin (E-2632 Sigma-Aldrich, Stockholm, Sweden) and phosphatase substrate (Sigma 104®, Sigma-Aldrich, Stockholm, Sweden) the amount of nAAL or rAAL binding to each well were measured at 405 nm using a $VERSA_{max}$ microplate reader (Molecular Devices Corporation, Sunnyvale, CA). In one set of experiments rAAL was pre-treated with L-fucose before analysis. Purified rAAL was dissolved in 3 ml of 50 mM L-fucose in PBS to a final concentration of 1.5 μM. This was incubated for 3 h in 4°C. The lectin fucose suspension was then dialyzed against two changes of 2 L of PBS prior to the ELLA experiments as above. In another set

of experiments thrombin protease was used to cleave the His-tag from biotinylated rAAL. To 50 μg of biotin labelled rAAL and nAAL in PBS 2 units of thrombin protease (Amersham Biosciences, Uppsala, Sweden) was added and incubated for 2 h in room temperature. ELLA analysis using thrombin treated rAAL was performed as described above.

Surface Plasmon Resonance (SPR)

SPR measurements were performed using a Biacore 2000 (BiacoreAB, Uppsala, Sweden) at 25°C with PBS as running buffer and a flow rate of 5 μl/min. Measurements were carried out simultaneously on three of the four channels. Channel one contained nAAL (9000 RU) and channel two rAAL (11000 RU) whereas the third channel was used as the control flow cell. A research grade CM5 sensor chip was activated with a 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide/*N*hydroxysuccinimide solution for 7 min. Then 100 μl of 10 μM nAAL in acetate buffer (pH 5.0) was injected into flow cell one and 35 μl of 0.3 μM rAAL in acetate buffer was injected into flow cell two. The unreacted species on the sensor surface were blocked by a 35 μl injection of 1 M ethanolamine. The blank channel was treated identically except for the lectin injection. Then 25 μl of oligosaccharide solutions (concentrations between 0.01 and 100 μM) in running buffer were injected into the flow cells using the kinject mode. The equilibrium response (after subtraction from the response of the reference surface) of each experiment was used to create curves of analyte binding, which were fitted to a 1:1 steady-state affinity model or a two site binding model using Scrubber version 2.0 software (BioLogic Software Pty Ltd, Campbell, Australia). In another set of experiments 25 μl of 100 mM free fucose was injected over the nAAL and rAAL surfaces prior to injection of oligosaccharides. After the fucose injection the surfaces was washed extensively with running buffer and the oligosaccharide binding studies were performed as described above. Injections with same concentrations of the non-fucosylated oligosaccharide LNT was performed as a negative control.

Tryptophan fluorescence

Tryptophan fluorescence measurements were preformed on a Hitachi F4500 spectrofluorophotometer (Hitachi, Tokyo, Japan). Spectra were recorded at 22°C. Emission spectra were obtained by excitation at 295 nm with emission monochromator scanning between 310-360 nm with increment of 1 nm and integration time of 0.5 s. Both the excitation and emission slits were set to 5 nm.

Purified nAAL and rAAL was diluted in PBS to a concentration of 0.1 μM. Aliquots of 5 μl of LNF I, LNF II, LNF III and NA2F in PBS was added in increased concentrations generating final concentrations from 0 to 55 μM. After each addition, the solution was mixed and the tryptophan fluorescence measured. Signals were corrected for background fluorescence using addition of the non-fucosylated oligosaccharide LNT as a negative control.

The change in total spectral fluorescence ($\Delta F = F - F_0$) at each oligosaccharide (os) concentration compared with no addition of antigen (F_0) were used to create binding curves. The data were adjusted to an adsorption isotherm (Langmuir-type) according to the equation: $\Delta F = \Delta F_{max} \times [os]_{free} / (K_d + [os]_{free})$, where $[os]_{free} = [os]_{tot} -$ [available binding sites $\times \Delta F / \Delta F_{max}$]. GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA) was used to analyze the data obtained from the oligosaccharide titrations.

Hemagglutination and hemagglutination inhibition

The hemagglutination activity of the lectins was determined by serial dilutions of the lectins in PBS and mixing with an equal volume (50 μl) of 2 % human type O erythrocytes suspended in PBS. After incubation at room temperature for 1 h the minimum lectin concentration that gave a positive reaction was determined. For the inhibition assay four times the minimal hemagglutinating lectin concentration of nAAL and rAAL were incubated for 1 h at room temperature with fucose, lactodifucotetraose and glucose which had been serially diluted with PBS and then assayed for hemagglutinating activity. The minimum concentration of sugar that completely inhibited the hemagglutinating activity was determined.

Monosaccharide analysis

Monosaccharide analysis was performed essentially as previously described [21]. In brief, 200 μg of nAAL or rAAL was hydrolyzed in 4 M trifluoro acetic acid at 100°C for 4 h with 5 μg perseitol added as an internal standard. The generated monosaccharides were converted into their corresponding alditiol acetates. The samples were analyzed by gas-liquid chromatography-mass spectrometry (Hewlett-Packard 5890 gas chromatograph and Hewlett-Packard 5972 mass selective detector) on a DB-1MS column, 30 m×0.25 mm (Agilent Technologies, USA).

Results and discussion

Expression and purification of His-tagged recombinant AAL (rAAL)

Traditionally nAAL is purified by affinity chromatography on fucose-starch columns with elution of nAAL with free L-fucose. To avoid the use of L-fucose in the purification

procedure we constructed a His-tagged variant of AAL (rAAL) in *E. coli* which was purified by affinity chromatography on an IMAC-column loaded with Ni-ions. The purified rAAL showed a single band with an approximate molecular weight of 32 kDa when analyzed by SDS-PAGE (Fig. 1). Purified rAAL migrated slightly slower than nAAL due to the presence of the His-tag. The yield of rAAL was about 5 mg from 500 ml of bacterial culture.

Analysis of secondary structure

Circular dichroism studies showed almost identical spectra for nAAL and rAAL in both far- UV (200–250 nm) and near-UV (250–310 nm) (Fig. 2) and they were also very similar to previously published corresponding spectra for nAAL [7]. Both proteins showed a minimum at 218 nm indicative of proteins with a high proportion of β-sheet. The far-UV spectrum of nAAL showed a slightly higher intensity around 230 nm compared to the spectrum of rAAL, which may indicate the presence of fucose in nAAL since a shift in this region was previously noted when comparing CD spectra of nAAL with and without addition of fucose [7]. Minor spectral differences between nAAL and rAAL around 270–280 nm may also reflect tryptophans in the binding site being affected by bound fucose in nAAL.

Enzyme linked lectin assay (ELLA) analysis

ELLA analysis showed that both nAAL and rAAL had the ability to bind to glycoconjugates with α1-2, α1-4 and α1-3 linked fucose (LNnF I-, LNF II- and LNF III-BSA conjugates, respectively). However, the amount of bound rAAL was constantly three to five times higher than the binding of nAAL (Fig. 3). These results suggested presence of high affinity binding sites in rAAL that was not present in nAAL.

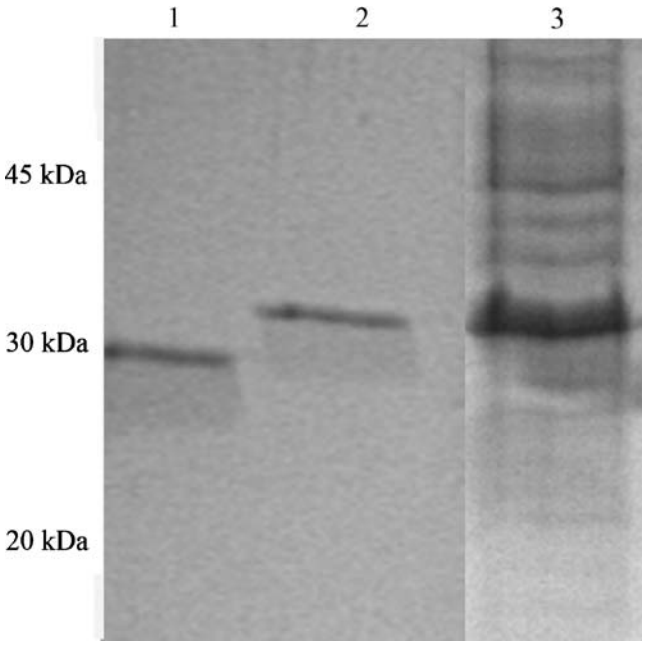

Fig. 1 Silver stained SDS-PAGE of native AAL (*lane 1*), purified rAAL (*lane 2*) and cytosolic fraction of BL21/DE3-AAL (*lane 3*)

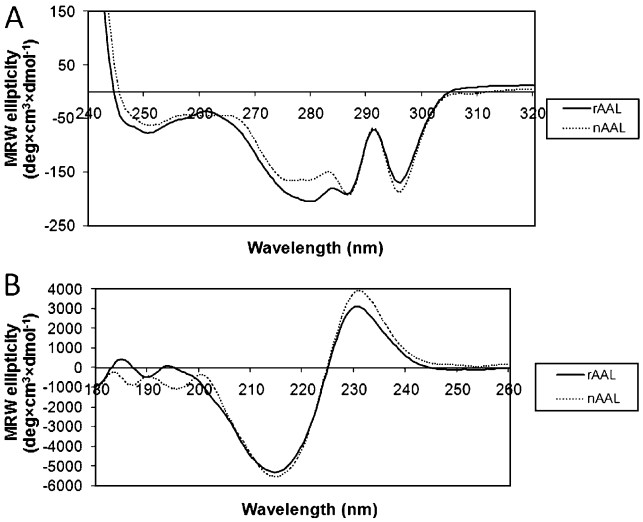

Fig. 2 Circular dichroism spectra of nAAL (*dashed line*) and rAAL (*solid line*). **a** Near-UV spectra of nAAL and rAAL showing characteristic tryptophan peaks around 290 nm. **b** Far-UV spectrum of nAAL and rAAL with a peak at 218 nm indicating β-sheet structure. All spectra are an average of three scans

To analyze whether the His-tag on rAAL affected the fucose binding the ELLA analysis was also performed using rAAL pretreated with thrombin protease to remove the His-tag. Both rAAL and nAAL was incubated with thrombin protease. SDS-PAGE analysis of rAAL after thrombin treatment showed a single band with a molecular weight about 1,000 Da lower than for non treated rAAL

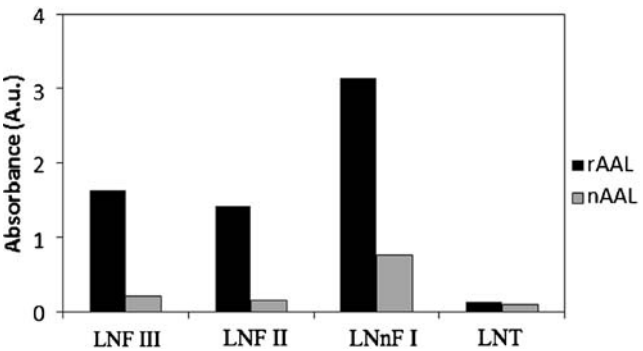

Fig. 3 ELLA showing binding of nAAL and rAAL to different fucosylated oligosaccharides. The non-fucosylated oligosaccharide LNT was used as a negative control. The amount of biotin label/mole protein was identical for nAAL and rAAL, and identical concentrations of nAAL and rAAL were used. *Bars* represent mean values from triplicate analysis. Figure shows representative results from one of five experiments

confirming complete cleavage of the His-tag from rAAL, whereas migration of nAAL (as expected) was not affected by thrombin treatment (data not shown). ELLA analysis using the thrombin treated rAAL showed identical binding as non-treated rAAL to LNnF I-, LNF II- and LNF III-BSA conjugates indicating that the His-tag does not contribute to the enhanced binding of rAAL to fucosylated oligosaccharides compared to nAAL (Fig. 4). As a control it was also shown that thrombin treatment did not affect oligosaccharide binding of nAAL (data not shown).

Surface plasmon resonance studies

Apparent equilibrium dissociation constants (K_d) for nAAL and rAAL binding to L-fucose and a series of fucose-containing oligosaccharides were estimated using surface plasmon resonance.

When fucosylated oligosaccharides were passed over nAAL immobilized to the sensor surface typical "pulse shaped" sensorgrams with rapid association and dissociation phases were obtained. A typical sensorgram obtained after injection of a fucosylated analyte over nAAL is shown in Fig. 5a. Binding curves for all analytes were generated using the steady-state part of the sensorgram. Apparent K_d values were estimated by non-linear curve fitting of the data to the Langmuir binding isotherm using a simple 1:1 binding model (Fig. 5a). The affinity data are summarized in Table 2. The K_d values obtained for fucose and fucose-containing oligosaccharides towards nAAL were in the micromolar range and consistent with previously published data [8, 22]. Binding of an oligosaccharide containing α1-6 linked fucose (NA2F) and a difucosylated oligosaccharide showed approximately tenfold higher affinities compared to oligosaccharides with α1-2 (LNF I), α1-4 (LNF II) and α1-3 linked (LNF III) fucose (Table 2), whereas injection of a non-fucosylated oligosaccharide, LNT, did not show any binding response (data not shown).

In contrast, when fucosylated oligosaccharides were injected over a sensor surface with immobilized rAAL distinct association and dissociation phases were seen, indicating a higher binding affinity towards fucosylated oligosaccharides compared to nAAL (Fig. 5b).

To obtain approximate K_d values the RU values at the end of the injection phase, representing pseudo-steady state plateau values, were fitted to a Langmuir binding isotherm. For LNF I, LNF II and LNF III and free fucose the best fit was obtained using a two-site binding model revealing apparent K_{d1} and K_{d2} values (Table 2). The K_{d1} values were very similar to the affinities obtained with nAAL, whereas the K_{d2} values revealed a binding site in rAAL with affinities in the nanomolar range. Interestingly, the binding data obtained with the oligosaccharide containing α1-6 linked fucose (NA2F) was similar to the binding data obtained for nAAL and could be fitted using a simple 1:1 binding model.

Taken together the surface plasmon resonance analyses revealed that rAAL has one binding site with high affinity towards fucose and fucosylated oligosaccharides that is not available in nAAL. But in contrast to the other binding sites this site did not seem to recognize oligosaccharides with fucose in an α1-6 position.

Tryptophan fluorescence studies of binding specificity of nAAL and rAAL

Since tryptophan residues have been implicated in fucose-binding of nAAL we used tryptophan fluorescence spectroscopy to further analyze the binding properties of rAAL. Addition of LNF I, LNF II or LNF III to rAAL induced dose dependent quenching of the observed tryptophan fluorescence, but no shifts in the emission spectra. The oligosaccharides did not show any fluorescence of their own that could influence the obtained results. The effect of the added oligosaccharides on the tryptophan fluorescence emission spectrum of rAAL at saturation is shown in Fig. 6. The tryptophan emission fluorescence spectrum of nAAL had lower intensity compared to the rAAL spectrum and was similar to the spectra obtained from rAAL with saturating concentrations of LNF I, LNF II and LNF III. However when NA2F was added to rAAL there was no change in tryptophan fluorescence.

None of the different fucose-containing oligosaccharides affected the tryptophan fluorescence spectra of nAAL.

Titration was performed using oligosaccharide concentrations in the range of 0–55 μM. Saturating concentrations for all analyzed oligosaccharides (LNF I, LNF II, LNF III) were reached at 200 nM or lower, thus supporting affinity constants in the nM region. The change in total spectral fluorescence ($\Delta F = F - F_0$) at each oligosaccharide concentration compared with no addition of oligosaccharide (F_0) were corrected for background fluorescence and adjusted to an adsorption isotherm (Langmuir-type). This

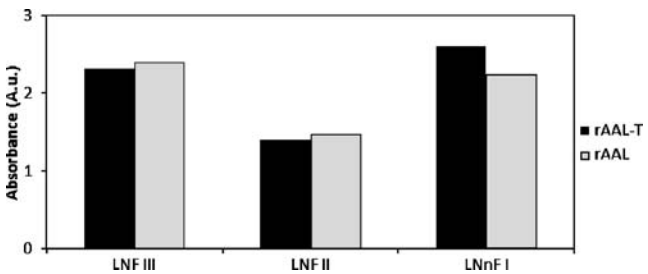

Fig. 4 ELLA comparing binding of thrombin protease treated rAAL (rAAL-T) with non treated rAAL to different fucosylated oligosaccharides. Identical concentrations of rAAL-T and rAAL were used. *Bars* represent mean values from triplicate analysis

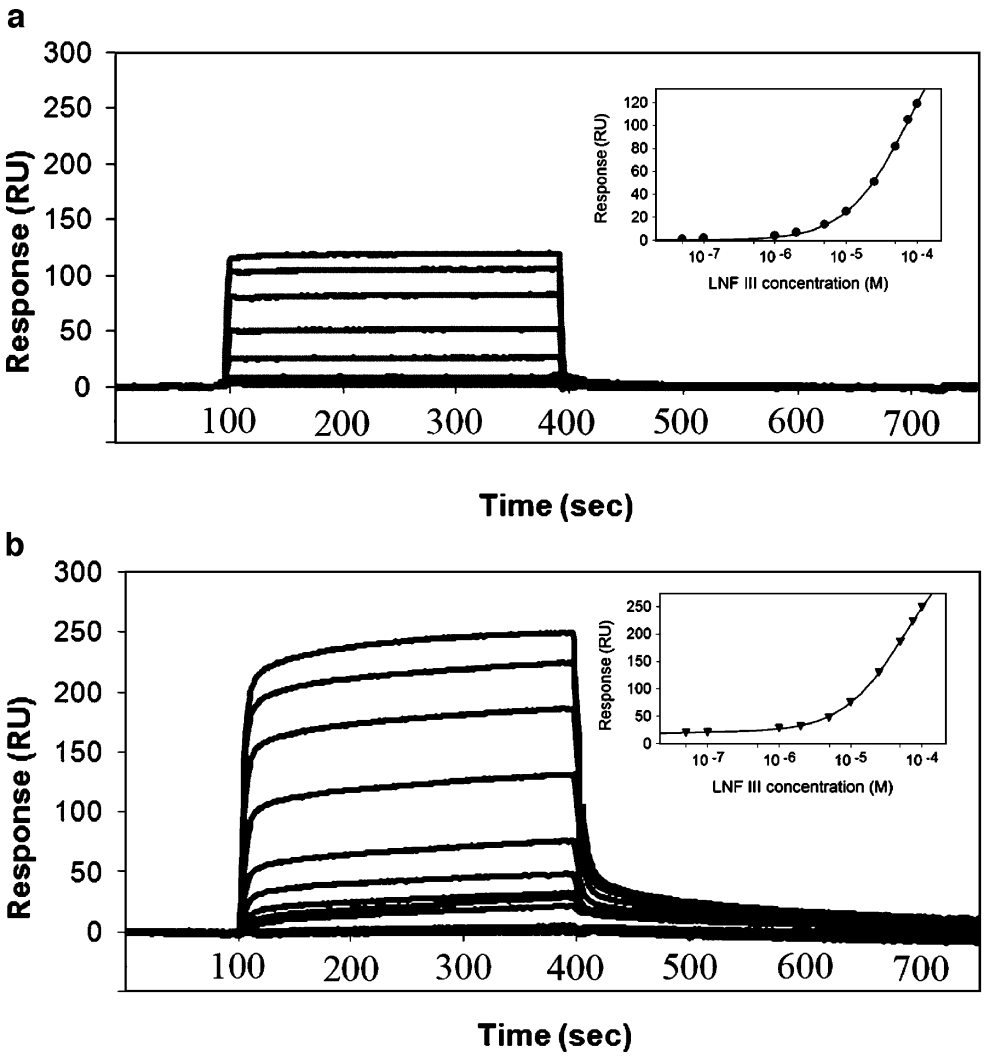

Fig. 5 Biacore sensorgrams showing titration with increased concentrations of LNF III over immobilized **a** nAAL and **b** rAAL at 25°C. The amount of immobilized nAAL was 9,000 RU and 11,000 RU for rAAL. The LNF III concentrations used were (from bottom to top curve) 0.05, 0.1, 1, 2, 5, 10, 25, 50, 75, and 100 μM. Injection time was 300 s at a flow rate of 5 μl/ml. *Inset* in **a** show the steady state analysis of the interaction of LNF III with sensor-bound nAAL. ΔRU values determined from the steady state plateau region were plotted as a function of analyte concentration, and the data were fit by nonlinear regression according to the single site Langmuir binding model. Inset in **b** show the steady state analysis of the interaction of LNF III with sensor-bound rAAL. ΔRU values derived from the end of the analyte injection phase were plotted as a function of analyte concentration, and the data were fit by nonlinear regression according to the two-site Langmuir binding model. The K_d values derived from these data are given in Table 2

Table 2 Equilibrium dissociation constants (K_d) for the interaction of fucose or fucose-containing oligosaccharides with nAAL or rAAL measured by SPR

Ligand	K_d nAAL (μM)	K_{d1} rAAL (μM)	K_{d2} rAAL (nM)	After 100 mM fucose injection		
				K_d nAAL (μM)	K_{d1} rAAL (nM)	K_{d2} rAAL (nM)
L-fucose	15.0±5	22.4±5	3.8±2	–[a]	–[a]	–[a]
LNF I → Fucα1-2Galβ1-3GlcNAcβ1-4Glc	118±5	128±2	14±1	–[b]	–[b]	–[b]
LNF II → Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc	106±8	92±6	23±7	102±4	119±8	–
LNF III → Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc	77±1	77±2	44±5	120±10	125±5	–
NA2F → -4GlcNAcβ1-4[Fucα1-6]GlcNAc	5.7±1	3.9±2	–	8.1±4	5.9±1	–
LDFT → Fucα1-2Galβ1-4[Fucα1-3]Glc	14.0±3	10.0±5	10±6	–[a]	–[a]	–[a]

The steady state binding values of analytes bound to nAAL were fit by nonlinear regression according to the single site Langmuir binding model. The steady state binding values of analytes bound to rAAL were fit by nonlinear regression according to the two-site Langmuir binding model except for NA2F where single site Langmuir binding model had to be used. For the experiments with fucose injected prior to the oligosaccharide injections the steady state binding values of analytes bound to both nAAL and rAAL were fit by nonlinear regression according to the single site Langmuir binding model. The standard deviations of the fit are shown for each K_d value

[a] Not determined

[b] Affinity data could not be obtained

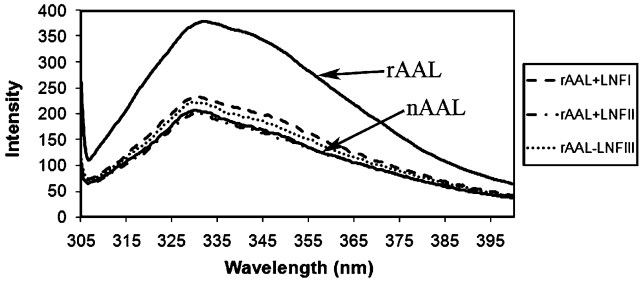

Fig. 6 Tryptophan fluorescence spectra of nAAL, rAAL (*solid lines*) and of rAAL with different oligosaccharides at saturating concentrations (*dashed lines*, as indicated in the figure) at 25°C. The lectin concentration was 0.2 μM in all experiments

revealed approximate affinities for all three oligosaccharides in the range of 5–50 nM. As an example the adsorption isotherm for LNF III is shown in Fig. 7. An optimal fit of the adsorption isotherm was obtained at an available binding site concentration of 0.02 μM. The K_d value obtained from the tryptophan fluorescence experiments was 20 nM which is in excellent agreement with the K_d value of 44 nM which was obtained from surface Plasmon analysis, considering the difficulties to determine accurate K_d values in the low nanomolar range [23]. Thus the striking similarity between K_d values determined by fluorescence measurements and by surface plasmon resonance analysis strongly supports the presence of a high affinity binding site in rAAL.

From the crystal structure of nAAL, site 2 seems to be the strongest binding site generating seven hydrogen-bonds to fucose [8]. This site also has a cluster of three tryptophans making it highly possible that oligosaccharide binding to this site is detected in the tryptophan fluorescence experiments, whereas oligosaccharide binding to the other fucose-binding sites in AAL does not generate a change in tryptophan fluorescence. This would be consistent with that this site is also the high-affinity site detected in the surface plasmon resonance analyses, that it does not bind NA2F and that it is blocked or inactivated in nAAL.

Hemagglutination studies

Hemagglutination assays showed that rAAL agglutinated erythrocytes at a lower concentration than nAAL (2.5 μg/ml and 5.0 μg/ml, respectively, Table 3). The lower hemagglutinating concentration of rAAL compared to nAAL indicated that there are high-affinity binding sites available on rAAL that are not accessible on nAAL.

Interestingly, the minimum inhibiting concentration (MIC) of fucose was 0.4 mM for both nAAL and rAAL, and when a difucosylated oligosaccharide (LDFT) was used the MIC-value was 0.8 mM for both lectins. The observed MIC value of fucose was similar to previously reported values for nAAL [5]. Glucose was used as a negative control and did not inhibit agglutination in concentrations up to100 mM. However, these data does not exclude the presence of a high affinity binding site in rAAL. There are several potential binding sites in AAL that may be involved in the hemagglutination reaction and these sites have been shown to differ in affinity for fucose [5, 8, 14]. A possible explanation for the similar MIC-values between nAAL and rAAL is that the two high-affinity binding sites are present in such close proximity in the dimeric form of the lectin [8] that involvement of both high-affinity sites in crosslinking of erythrocytes is impossible. Thus crosslinking and subsequent hemagglutination with rAAL will always be the result of interaction with at least one low-affinity binding site.

Role of free fucose in purified nAAL and rAAL

Crystallization studies of nAAL have shown the presence of L-fucose in some of the binding sites (site 1, site 2 and site 4), also after extensive dialysis, indicating that free fucose is bound with relative high affinities to these sites [8, 14]. Since all commercial nAAL preparations are purified using fucose-starch column chromatography it is possible that commercial nAAL contain bound fucose that have not been removed during dialysis potentially blocking the high affinity binding site. To investigate this hypothesis, monosaccharide analysis was performed on nAAL and rAAL. The monosaccharide analysis showed that nAAL contained fucose at a molar ratio of 2:1 (L-fucose-protein) whereas L-fucose could not be detected in the rAAL preparation (data not shown).

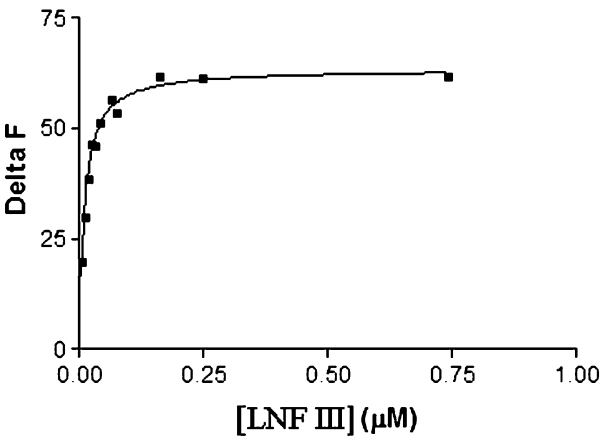

Fig. 7 Fluorescence quenching titration of rAAL upon binding to LNF III at 25°C. The data were fit to the hyperbolic equation defined in material and methods using non-linear regression. The calculated K_d value for LNF III was 20 nM

Table 3 Hemagglutination analyses

Lectin	Minimum hemagglutinating lectin concentrations	Minimum inhibiting fucose concentration	Minimum inhibiting LDFT concentration	Minimum inhibiting glucose concentration
rAAL	2.5 μg/ml	0.4 mM	0.8 mM	No inhibition
nAAL	5 μg/ml	0.4 mM	0.8 mM	No inhibition

To investigate whether bound L-fucose could affect binding of nAAL to fucosylated oligosaccharides rAAL was incubated with L-fucose and then dialyzed for 2 days against 2×2 l PBS, before ELLA analysis. The analysis showed that fucose treated-rAAL bound less efficiently to all tested fucosylated oligosaccharides compared to non-treated rAAL (Fig. 8). In the experiment we used a 3 ml solution of 50 mM fucose and 1.5 μM rAAL. During the dialysis the fucose will be diluted to an approximate concentration of 0.1 μM. The fact that this concentration gives a decreased signal from rAAL in the ELLA is consistent with a K_d value below 1 μM between rAAL and L-fucose.

To further analyze whether free L-fucose could block subsequent high-affinity binding of fucosylated oligosaccharides, surface plasmon resonance studies were performed where a pulse of 100 mM fucose was injected prior to the oligosaccharide injections. Affinity analysis of LNF II, LNF III and NA2F using Langmuir binding isotherms showed data that fitted to a 1:1 binding model with no detection of a high-affinity binding site. The observed affinities for fucose-treated rAAL was similar to the data obtained with fucose treated nAAL (Table 2). This indicates that free fucose blocks the high affinity binding site in rAAL and thereby makes it unable to bind to the fucosylated oligosaccharides. Data obtained with LNF I after fucose treatment was inconclusive, and may indicate that binding to LNF I is different from the other oligosaccharides.

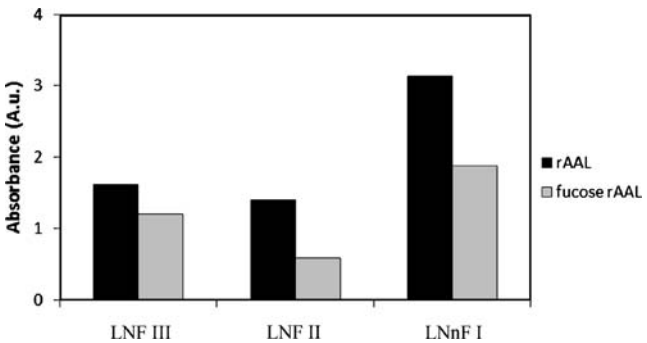

Fig. 8 ELLA comparing the binding capacity between rAAL and rAAL pre-treated with fucose. Bars represent mean values from triplicate analysis. Figure shows representative results from one of three experiments

Although it is possible that free L-fucose blocks the high affinity binding site in nAAL it is worth noting that binding of oligosaccharides to nAAL or fucose treated rAAL immobilized to the biosensor chip did not improve even after extensive washing with injection buffer. This indicates that regeneration of the high affinity binding site from commercial nAAL may not be possible. Perhaps binding of free L-fucose to the high affinity binding site permanently alters the structure in this site so that the high affinity binding site can not be regenerated.

Conclusion

Detection of carbohydrate epitopes has become increasingly important in the development of techniques to identify disease-specific glycosylation. Furthermore reliable glycosylation detection methods for quality control of recombinantly produced glycoproteins for use in therapeutics are needed. The use of monoclonal antibodies for detecting carbohydrates often suffers from low affinity. Production of recombinant lectins offers new possibilities to produce large quantities of lectins and also to use recombinant technology to study and modify binding characteristics and potentially produce specific high-affinity carbohydrate binding reagents. Using two independent methods (SPR and tryptophan fluorescence) we have identified a high affinity binding site in the recombinant form of AAL with affinities in the nanomolar range towards fucose and fucosylated oligosaccharides. In contrast to the other binding sites in the molecule this high-affinity binding site has a somewhat more restricted specificity and does not seem to bind oligosaccharides with α1-6 linked fucose such as NA2F. This high affinity binding site is not observed in nAAL, a partial explanation may be that this site is blocked by free fucose in commercial preparations of nAAL.

Further studies using mutational and crystallization studies will be needed to fully determine the fine details in the specificity of the different binding sites of rAAL. Knowledge of the differences in binding specificity in the different binding sites of rAAL would be important in understanding the basis of protein carbohydrate recognition and to use rAAL as a molecular scaffold to alter binding specificity by protein engineering.

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