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Short communication

Application of reinforced frontal affinity chromatography and advanced processing procedure to the study of the binding property of a *Caenorhabditis elegans* galectin

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Abstract

Frontal affinity chromatography is a very useful and simple method to analyze molecular interactions between an analyte and an immobilized ligand by calculating the extent of ''retardation'' of the elution front. We developed a very simple and efficient data-processing procedure that enables the measurement of very small differences in retardation with precision. This procedure was successfully applied to comparison of the binding properties of recombinant *C. elegans* galectins for their ligand.

Keywords: Frontal affinity chromatography; *Caenorhabditis elegans*; Galectin; Proteins; Lectins

1. Introduction

We have demonstrated that frontal affinity chromatography [1] is very useful as an analytical tool to elucidate quantitatively the ligand-binding property of proteins such as proteases [2,3], nucleases [1], and lectins [4,5]. When a large amount of an analyte solution is applied continuously to a column packed with affinity adsorbent that immobilizes a counter ligand, the extent of retardation of the elution front of the analyte reflects the strength of the interaction [1]. Recently, we applied this procedure to the study of the binding properties of two lectin domains of a tandemly-repeated M_r 32 000 galectin (β-galactoside binding lectin) from the nematode *Caenorhabditis elegans* (*C. elegans*) [6]. Galectins are metal-independent, β-galactoside-binding lectins and now known to be widely distributed throughout the animal kingdom [7–9]. The M_r 32 000 galectin (LEC-1 or N32) is composed of two tandemly-repeated domains, each of which is homologous to typical vertebrate M_r 14 000-type galectins [10,11]. We prepared recombinant proteins composed of the whole protein (LEC-1), the N-terminal lectin domain (Nh), and the C-terminal lectin domain (Ch) in *Escherichia coli* [5]. Comparison of the affinity of them for asialofetuin-Sepharose 4B by frontal affinity chromatography using an open column showed that Ch had seven-fold weaker affinity than LEC-1 and that Nh had even weaker affinity [5]. Since frontal analysis by using an open column is very time-consuming (>10 h) and requires a relatively large amount of the lectin protein (1–3 mg per run), we wished to improve it, and so we devised a

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reinforced system incorporating various merits of a contemporary liquid chromatography system [12]. Here we report a very simple, sensitive, and reproducible procedure to evaluate extremely small differences in the ''retardation'' of an analyte in frontal affinity chromatography by combining an HPLC apparatus and commercially available computer and software. The combination of the newly constructed equipment and the present data-analyzing procedure is very promising as a research tool for molecular recognition.

In this method, an analyte protein solution is applied by means of a sample loop to a small column packed with an affinity adsorbent. Elution of protein is then monitored continuously by a fluorescence detector measuring the fluorescence derived from tryptophan residues in the protein, thus allowing a great reduction in the amount of the protein needed for the analysis (5–10 μg per run). Furthermore, the time required for one run can be reduced to about 30 min. The extent of retardation of elution front of an analyte is calculated from the area under the elution curve by using a commercially available software. As a consequence of these reinforcements, analysis of binding properties of recombinant galectins is made extremely efficient.

2. Experimental

Fetuin purchased from Gibco was treated in 0.1 m*M* HCl for 2 h at 80°C in order to remove sialic acids, which block galactose residues of the sugar chains [13]. The reaction mixture was dialyzed against first 0.1 *M* $NaHCO_3$ and then against 0.1 *M* sodium phosphate buffer, pH 7.0. Asialofetuin thus obtained was coupled to NHS (*N*-hydroxysuccinimide)-activated Sepharose-4FF (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. The remaining NHS groups were blocked with ethanolamine. The amount of the protein immobilized was 2.0 mg/ml gel. This asialofetuin Sepharose-4FF gel was packed into a column (10 mm×4 mm I.D.) (GL Science).

Expression and purification of recombinant nematode proteins were performed as described before [5]. Briefly, DNA fragments encoding M_r 32 000 galectin (N32 or LEC-1), its N-terminal half domain (Nh), and its C-terminal half domain (Ch) were amplified by polymerase chain reaction (PCR) using cloned cDNA [10] as a template. The amplified fragments were ligated into digested pET21a (Novagen) and used to transform *E. coli* BL21(DE3) cells. The recombinant proteins were induced in the presence of 1 m*M* IPTG in the culture medium. LEC-1 and Ch were affinity purified by use of asialofetuin-Sepharose 4B, and Nh, with DEAE-Toyopearl 650S (Tosoh, Tokyo, Japan).

The analytical system is shown in Fig. 1. An analyte protein was dissolved in EDTA–phosphate-buffered saline (PBS) (20 m*M* sodium phosphate, pH 7.2, containing 150 m*M* NaCl and 2 m*M* EDTA) to make 1 μg/ml, degassed, and applied to the asialofetuin Sepharose-4FF column through a 5-ml sample loop connected to the Rheodine 7725 Injector. The flow-rate was 0.25 ml/min. The sample loop and the column were immersed in a 20°C waterbath. Elution of the protein from the column was monitored by a Shimadzu fluorescence detector RF10AxL that measured the fluorescence at 350 nm (excitation

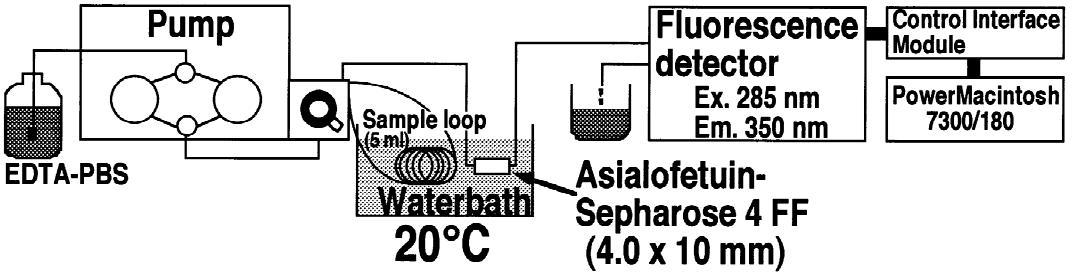

Fig. 1. System diagram of the reinforced frontal affinity chromatography system. An analyte solution (5 ml) is injected through a 5-ml sample loop. Elution of lectin protein is monitored by measuring the fluorescence at 350 nm (excitation at 285 nm). The fluorescence signals are collected at 2-s intervals and sent to a computer through a control interface module, and the data are processed by Microsoft Excel.

at 285 nm). The signal from the detector was sent to a computer (Power Macintosh 7300/180) through a control interface module (Varian Chromatography Systems, USA) at 2-s intervals and the collected data were processed by Dynamax Compare Module software (Rainin, USA) and by a table-calculating software, Microsoft Excel.

The basic concept of frontal affinity chromatography [1,5] is shown in Fig. 2A. Elution of an analyte will be retarded by specific interaction with the immobilized ligand (curve I in Fig. 2A) as compared to that of a control substrate which has no affinity for the immobilized ligand (curve II in Fig. 2A).

Eq. (1) is the basic equation of frontal affinity chromatography [1,4]:

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

where V_f is the elution volume of the analyte, $[A]_0$ is the concentration of the added analyte, V_0 is the elution volume of a control substance having no affinity (in this paper, ovalbumin was used), B_t is the total amount of immobilized ligand (saccharide chains attached to asialofetuin recognized by the applied protein), and K_d is the dissociation constant. The smaller is $[A]_0$, the larger is V_f. If $[A]_0 \ll K_d$, i.e., if $[A]_0$ is negligibly small compared with K_d, V_f approaches its maximum value, V_m, which is independent of $[A]_0$ and the following equation is obtained:

$$V_m - V_0 = \frac{B_t}{K_d} \quad (2)$$

This means that the value of $(V_m - V_0)$ is proportional to the affinity. Therefore, if we measure elution volumes for the recombinant proteins at adequately low concentrations, these values are inversely proportional to their dissociation constants (or proportional to their association constants).

V_f can be considered as the volume where the hypothetical boundary of the analyte solution would appear if the boundary were not disturbed at all. Therefore, V_f is the point where the area under the elution curve is equal to the area of the rectangle ($[A]_0$ for height and $(\Sigma\Delta S_i)/[A]_0$ for base) shown in the bottom panel of Fig. 2B. If fluorescence intensity

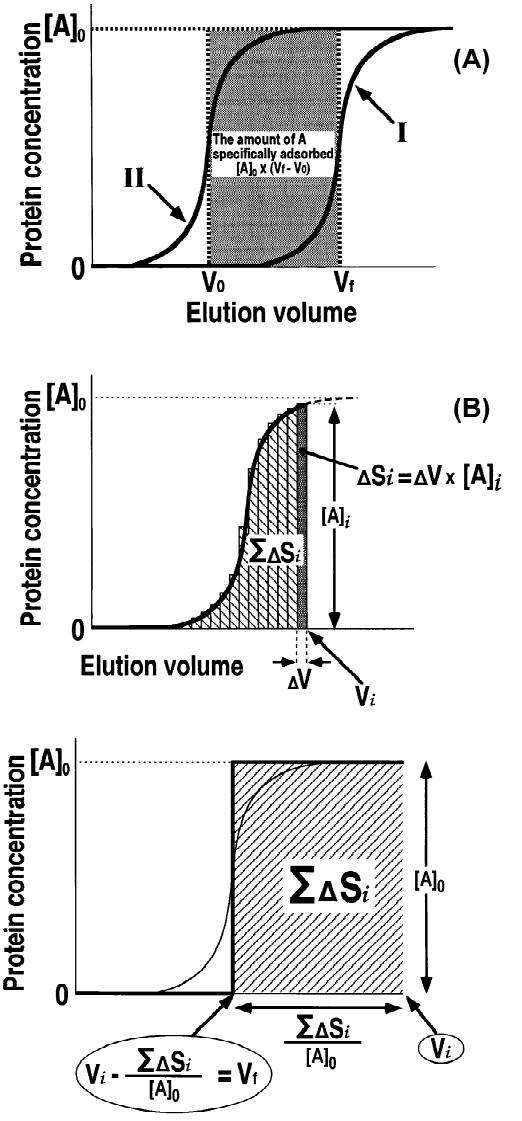

Fig. 2. (A) Elution profiles in frontal affinity chromatography. Curve I: Elution profile of an analyte, in the present case one of the recombinant lectins that specifically interacts with the immobilized ligand (elution volume $= V_f$). Curve II: Elution profile of the analyte having no specific interaction with the ligand, in this case ovalbumin (elution volume $= V_0$). (B, top panel) To calculate the value V_f, the area under the curve was calculated first. The fluorescence intensity was measured every 2 s, and the small area (ΔS_i) with ΔV (eluted volume at flow-rate 0.25 ml/min for 2 s, i.e., 8.33 μl) for the base and $[A]_i$ for height was summed ($\Sigma\Delta S_i$). The value of the base of the rectangle that makes the same area value of the added area ($\Sigma\Delta S_i$) was calculated (i.e., $\Sigma\Delta S_i/[A]_i$). (B, bottom panel) When $[A]_i$ reaches the plateau $[A]_0$, the value $V_i - \Sigma\Delta S_i/[A]_i$ will converge to V_f, which that makes the two area-under-the-curve values equal.

data are collected periodically, the area under the elution curve becomes the sum of the areas of small rectangles with ΔV for base and $[A]_i$ for height, i.e., $\Delta S_i = \Delta V \times [A]_i$ (Fig. 2B, top panel). If ΔV is small enough, $\Sigma \Delta S_i$ (shaded in Fig. 2B, top panel) is close enough to the actual area. If the column i is located at the plateau, dividing $\Sigma \Delta S_i$ by $[A]_i$ gives the length of the base of the large rectangle. Subtracting this value from V_i {i.e., $V_i - (\Sigma \Delta S_i)/[A]_i$} gives the point where the area below the elution curve and the rectangle become equal. If we make the computer to calculate this value $V_i - (\Sigma \Delta S_i)/[A]_i$ continuously and when the elution curve reaches plateau ($[A]_0$), this value converges to a certain figure that should be V_f; the point where the hypothetical boundary of the analyte appears (Fig. 2B, bottom panel). By this procedure, reliable values can be obtained even if the elution curve is not symmetrical (we often encounter such a case).

The calculation procedure is as follows: Collected data were saved as text file by using ChromData (Dynamax Compare Modules software, Rainin Instrument Company, Inc., USA), transferred to an Excel format and calculated automatically. When V_f converges to a certain value, we consider it true V_f. Table 1 shows an example of such a calculation. In this case, V_f was estimated as 0.628 ml. Although the V_f value includes the volume of the tubing from the outlet of the column to the fluorescence detector, this can be compensated because we always consider values relative to V_0, which is the elution volume of a protein without specific interaction with the affinity adsorbent (in this paper, ovalbumin).

3. Results and discussion

We applied the present reinforced frontal affinity chromatography to recombinant proteins derived from *C. elegans* galectin (LEC-1, Nh, Ch) in order to assess its efficiency, because we had already found them to have rather weak affinity by the classical open column method.

A 5-ml solution of 1 μg/ml ovalbumin or one of the recombinant galectins dissolved in EDTA–PBS was applied to the asialofetuin Sepharose-4FF

Table 1
An example of processing of data by Excel[a]

Time (min)	V_i	ΔV (ml)	$[A]_i$ (Signal intensity)	ΔS_i ($\Delta V \times [A]_i$)	$\Sigma \Delta S_i$	$\frac{\Sigma \Delta S_i}{[A]_i}$	$V_i - \frac{\Sigma \Delta S_i}{[A]_i}$
0.000	0.00000		0	0	0	0	0
0.033	0.00825	0.00825	0	0	0	0	0
0.066	0.01650	0.00825	0	0	0	0	0
0.100	0.02500	0.00850	0	0	0	0	0
0.133	0.03325	0.00825	0	0	0	0	0
.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.
1.766	0.44150	0.00825	607	5.008	48.067	0.079	0.362
1.800	0.45000	0.00850	647	5.500	53.567	0.083	0.367
1.833	0.45825	0.00825	683	5.635	59.202	0.087	0.372
1.866	0.46650	0.00825	723	5.965	65.166	0.090	0.376
1.900	0.47500	0.00850	764	6.494	71.660	0.094	0.381
.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.
5.666	1.41650	0.00825	2560	21.120	2021.616	0.790	0.627
5.700	1.42500	0.00850	2565	21.802	2043.418	0.797	0.628
5.733	1.43325	0.00825	2565	21.161	2064.579	0.805	0.628
5.766	1.44150	0.00825	2565	21.161	2085.741	0.813	0.628
5.800	1.45000	0.00850	2565	21.802	2107.543	0.822	0.628

[a] As the $V_i - (\Sigma \Delta S_i/[A]_i)$ value becomes constant, we define it as V_f (present case; 0.628 ml).

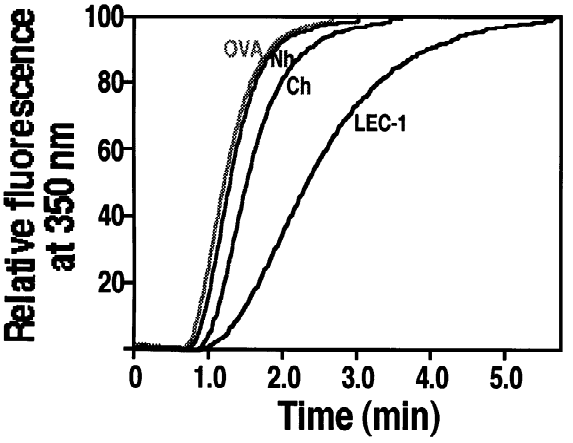

Fig. 3. Elution profiles of LEC-1, Nh, Ch and ovalbumin in frontal affinity chromatography.

through a 5-ml sample loop. This concentration is low enough ($3.2 \cdot 10^{-8}$ *M*, $6.2 \cdot 10^{-8}$ *M* and $6.6 \cdot 10^{-8}$ *M* for LEC-1, Nh and Ch, respectively) to consider the obtained V_f value as V_m. The elution profiles of LEC-1, Nh, Ch and ovalbumin are shown in Fig. 3. From these profiles, the order of the binding strength of recombinants can be roughly estimated as LEC-1 $\gg$ Ch $>$ Nh. As shown in Table 2, the calculated values of $(V_m - V_0)$ for LEC-1, Nh and Ch were 0.303, 0.011 and 0.078 ml, respectively. The values obtained previously by the conventional frontal affinity chromatography using an open column were 100, 1.14 and 14.6 ml [5]. Whereas the previous experiments were carried out at 4°C, the present experiments were conducted at 20°C, the same temperature at which *C. elegans* is usually cultivated. This temperature seems to be more suitable to analyze the binding properties of *C. elegans* galectins. In the previous experiments [5], it took more than 10 h for one run of frontal analysis and the whole equipment was set in a cold room to avoid possible protein denaturation during the relatively long run. The present method [12] allowed us to reduce the time to 30 min for one run including the time required for washing of the column (application of 5-ml sample at the flow-rate of 0.25 ml/min). Furthermore, by introducing the reproducible procedure using a commercially-available software, we were able to evaluate extremely small differences in the retardation of the analyte. This allowed us to collect multiple data for each protein and resulted in a great increase in accuracy and reproducibility, as shown in Table 2.

If the B_t value for the column is known, K_d values for LEC-1, Nh and Ch can be calculated. However, for the relatively weak binding of nematode galectins to the column, it was not possible to saturate the column by nematode galectin and calculate the B_t value. This was probably because these galectins may have a more preferable sugar structure than the Galβ1-4GlcNAc structure expressed on asialofetuin, although these galectins do interact with this structure. Therefore, the amount of the Galβ1-4GlcNAc structure on asialofetuin-Sepharose 4FF should be determined by measuring the amount of binding by a lectin that strongly interacts with this structure. For this purpose, castor bean (*Ricinus communis*) lectin RCA120, which shows very strong affinity for Galβ1-4GlcNAc structure [14] and should saturate them on asialofetuin-Sepharose 4FF, was used. The amount of binding sites for RCA120 on the gel packed in the column (10 mm×4 mm I.D.) was calculated to be 40.2 nmol (for 5.6 nmol of immobilized asialofetuin). If we assume that recombinant

Table 2
Results of frontal affinity chromatography of ovalbumin, LEC-1, Nh and Ch[a]

	Elution volume V_m (ml)				$V_m - V_0$ (ml)	Relative binding strength
	Experiment 1	Experiment 2	Experiment 3	Average±S.D.		
OVA	0.322	0.322	0.320	0.321±0.001		
LEC-1	0.618	0.628	0.628	0.625±0.005	0.303	1.00
Nh	0.336	0.329	0.331	0.332±0.003	0.011	0.04
Ch	0.406	0.396	0.395	0.399±0.005	0.078	0.26

[a] V_f for each protein was measured three times and the average was calculated. V_0 was determined by applying ovalbumin (OVA). Relative binding strengths of Ch and Nh are the ratio of their $(V_m - V_0)$ to that of LEC-1.

galectins also interact with these binding sites, that is, $B_t = 40.5$ nmol, K_d values for LEC-1, Nh, Ch can be calculated to be $1.3 \cdot 10^{-4}$ *M*, $3.7 \cdot 10^{-3}$ *M* and $5.2 \cdot 10^{-4}$ *M*, respectively.

Recently, Shriemer et al. reported a frontal affinity chromatography system applicable to screening specific compounds synthesized by combinatorial chemistry [15]. They introduced an electrospray mass spectrometer as an on-line detector, making it possible to monitor multiple analytes in one run of frontal affinity chromatography. This system seems extensively versatile, though it will be rather difficult to become commonly used because of the cost.

This reinforced frontal affinity chromatography system proved to be very useful and will surely contribute to the study of lectin–sugar interactions as much as other methods recently developed, such as affinity capillary electrophoresis [16,17] and a biosensor method based on surface plasmon resonance (SPR) [18,19]. This system is rapid and sensitive and the results are reproducible and reliable. Furthermore, the lack of a need for column regeneration makes it possible to carry out many chromatographic runs to obtain the retardation values in a short period. Reductions in the amount of required sample and the run time allow multiple measurements and result in a great increase in accuracy. Only a slight modification of the HPLC system (such as addition of a control interface module) is required. Introduction of a highly expensive device (such as an SPR biosensor) is not necessary. The theory is very simple because we apply an isocratic elution program and describe this system in terms of a simple equilibrium problem. One of the greatest advantages of this approach is that this method is suitable for analyzing weak interactions. We can detect a slight retardation by analyzing the elution profile by using commercially-available software. Therefore, this reinforced frontal affinity chromatography system can easily be constructed in laboratories of moderate economical condition and can be flexibly modified depending on the researcher's demands.

As a result of the reinforcement presented in this report, speed, sensitivity and accuracy were greatly improved. The data-processing procedure presented here made it possible to discriminate very small differences in the elution volumes (for example, the elution front of Nh was retarded only 11 μl from that of ovalbumin), thus making frontal affinity chromatography much more advantageous for analysis of weak interactions. The present system is expected to become widely used by all researchers interested in specific interactions between biomolecules because it is made of ordinary, low-cost chromatographic equipment and easily-available computer software programs.

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