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Comparative analysis of oligosaccharide specificities of fucose-specific lectins from *Aspergillus oryzae* and *Aleuria aurantia* using frontal affinity chromatography

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ABSTRACT

Aleuria aurantia lectin (AAL) is widely used to estimate the extent of α1,6-fucosylated oligosaccharides and to fractionate glycoproteins for the detection of specific biomarkers for developmental antigens. Our previous studies have shown that *Aspergillus oryzae* lectin (AOL) reflects the extent of α1,6-fucosylation more clearly than AAL. However, the subtle specificities of these lectins to fucose linked to oligosaccharides through the 2-, 3-, 4-, or 6-position remain unclear, because large amounts of oligosaccharides are required for the systematic comparative analysis using surface plasmon resonance. Here we show a direct comparison of the dissociation constants (K_d) of AOL and AAL using 113 pyridylaminated oligosaccharides with frontal affinity chromatography. As a result, AOL showed a similar specificity as AAL in terms of the high affinity for α1,6-fucosylated oligosaccharides, for smaller fucosylated oligosaccharides, and for oligosaccharides fucosylated at the reducing terminal core GlcNAc. On the other hand, AOL showed 2.9–6.2 times higher affinity constants (K_a) for α1,6-fucosylated oligosaccharides than AAL and only AAL additionally recognized oligosaccharides which were α1,3-fucosylated at the reducing terminal GlcNAc. These results explain why AOL reflects the extent of α1,6-fucosylation on glycoproteins more clearly than AAL. This systematic comparative analysis made from a quantitative viewpoint enabled a clear physical interpretation of these fucose-specific lectins with multivalent fucose-binding sites.

α-L-Fucopyranosyl residues are widely distributed in cell-surface sugar chains and often play important roles in biological phenomena [1,2]. Increased levels of fucosyl residues and changes in fucosylation patterns as a result of different expression levels of various fucosyltransferases act as specific biomarkers for developmental antigens, particularly in inflammatory processes and in various cancers [3,4]. In particular, the α1,6-fucosylated oligosaccharide content of both liver and serum glycoproteins is elevated during the development of malignant liver diseases because the activity of α1,6-fucosyltransferase is increased [5]. Meanwhile, lectins are specific carbohydrate-binding or carbohydrate-crosslinking proteins and some of them have particular value as specific probes for investigating the distribution, structure, and biological function of carbohydrate chains on the cell surface because of their specificity for defined carbohydrate structures [6,7]. *Aleuria aurantia* lectin (AAL)[1] is a commercially available lectin that is known for its high affinity for α1,6-fucosylated oligosaccharides [8], and it is widely used to estimate the extent of α1,6-fucosylation on glycoproteins and to fractionate glycoproteins [9]. However, AAL also exhibits broad specificity for α1,2-, α1,3-, and α1,4-fucose-containing oligosaccharides [10]. Therefore, another lectin that recognizes α1,6-fucosylated oligosaccharides would be a valuable tool in glycobiology research because only a few lectins have been identified which are specific for the α1,6-fucosyl residue. We have identified a unique lectin (AOL) from the filamentous fungus, *Aspergillus oryzae* [11]. AOL is predicted to have a similar sixfold β-propeller structure containing multivalent carbohydrate recognition sites as AAL from the alignment of their amino acid sequence repeats [10]. Our previous studies have shown that AOL recognizes the α1,6-fucosylation on glycoproteins more specifically than AAL using surface plasmon resonance (SPR) analysis, lectin affinity chromatography, lectin blot analysis, and immunocytochemical staining [12], so that AOL was recently used to estimate the extent of α1,6-fucosylation on glycoproteins and to fractionate glycoproteins [13–16]. However, their subtle specificities to fucose linked in oligosaccharides through the 2-, 3-, 4-, or 6-position remain unclear because large amounts of oligosaccharides are required for the systematic comparative analysis using SPR. Furthermore, because AOL and

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[1] *Abbreviations used:* AAL, *Aleuria aurantica* lectin; AOL, *Aspergillus oryzae* lectin; FAC, frontal affinity chromatography; SPR, surface plasmon resonance; PA, pyridylaminated; pNP, *p*-nitrophenyl.

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AAL display additive multivalent carbohydrate recognition which is reflected in the multivalent fucose-binding sites with different affinities for fucose, it is difficult to analyze individual fucose-binding sites. Therefore, a systematic comparative analysis made from a quantitative viewpoint is required for a clear physical interpretation of these lectins with multivalent fucose-binding sites. In this study, by combining 113 pyridylaminated (PA) oligosaccharides with a high-throughput system for frontal affinity chromatography (FAC), we have directly compared the dissociation constants (K_d) values of AOL and AAL. Thus we have completed a more detailed analysis of oligosaccharide-binding affinity and specificity of this lectin family. The information of affinity and specificity obtained here should be helpful for understanding why these lectins show the highest specificity for α1,6-fucosylation within a diversified group of oligosaccharides and why AOL exhibits a higher degree of selectivity for the detection of the α1,6-fucosyl residue than AAL.

Materials and methods

Materials

p-Nitrophenyl α-L-fucopyranoside (Fuc-α-pNP) was purchased from Funakoshi (Tokyo, Japan). PA oligosaccharides used in this study are listed in Supplementary Figs. S1 and S2. PA-*N*-linked oligosaccharides numbered 001–014, 103, 105, 107, 108, 307, 313, 314, 323, 405, 410, 418, 419, 420, and 503 were from Takara Bio. (Kyoto, Japan); and the others were purchased from Seikagaku Corporation. Glycolipid-type oligosaccharides numbered 701–703, 705–713, 715–721, 724, 726, and 728–731 were obtained from Takara Bio. The sources of nonlabeled oligosaccharides were as follows: 727 from Funakoshi Co.; 733, 734, and 908 from Dextra Laboratories, Ltd. (Reading, UK); 725, 909, 910, 931, 932, and 933 from Calbiochem (San Diego, CA); and 906 and 907 from Seikagaku Corporation. Oligo-lactosamines 901, 902, 903, and 905 and milk oligosaccharides 722, 723, 732, and 735–739 were generous gifts from K. Yoshida (Seikagaku Corporation) and from T. Urashima (Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan), respectively. The nonlabeled oligosaccharides were pyridylaminated with GlycoTag (Takara Bio) before use.

Horseradish peroxidase (Sigma; St. Louis, MO) was subjected to hydrazinolysis to liberate *N*-oligosaccharide with Hydraclub (J-OIL MILLS; Yokohama, Japan). After pyridylamination, the derived oligosaccharide 017 was purified by RP-HPLC on a PALPACK Type-R column (Takara Bio) using 10 mM ammonium acetate buffer, pH 4.0, containing 0.1% (v/v) *n*BtOH, at 30 °C. Oligosaccharides 937 and 938 were obtained by digestion of 015 (72.5 pmol) and 017 (300 pmol), respectively, with jack bean α-mannosidase (0.2 and 0.6 U, respectively) (GLYKO; Novato, CA). Among them, 938 (160 pmol) was digested with β1,2-xylosidase (0.2 U) (Calbiochem) to obtain 939. Oligosaccharides 940 and 941 were obtained by further digestion with β-mannosidase (6 mU) from 939 and 937, respectively. Each oligosaccharide (937, 938, 939, 940, and 941) was purified by RP-HPLC as described above.

Preparation of lectin columns

Recombinant AOL was purified according to the methods as described previously [12]. AAL was purchased from Vector Laboratories (Peterborough, UK). We prepared lectin-immobilized columns, according to the methods described previously [17]. Briefly, AOL and AAL were dissolved in 10 mM phosphate buffer, pH 7.4, containing 0.7% NaCl coupled to NHS-activated Sepharose 4FF (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). The amount of immobilized protein was determined by measuring the amount of uncoupled protein in the above wash fraction by the method of Bradford [18]. The slurry was packed into a capsule-type miniature column (φ 2 × 10 mm, 31.4 μL) and then connected to the FAC-1 machine (Shimadzu, Kyoto, Japan).

FAC analysis

FAC analysis was carried out as described previously [19]. Frontal affinity chromatography was performed using a machine for automated FAC (FAC-1) [20]. Lectin columns were equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 0.8% NaCl (TBS) and the flow rate and the column temperature were kept at 0.125 mL/min and 25 °C, respectively. The *N*-oligosaccharides (2.5 nM), glycolipid-type oligosaccharides, and others (5.0 nM) were dissolved in TBS, and an excess volume (0.5–1.0 mL) of each oligosaccharide solution was successively injected into a pair of lectin columns by an autosampling system. Elution of pNP glycosides and PA oligosaccharide was monitored by measuring UV (280 nm) and fluorescence (ex/em = 310/380 nm), respectively.

When the amount of the applied analyte molecules exceeded the retention ability of the column, leakage occurred and the concentration of oligosaccharides in the elution reached a plateau, where the concentration was equal to that of the initial solution. The volume of elution front of each PA oligosaccharide (V) was determined as described previously [19]. The elution volume of each PA oligosaccharide, which has no affinity to the lectins (V_0), was determined using PA lactose. The total amount of immobilized lectin in the column, B_t, is first determined by Fuc-α-pNP using the equation described previously, where K_d is the dissociation constant between interacting biomolecules, B_t is the total amount of immobilized ligand, $[A]_0$ is the initial concentration of the PA oligosaccharide (A), V is the elution volume of A, and V_0 is the elution volume of PA lactose, which has no affinity toward the lectins.

$$[A]_0(V - V_0) = B_t K_d (V - V_0) \quad (1)$$

$[A]_0$, the initial concentration of the PA oligosaccharides, was 10 nM, which was small compared with K_d. Thus we used the following equation to obtain the values of K_d of each lectin toward a PA oligosaccharide:

$$V - V_0 = B_t / K_d \quad (2)$$

Evaluation of lectin columns

For the determination of effective ligand content, B_t, a concentration-dependent analysis and subsequent Woolf-Hofstee-type plot were performed as described previously [19]. Here, various concentrations ($[A]_0$) of Fuc-α-pNP dissolved in TBS were applied to the miniature column, and the elution was monitored by the absorbance at 280 nm. Woolf-Hofstee-type plots, i.e., $(V - V_0)$ vs $(V - V_0)[A]_0$, were made to determine B_t and K_d values from the intercept and the slope, respectively, of the fitted curve.

Results

Evaluation of the lectin columns

To evaluate the prepared columns, it was necessary to determine the effective ligand content (B_t) based on concentration-dependent analysis [19,21]. The amounts of immobilized AOL and AAL were determined to be 1.9 and 0.5 mg/mL gel, respectively. Since AOL and AAL are fucose-specific lectins, Fuc-α-pNP is a useful substrate for evaluating the affinity of the lectins to the columns. A concentration-dependent analysis of AOL and AAL was performed with Fuc-α-pNP at various concentrations ranging from 1.5 to 20 μM and from 3.0 to 20 μM, respectively (Fig. 1).