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Journal of Chromatography A, 890 (2000) 261–271

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Reinforcement of frontal affinity chromatography for effective analysis of lectin–oligosaccharide interactions

Jun Hirabayashi*, Yoichiro Arata, Ken-ichi Kasai

Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-0195, Japan

Received 7 March 2000; received in revised form 1 May 2000; accepted 1 May 2000

Abstract

Frontal affinity chromatography is a method for quantitative analysis of biomolecular interactions. We reinforced it by incorporating various merits of a contemporary liquid chromatography system. As a model study, the interaction between an immobilized *Caenorhabditis elegans* galectin (LEC-6) and fluorescently labeled oligosaccharides (pyridylaminated sugars) was analyzed. LEC-6 was coupled to *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow (100 μm diameter), and packed into a miniature column (e.g., 10×4.0 mm, 0.126 ml). Twelve pyridylaminated oligosaccharides were applied to the column through a 2-ml sample loop, and their elution patterns were monitored by fluorescence. The volume of the elution front (V) determined graphically for each sample was compared with that obtained in the presence of an excess amount of hapten saccharide, lactose (V_0); and the dissociation constant, K_d, was calculated according to the literature [K. Kasai, Y. Oda, M. Nishikawa, S. Ishii, J. Chromatogr. 376 (1986) 33]. This system also proved to be useful for an inverse confirmation; that is, application of galectins to an immobilized glycan column (in the present case, asialofetuin was immobilized on Sepharose 4 Fast Flow), and the elution profiles were monitored by fluorescence based on tryptophan. The relative affinity of various galectins for asialofetuin could be easily compared in terms of the extent of retardation. The newly constructed system proved to be extremely versatile. It enabled rapid (analysis time 12 min/cycle) and sensitive (20 n*M* for pyridylaminated derivatives, and 1 μg/ml for protein) analyses of lectin–carbohydrate interactions. It should become a powerful tool for elucidation of biomolecular interactions, in particular for functional analysis of a large number of proteins that should be the essential issues of post-genome projects.

Keywords: Frontal chromatography; Affinity adsorbents; Dissociation constants; Lectins; Oligosaccharides; Galectins

1. Introduction

Affinity chromatography is extremely powerful for not only preparative but also analytical purposes. For the latter, frontal affinity chromatography (FAC) was established by Kasai and Ishii, a method that enables the determination of the dissociation constant (K_d) between an analyte (A) present in a solution and an immobilized ligand (B) (for review, see Ref. [1]). It proved to be very useful for analysis of a variety of biomolecular interactions, such as enzyme–substrate analog [2,3], and lectin–oligosaccharides [4–6]. Other techniques applicable to analysis of biomolecular interactions, such as affinity capillary electrophoresis [7,8] and a method using biosensors based on surface plasmon resonance have emerged [9,10]. A most distinct feature of FAC is that an excess volume of the analyte solution is continuously

*Corresponding author. Tel.: +81-426-85-3741; fax: +81-426-85-3742.

E-mail address: j-hira@pharm.teikyo-u.ac.jp (J. Hirabayashi).

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00545-8

applied to a column packed with an affinity adsorbent mostly based on agarose, to which ligand B is immobilized. This procedure is very easy to perform. The theory is straightforward, essentially the same as that of the Michaelis–Menten equation [1]. From a contemporary viewpoint, however, FAC has a few drawbacks; it is time-consuming, requires a relatively large amount of analyte, and has not been automated. In a previous study, for example, more than 100 ml of a protein solution (5 μg/ml) was applied to a relatively large affinity adsorbent column (bed volume, a few ml), and one run took several hours [6].

Since we felt keenly the importance of reinforcing this method, we sought to improve it by the following means: (i) use of mechanically stable chromatographic media for packing. For this purpose, *N*-hydroxysuccinimide (NHS)-activated Sepharose 4 Fast Flow (100 μm in diameter, Pharmacia Biochemicals) was used. (ii) Adoption of fluorescence-based detection for raising the sensitivity. In the present study, either fluorescent sugars {pyridylaminated (PA) oligosaccharides; 320 nm and 400 nm for excitation and emission, respectively; [11]} or lectin proteins were used as analytes. In the latter case, fluorescence based on tryptophan (285 nm and 350 nm for excitation and emission, respectively) was used for detection. (iii) Design of a simplified application system of analyte solution by using a relatively large sample loop (2–5 ml), and the use of miniature columns (e.g., 10×4.0 mm; bed volume, 0.126 ml, and 35×4.6 mm; bed volume, 0.582 ml).

Since we have been interested in the specific binding ability of lectins, we firstly applied this newly reinforced system to the study of galectins, galactose-binding lectins, from the nematode *Caenorhabditis elegans*. Galectins are a group of soluble, metal-independent animal lectins, the binding specificity of which is maintained for galactose through their evolutionarily conserved carbohydrate-recognition domains (CRDs) [12]. We previously identified two types of galectins from *C. elegans*. One is an M_r 32 000 galectin (renamed LEC-1) consisting of a single polypeptide chain containing two homologous carbohydrate-recognition domains [13], and the other is an M_r 32 000 galectin (LEC-6) which exists as a dimer of two identical M_r 16 000 subunits [14]. More recently, many other *C. elegans* galectin genes have been found as a result of the genome project [15]. Almost all galectins studied so far have shown preferential binding to disaccharide units *N*-acetyllactosamine (LacNAc; Galβ1-4GlcNAc) and its linkage isomer, lacto-*N*-biose (Galβ1-3GlcNAc). Three OH groups are known to be important for carbohydrate recognition by mammalian galectins, i.e., the 4-OH and 6-OH groups of galactose, and the 3-(4)-OH group of GlcNAc [12,16–20]. However, the sugar-binding properties of *C. elegans* galectins have not been extensively studied. Therefore, we attempted to elucidate the nature of molecular recognition by *C. elegans* galectins by the newly reinforced FAC. As the first target, we chose LEC-6.

We took two approaches: first, we analyzed the behavior of various PA-oligosaccharides on an immobilized LEC-6 column. Recombinant LEC-6 was produced in *Escherichia coli* as a fusion protein with *E. coli* β-galactosidase α-peptide (43 amino acids). This is to facilitate coupling to NHS-activated Sepharose, because LEC-6 has originally only one lysine residue, whereas the α-peptide has three amino groups available for immobilization. Next, we used an opposite system; analysis of the behavior of galectins on an immobilized sugar column. For this, we prepared a column of asialofetuin-Sepharose 4 Fast Flow, and the elution profile of various recombinant *C. elegans* galectins was analyzed. The newly reinforced system proves to be very simple and useful for rapid and sensitive FAC analysis.

2. Materials and methods

2.1. Materials

Twelve PA-oligosaccharides, 01, 21, 22, 23, 26, 27, 28, 40, 41, 42, 45, 47 (for structures, see Table 1; the numbers denote commercially assigned ones, and will be used throughout in this and future works for the sake of unity) and PA-rhamnose were purchased from Takara Biomedicals (Tokyo, Japan). Bovine serum albumin (BSA) and *p*-aminophenyl-β-lactoside were from Sigma. Stainless steel empty columns (10×4.0 mm; bed volume, 0.126 ml, and 35×4.6 mm; 0.582 ml) were obtained from GL Sciences (Tokyo, Japan); and polyether ether ketone

Table 1
Structure of PA-oligosaccharides used in this study and the obtained dissociation constants (K_d's) for LEC-6

No.	Common name	Structure	V (ml)	$V-V_0$ (ml)	K_d for LEC-6 (M)	Relative affinity[a]
01	Biantennary complex	Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3$^{Man\beta R^b}$	0.92	0.65	$4.2\cdot10^{-5}$	6.4
21	Monosialylated biantennary complex	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3$^{Man\beta R^b}$	0.38	0.11	$2.5\cdot10^{-4}$	1.1
22	Monosialylated biantennary complex	Galβ1-4GlcNAcβ1-2Manα1-6 NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3$^{Man\beta R^b}$	0.35	0.08	$3.2\cdot10^{-4}$	0.83
23	Disialylated biantennary complex	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6 NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3$^{Man\beta R^b}$	0.29	0.02	$1.4\cdot10^{-3}$	0.2
26	Asialo GM3 (lactose)	Galβ1-4Glc-PA	0.34	0.07	$3.9\cdot10^{-4}$	0.69
27	Asialo GM2	GalNAcβ1-4Galβ1-4Glc-PA	0.31	0.04	$6.9\cdot10^{-4}$	0.39
28	Asialo GM1	Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA	0.37	0.10	$2.7\cdot10^{-4}$	0.98
40	Forssman pentasaccharide	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc-PA	0.32	0.05	$5.5\cdot10^{-4}$	0.49
41	Lacto-*N*-neotetraose	Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA	0.43	0.16	$1.7\cdot10^{-4}$	1.6
42	Lacto-*N*-tetraose	Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	0.53	0.26	$1.1\cdot10^{-4}$	2.6
45	Lacto-*N*-fucopeptaose III	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-PA Fucα1-3	0.31	0.03	$9.1\cdot10^{-4}$	0.29
47	A-Hexasaccharide	GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA Fucα1-3	0.33	0.06	$4.6\cdot10^{-4}$	0.59

[a] Relative affinity calculated on the basis of K_d for *p*-aminophenyl-β-lactoside.
[b] R=1-4GLcNAcβ1-4GlcNAc-PA.

(PEEK) sample loops (2 ml and 5 ml; 0.75 mm I.D.), from Rheodyne. NHS-activated Sepharose 4 Fast Flow was from Pharmacia Biochemicals. All other chemical reagents were of analytical grade.

2.2. Production of recombinant galectins

Recombinant LEC-6 as a fused protein with *E. coli* β-galactosidase α-peptide (LEC-6α) was prepared as described previously [14]. Production and purification of recombinant intact LEC-6 was performed by utilizing a recently developed expression system in fission yeast [21]. Another *C. elegans* galectin, LEC-1 (previously designated M_r 32 000 galectin) was produced in *E. coli* and purified as described previously [6]. An LEC-1 mutant, LEC-1/K68E, was obtained in the course of polymerase-chain reaction amplification of LEC-1 cDNA by a spontaneous mismatch incorporation (AAA originally encoding for Lys68 was substituted by GAA for glutamic acid; underlines denote the mutated position). The mutant cDNA was cloned in pET21a, expressed in *E. coli*, and purified by asialofetuin-agarose affinity chromatography as well as the parent LEC-1. The expression plasmid for another *C. elegans* galectin, LEC-4 (cosmid identification number, C44F1.3 [15]) was constructed by using the expression vector pET21a essentially similarly as for LEC-1.

2.3. Preparation of affinity adsorbents

For immobilization of LEC-6α, 11 mg of the recombinant protein was dissolved in 3 ml of 0.1 *M* sodium phosphate, pH 7.0, containing 0.1 *M* lactose, and was reacted with 1.4 ml of NHS-activated Sepharose 4 Fast Flow at 22°C for 3 h. The remaining activated groups were blocked with an excess amount of monoethanolamine at 4°C for 16 h. For immobilization of asialofetuin, 71 mg of asialofetuin dissolved in 10 ml of 0.1 *M* sodium phosphate, pH 7.0, was reacted with 6 ml of NHS-

activated Sepharose 4 Fast Flow at 22°C for 3 h. The remaining activated groups were blocked as described above. The resultant adsorbents were extensively washed on a glass filter with 0.5 *M* NaCl, and unreacted proteins in the filtrates were determined by Bradford's method using a Bio-Rad dye reagent.

Each adsorbent was mixed with EDTA-PBS (1 m*M* EDTA, 20 m*M* sodium phosphate, pH 7.2, 150 m*M* NaCl), and the resultant slurry was packed into a stainless steel column (10×4.0 mm for LEC-6α-Sepharose, and 35×4.6 mm for asialofetuin-Sepharose).

2.4. Operation of frontal affinity chromatography

The present FAC system consisted of a Tosoh CCPD pump, a Tosoh FS8000 fluorescence detector, and a Shimadzu CR-5A integrator. Sample solutions were injected through a Rheodyne 7125 injector equipped with either 2- or 5-ml PEEK sample loops. Prior to injections, the solvent contained in the loop was completely purged by air injection in order to avoid partial dilution of the sample. Sample solutions were sufficiently degassed immediately before application.

2.5. Principle of the analysis

The basic equation of FAC, Eq. (1), has been described [1–3], where K_d is the dissociation constant between interacting biomolecules, A (analyte) and B (immobilized ligand), B_t is the ligand content (mol) of the column, $[A]_0$ is the initial concentration (M) of A, V is the elution volume (ml) of A, and V_0 is that obtained in the case there is no interaction between A and B:

$$K_d = \frac{[A][B]}{[AB]} = \frac{B_t}{V - V_0} - [A]_0 \quad (1)$$

Eq. (1) is further simplified, if $[A]_0$ is small enough relative to K_d, as follows:

$$K_d = \frac{B_t}{V - V_0}, \text{ if } [A]_0 \ll K_d \quad (2)$$

For the determination of B_t and K_d, Eq. (1) is modified (Eq. (3)), and from a double reciprocal plot ($1/[A]_0(V - V_0)$ vs. $1/[A]_0$) using various $[A]_0$, B_t and K_d were obtained from the intercepts on the ordinate and abscissa, respectively:

$$\frac{1}{[A]_0(V - V_0)} = \frac{K_d}{B_t} \cdot \frac{1}{[A]_0} + \frac{1}{B_t} \quad (3)$$

Elution volume of the analyte (V) was determined graphically as the volume corresponding to the eluting point, at the half value of the ''plateau'' of the elution curve. V_0 was determined as either V of an appropriate control sample devoid of affinity for the immobilized ligand, or V obtained in the presence of excess amount of inhibitor sugar. In the case of monitoring the elution of PA-oligosaccharides from the LEC-6α column, V of the oligosaccharide ''01'' in the presence of 20 m*M* lactose was taken as V_0, while in the case of monitoring protein elution from the asialofetuin column, V of BSA was regarded as V_0.

3. Results and discussion

3.1. System construction

Two affinity adsorbents were prepared for the present work: (i) LEC-6α-Sepharose 4 Fast Flow, and (ii) asialofetuin-Sepharose 4 Fast Flow. The former was used for application of PA-oligosaccharides, whereas the latter was used for that of recombinant galectins. These adsorbents were packed into a miniature column, and they were linked to a conventional high-performance liquid chromatography (HPLC) system (Fig. 1). The system consisted of a pump (Tosoh CCPD), a fluorescence detector (Tosoh FS8000), and an integrator (Shimadzu CR-5A). Major points for the present improvement include (i) use of a relatively large sample loop (2 or 5 ml) to enable application of an excess volume of analyte solution to the column (0.126 ml), and (ii) monitoring of the analyte elution by fluorescence. For the detection of PA-oligosaccharides, excitation and emission wavelengths of 320 nm and 400 nm, respectively, were used; whereas for that of lectins, the respective wavelengths were 285 nm and 350 nm. PA-oligosaccharides were detectable at a concentration as low as 10 n*M*, when the FS8000 detector was used. In the present study, the

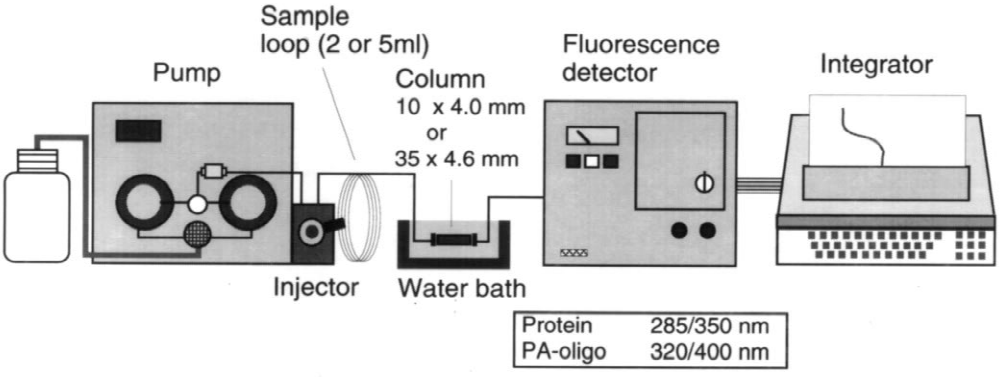

Fig. 1. Outline of the reinforced system for FAC. Principal components of this system are (i) a relatively large sample loop, (ii) a small column, and (iii) a fluorescence detector.

concentration of PA-oligosaccharides was settled at 20 n*M*. To maintain equilibrium between the stationary phase and the mobile phase, a flow-rate of 0.25 ml/min was adopted. In fact, there was no substantial difference in chromatograms and *V* values obtained at different flow-rates, i.e., 0.1 ml/min and 0.25 ml/min, when the combination of the LEC-6α-Sepharose 4 Fast Flow column and PA-oligosaccharide solutions was used (data not shown). Even at this flow-rate, only 12 min was required for each FAC analysis. However, care should be taken for macromolecules like proteins to maintain the equilibrium, because of their much slower diffusion. Columns were subjected to repeated (>10 times) analyses with no special regeneration procedures. For storage of the LEC-6α column, the elution buffer was substituted with EDTA-PBS containing 0.1% (w/v) BSA and 20 m*M* lactose, and were kept at 4°C.

3.2. Determination of ligand content, B_t, and dissociation constant, K_d

To obtain K_d values for various PA-oligosaccharides, the immobilized ligand content (B_t) for the prepared LEC-6α column was necessary. For this, *p*-aminophenyl-β-lactoside was applied to the LEC-6α column at various initial concentrations ($0.05 \leqq [A]_0 \leqq 1$ m*M*). Experiments were performed at 22°C at a flow-rate of 0.25 ml/min. V_0 was determined by applying PA-oligosaccharide 01 (''01''; for structure, see Table 1) in the presence of 20 m*M* lactose. According to Eq. (3) described by Kasai et al. [1], $1/[A]_0(V-V_0)$ versus $1/[A]_0$ plot was made (graph not shown; for practical values, see Table 2). The straight-line defined by the equation $y=9.772x+36.54$, where $x=1/[A]_0$ (mM^{-1}) and $y=1/[A]_0(V-V_0)$ (μmol) was derived. Hence, K_d ($2.7\cdot10^{-4}$ *M*) and B_t (27 nmol) were graphically obtained from the intercepts on the abscissa and ordinate, respectively. Hence, B_0 (ligand density) was calculated to be 27 nmol/0.126 ml gel (=164 nmol/ml gel). Since the protein content of the adsorbent suggested that 7.5 mg of LEC-6α was immobilized per ml gel, 58% of the immobilized LEC-6α was considered to retain binding ability (21 000 of the theoretical molecular mass of LEC-6α is assumed). The K_d value ($2.7\cdot10^{-4}$ *M*, at 22°C) obtained for *p*-aminophenyl-β-lactoside was comparable to those values reported for other galectins and lactose/*N*-acetyllactosamine (LacNAc), e.g., Chinese hamster galectin-1 for LacNAc ($K_d=1.6\cdot10^{-4}$ *M*, at 27°C; [22]), bovine

Table 2
Analysis of concentration-dependency of *p*-aminophenyl-β-lactoside on a LEC-6α-Sepharose 4 Fast Flow column

$[A]_0$ (m*M*)	$1/[A]_0$ (mM^{-1})	$V-V_0$ (ml)	$[A]_0(V-V_0)$ (μmol)	$1/[A]_0(V-V_0)$ (μmol^{-1})
0.05	20	0.082	0.0041	244
0.07	14.29	0.092	0.0064	155
0.1	10	0.072	0.0072	139
0.2	5	0.057	0.0114	87.7
0.5	2	0.037	0.0185	54.1
1	1	0.02	0.02	50

galectin-1 for lactose ($K_d = 1.5 \cdot 10^{-4}$ *M*, at 23°C) and LacNAc ($K_d = 4.5 \cdot 10^{-5}$ *M*, at 24°C; [23]).

3.3. Analysis of interaction between PA-oligosaccharides and a LEC-6α column

Results obtained for the interaction between the LEC-6α column and 12 PA-oligosaccharides are shown in Table 1. The first four (''01'', ''21'', ''22'' and ''23'') represent *N*-glycans derived from glycoproteins, whereas the remaining eight (''26''–''28'', ''40''–''42'', ''45'' and ''47'') are oligosaccharides derived from glycolipids. Among them, biantennary complex-type oligosaccharide ''01'' showed the strongest interaction with LEC-6α (V= 0.92 ml, Fig. 2). This is not attributable to non-specific interaction with the adsorbent, because such a retardation was completely lost (V=0.27 ml) when this oligosaccharide was mixed with 20 m*M* lactose and applied to the column. Since the latter value was exactly the same as that of PA-rhamnose, which did not show any affinity for LEC-6α (data not shown), the V value of 0.27 ml was regarded as V_0 throughout the series of experiments. Based on this assumption, K_d for each oligosaccharide was calculated (Table 1). For the sake of simplicity, however, affinity for these saccharides is discussed hereafter in terms of affinity relative to that for *p*-aminophenyl-β-lactoside (Table 1). We found that if either of the two non-reducing terminal galactose residues of the oligosaccharide ''01'' (relative affinity, 6.4) was masked with α2-6NeuAc (*N*-acetylneuraminic acid), the affinity for these saccharide derivatives was considerably reduced (1.1 for ''21'', and 0.83 for ''22''), becoming almost comparable to that for *p*-aminophenyl-β-lactoside. Moreover, the affinity for these branched saccharides was nearly completely lost when both non-reducing terminal galactosyl residues were modified with α2-6NeuAc (''23'';

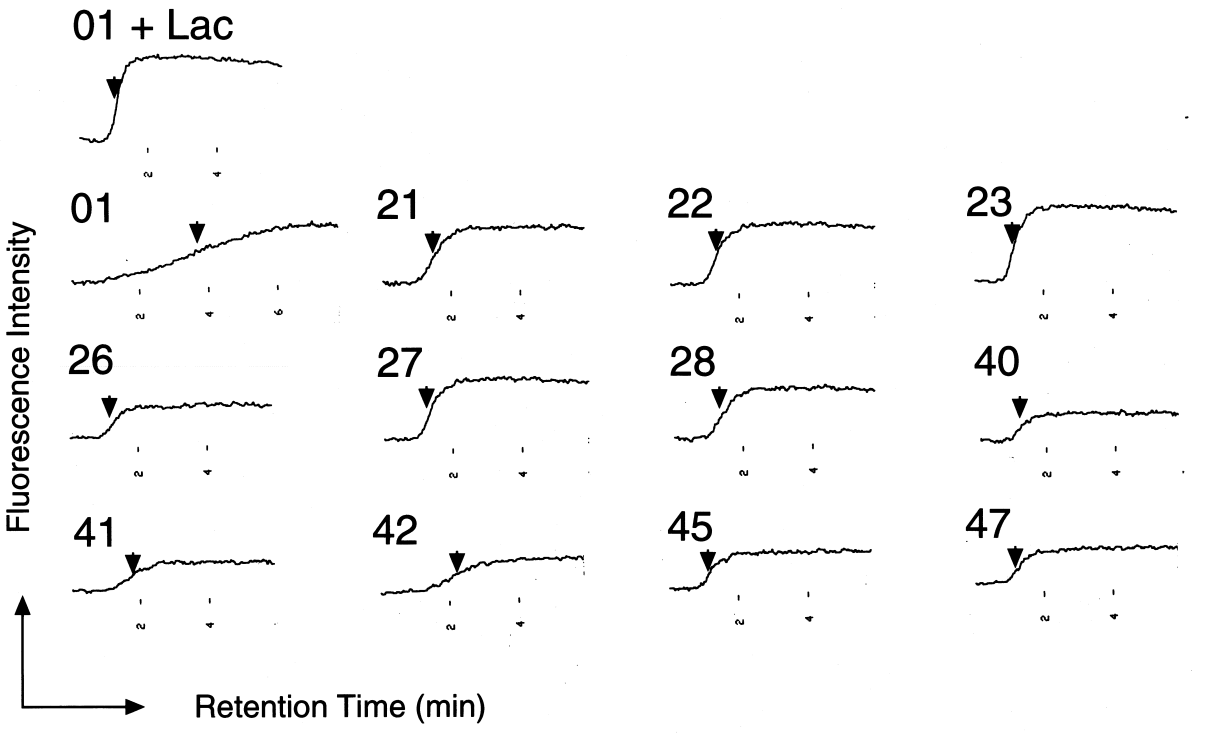

Fig. 2. Elution profiles of various PA-oligosaccharides (for structures, see Table 1) after application to a LEC-6α column. PA-oligosaccharides were dissolved in EDTA-PBS at a concentration of 20 n*M*, and 2 ml of each solution was applied to the column (10×4.0 mm, 0.126 ml) through a 2-ml loop (I.D. 0.75 mm) at a flow-rate of 0.25 ml/min at 22°C. Arrowheads indicate the positions of graphically determined V (volume of the elution fronts). For detailed data, see Table 1.

relative affinity, 0.2). These observations indicate that the 6-OH group of the non-reducing terminal galactose is critical for recognition by LEC-6. This finding is consistent with the common property of mammalian galectins [16–20].

On the other hand, glycolipid-derived, single-chain glycans having one lactose/LacNAc unit (''26''–''40'') showed relatively weak affinity for LEC-6. Asialo GM3 (lactose) showed somewhat lower affinity (0.69) than the above described ''21'' (1.1), ''22'' (0.83) and *p*-aminophenyl-β-lactoside (1), all of which have practically one lactose/LacNAc unit. This is possibly because the reducing terminal glucose of ''26'' has an open ring structure as the result of PA-derivatization, or because there is no *N*-acetylamino group on its glucose moiety, which is known to be responsible for strong binding [16,17]. On the other hand, substitution of the 4-OH group of the non-reducing terminal galactose with β-GalNAc resulted in a considerable decrease in affinity (''27''=asialo GM2; relative affinity, 0.39), as expected from the established consensus; that is, the 4-OH group of galactose is essential for galectin recognition. Further substitution of this β-GalNAc with β1-3Gal fully restored the affinity (''28''=asialo GM1; relative affinity, 0.98). Assuming that galectins recognize a non-reducing terminal disaccharide, this result implies that LEC-6 has acquired the ability to recognize another disaccharide unit besides LacNAc, i.e., Galβ1-3GalNAc, which is known as T antigen. In contrast, LEC-6 poorly recognized Forssman pentasaccharide having non-reducing terminal disaccharide, GalNAcα1-3GalNAc (''40''; relative affinity, 0.49), which is known as a strong heterophile antigen. On the other hand, two similar tetrasaccharides having non-reducing terminal LacNAc (lacto-*N*-neotetraose, ''41'') or lacto-*N*-biose (lacto-*N*-tetraose, ''42'') structures showed relatively high affinity for LEC-6 (1.6 for ''41'' and 2.6 for ''42''). The difference in their binding strength suggests that LEC-6 prefers lacto-*N*-biose (type 1) to LacNAc (type 2) structures. Notably, α1-3 fucosylation of the penultimate GlcNAc of ''41'' greatly reduced the binding ability (''45'', known as lacto-*N*-fucopentaose III; relative affinity, 0.29). This again confirmed the consensus that 3-OH group of GlcNAc is essential for the recognition by galectins. Lastly, A-hexasaccharide, ''47'', having the non-reducing terminal GalNAcα1-3Gal structure, showed relatively poor affinity (0.59), though it was bound strongly by mammalian galectin-3 [16]. Therefore, though further study is necessary, it seems that LEC-6 has adapted to Galβ1-3GalNAc as in ''28'', but not to GalNAcα1-3Gal as in ''47''.

In the present study, LEC-6 was shown to have the common sugar binding properties of the galectin family. In addition, some novel features of LEC-6 also became evident: it prefers a branched oligosaccharide (''01'') to linear oligosaccharides (''41'' and ''42'') both having the same number of lactose units, and it can also recognize the Galβ1-3GalNAc unit as well as *N*-acetyllactosamine/lacto-*N*-biose units.

3.4. Analysis of interaction between galectins and an asialofetuin-immobilized column

Next, we examined a system opposite to that used in the above experiment, i.e., application of galectin proteins to an immobilized glycan column. For this purpose, a miniature column of asialofetuin and three recombinant galectins, LEC-1, LEC-1/K68E and LEC-4 were prepared. Asialofetuin has three triantennary complex-type *N*-glycans [24], and is able to bind galectins. For immobilization, NHS-activated Sepharose 4 Fast Flow was used as in the case of the LEC-6α agarose column, and the derived gel was packed into a column (35×4.6 mm; volume, 0.582 ml). From the results obtained by preparative use of an asialofetuin-agarose, the mutant galectin LEC-1/K68E was shown to retain binding activity toward the adsorbent, whereas LEC-4 was found to have only weak affinity (Hirabayashi et al., unpublished results). Since almost all galectins identified so far conserve a single tryptophan residue (Trp^{68} in case of human galectin-1; [12]), it can be conveniently utilized for fluorescence detection.

LEC-1 showed the strongest affinity for asialofetuin, followed by LEC-1/K68E and LEC-4 at 22°C, as expected from the results of the preparative experiments. Their elution volumes (V) were 1.85 ml (LEC-1), 1.30 ml (LEC-1/K68E), and 0.99 ml (LEC-4), whereas that value for BSA was 0.60 ml (Table 3). In the present case, the assumption that $[A]_0$ is small enough relative to K_d is fully applicable, because the protein concentration used (1 μg/

Table 3
Result of FAC analysis using an asialofetuin-Sepharose 4 Fast Flow column and recombinant galectins

Sample	Temperature (°C)	V (ml)	$V-V_0$[a] (ml)	Relative affinity[b]
BSA	37	0.65	0	0
	30	0.67	0	0
	22	0.60	0	0
	15	0.65	0	0
LEC-1	37	1.08	0.41	0.33
	30	1.29	0.62	0.50
	22	1.85	1.25	1
+20 m*M* lactose[c]	22	1.00	0.40	–
	15	2.70	2.05	1.64
LEC-1/K68E	37	0.85	0.20	0.16
	30	0.98	0.31	0.25
	22	1.30	0.70	0.56
+20 m*M* lactose[c]	22	0.95	0.35	–
	15	2.13	1.48	1.18
LEC-4	37	0.80	0.15	0.12
	30	0.92	0.25	0.2
	22	0.99	0.39	0.31
+20 m*M* lactose[c]	22	0.65	0.05	–
	15	1.25	0.60	0.48

[a] V_0 was regarded as V obtained for bovine serum albumin at each temperature.
[b] Relative affinities were calculated on the basis of $V-V_0$ obtained for LEC-1 at 22°C.
[c] Inhibition experiments were carried out at 22°C for each galectin in the presence of 20 m*M* lactose.

ml) corresponds to $3\cdot10^{-8}$ *M* in the case of LEC-1 (M_r 32 000), which is far smaller than the K_d values generally observed for galectins and oligosaccharides (not less than 10^{-6} *M*). Hence, the relative affinities of the galectins to asialofetuin can be discussed in terms of $V-V_0$ according to Eq. (2) (see Materials and Methods section); on the basis of LEC-1, the relative affinities of LEC-1/K68E and LEC-4 were 0.56 and 0.31, respectively. Under the same conditions, LEC-6 showed still stronger affinity for asialofetuin, with a relative affinity of 2.3 ($V-V_0$, 2.82 ml; data not shown), as expected from the above experiment (Fig. 2), which showed especially high affinity of LEC-6 for the branched saccharide ''01''. Retardations observed for LEC-4 and LEC-6 were almost completely abolished in the presence of 20 m*M* lactose ($V-V_0=0.05$ ml, both), whereas the effect of lactose was only partial for LEC-1 and LEC-1/K68E ($V-V_0=0.40$ ml for LEC-1, and $V-V_0=0.35$ ml for LEC-1/K68E). Since LEC-1 has two homologues, but distinct (~35% amino acid identity) CRDs [6], this feature may explain this incomplete effect of lactose.

In this series of experiments, we also examined the effect of temperature. The results of the present FAC analysis are shown in Fig. 3 (for detailed data, see Table 3). Obviously, all galectins showed stronger retardation at lower temperature, whereas the control protein, BSA, showed no significant temperature-dependency. In the case of LEC-1, the observed $V-V_0$ increased as temperature decreased; i.e., 0.41 ml (37°C), 0.62 ml (30°C), 1.25 ml (22°C) and 2.70 ml (15°C). Some reports on the effect of temperature have been published. Isothermal titration microcalorimetry revealed significant temperature-dependency of galectin binding to oligosaccharides, but the results of two groups of investigators are inconsistent: Gupta et al. reported four-times stronger affinity of hamster galectin-1 for LacNAc at a higher temperature (27°C) than at a lower temperature (9°C) [22], whereas Schwarz et al. reported 3–5-times stronger affinity of bovine galectin-1 to

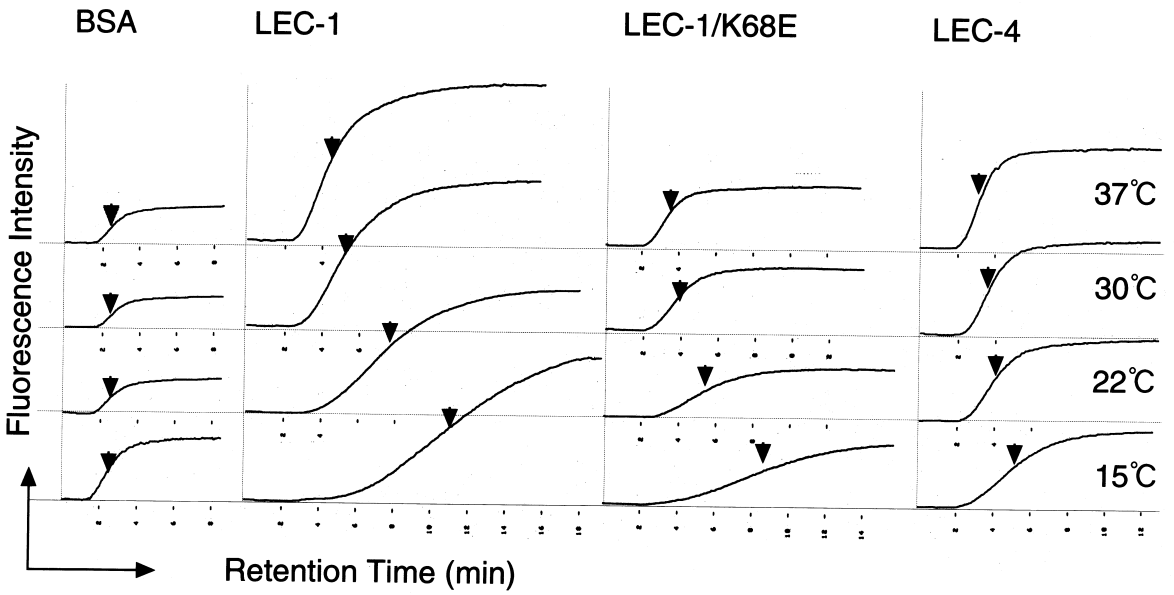

Fig. 3. Elution profiles of recombinant galectins originally derived from *C. elegans* after application to an asialofetuin column. Effect of temperature was also examined. Recombinant galectins, i.e., LEC-1, LEC-1/K68E and LEC-4, were dissolved in EDTA-PBS at a concentration of 1 μg/ml, and 2 ml of each solution was applied to the column (35×4.6 mm, 0.582 ml) at a flow-rate of 0.25 ml/min at the various temperatures indicated (15, 22, 30 and 37°C). Both the column and the 5-ml sample loop (I.D. 0.75 mm) were immersed in a water bath.

LacNAc at a lower temperature (7°C) than at a higher temperature (23°C) [23]. Our result is consistent with the data obtained by Schwarz et al. [23], though the molecules used for these experiments are different; i.e., we used galectins from *C. elegans*, and Schwarz et al. used bovine galectin-1. It is noteworthy that *C. elegans* galectins bind significantly more strongly to the glycoprotein asialofetuin at lower temperature, consistent with the optimum growth temperature (20°C) of this soil organism. In this context, there is a similar observation for polypeptide GalNAc transferase cloned from *C. elegans* [25], which showed only poor transferase activity at 37°C, while it maintained high activity at lower temperatures (16–25°C).

3.5. Comparison with other methods and possible problems

The reinforced FAC system apparently has an economical merit over other methods recently developed, such as affinity capillary electrophoresis [7,8] and a biosensor method based on surface plasmon resonance [9,10]. Nevertheless, the present system can provide us with reproducible (e.g., as regards determination of V values, within ±5% SD) and reliable data within a short period. Moreover, no special skill is required for column handling and sample preparations. However, care is necessary to solve possible problems. Firstly, immobilized ligands, in particular in case of proteins, may have altered their properties (e.g., K_d and specificity) compared with those in their free states, as has been pointed for biosensor analyses. In this context, determined K_d values should better be confirmed by the opposite approach in FAC (i.e., the immobilized ligand and analyte molecules are exchanged) or by other quantitative methods, such as capillary affinophoresis [26]. Sometimes, immobilized proteins may have only low capacity due to masking of their binding sites by the supporting matrices. In the presence study, a short α-peptide was genetically introduced expecting so-called oriented immobilization.

Secondly, it may not always be easy to carry out frontal analysis by varying protein concentration in

order to determine B_t according to Eq. (3), because of increase in viscosity, possible bubble generation at a high concentration of protein and also increase in the cost. Another approach to determine B_t for galectin binding sites will be use of other lectins (e.g., castor bean lectin, RCA120), which have an affinity strong enough to saturate the binding sites on the affinity adsorbent at a low protein concentration [27]. On the other hand, *p*-aminophenyl-β-lactoside was used in the present work to perform a concentration-dependency analysis on the LEC-6α column. However, relatively few oligosaccharides are commercially available for this purpose, i.e., those having an appropriate aromatic aglycon which enables UV detection. In this regard, an attempt has already been in progress which utilizes non-labeled oligosaccharides (e.g., Asn-linked complex type, biantennary oligosaccharide) by mixing with a constant concentration of the corresponding PA-oligosaccharide (''01''). Though this approach assumes that both labeled and non-labeled oligosaccharides are equally recognized by lectins, it is expected that PA-derivatization has essentially only little effect on lectin binding because most lectins recognize outermost few saccharide units.

Thirdly, for the present fluorescence-based detection, analyte proteins must contain at least one tryptophan residue. If they lack tryptophan, they need to be modified with an appropriate fluorescent reagent prior to analysis, or expressed as tryptophan-containing mutant proteins by a genetic manipulation.

4. Conclusions

FAC using a conventional HPLC system proved to be versatile. Though only ordinary devices were utilized, speed, sensitivity and accuracy were greatly improved. Merits of the reinforced FAC are summarized as follows:

(i) No need for column regeneration
(ii) No drift of the baseline
(iii) No need for a special device
(iv) Simplified operation
(v) High speed
(vi) High sensitivity

Firstly, (i–iv) are apparent benefits of the application of an isocratic elution program. Secondly, by the combination of a miniature column and a large sample loop, FAC becomes much easier to operate (iv), and an analysis time becomes much shorter (v). Lastly, by using a fluorescence detector, both sensitivity and accuracy are greatly improved (vi). Actually, the reinforced FAC made it possible to carry out more than 30 runs of chromatography in a single day. This system proved to work for both immobilized lectin columns (LEC-6α-Sepharose 4 Fast Flow) and immobilized glycan columns (asialofetuin-Sepharose 4 Fast Flow). By using the former system, binding properties of many PA-oligosaccharides could be analyzed; and by using the latter system, binding properties of various galectin proteins could be compared. Recently, Schriemer et al. reported a reinforced FAC system in which electrospray ionization-mass spectrometry was used for detection [28]. Their system should be extremely useful because multiple analytes can be applied simultaneously. However, such a system is very costly and therefore probably will not become very popular. In contrast, we use a fluorescence detector, which is more easily afforded by most laboratories. It will be possible to further raise the sensitivity by technical improvement of the fluorescence detectors. This system will hopefully be useful for other fields analyzing biomolecular interactions, and not restricted to lectin–carbohydrate interactions. Because of its particular simpleness and convenience, the presently reinforced FAC method will exert its maximal power for functional analyses required for various post-genome projects, and also for the forthcoming glycome projects [29].

Acknowledgements

The studies described herein were supported by a grant (No. 10178102) from the program Grants-in-Aid for Scientific Research on Priority Areas of the Ministry of Education, Science, Sports and Culture of Japan.

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