Binding mechanism of proteins and DNAs in electrostatic interaction chromatography –Ion Exchange Chromatography and Hydroxyapatite Chromatography-

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1. Introduction

It is well known that very difficult separation of protein variants can be achieved by electrostatic interaction chromatography (ion-exchange chromatography, IEC) although the mechanism has not yet been fully clarified. DNAs are also efficiently separated not only by IEC but also by hydroxyapatite chromatography (HAC), which is known as a bi-modal or mixed mode interaction system.

In this study we investigated the binding mechanism of proteins and DNAs in IEC and HAC. Especially oligoDNA separation behavior was carefully examined by using different mobile phases for HAC in order to understand its unique property for DNA binding and elution. For this purpose the number of binding sites *B* values were determined from linear (salt) gradient elution (LGE) experiments as well as the peak salt concentration.

2. Materials and Methods

2.1. Chromatography column

Poly(glycidyl methacrylate-co-ethylene dimethacrylate) disks (12 mm diameter x 3mm thickness) with strong anion exchange group were contained in a specially designed disk holder column from BIA Separations (Ljubljana, Slovenia) This disk is called hereafter QA-CIM.

Two Hydroxyapaptite chromatography columns were used: A pre-packed ceramic hydroxyapatite bead column from Bio-Rad (CHT Type I) and an agarose-hydroxyapatite composite gel (HA-Ultrogel) column packed according to the supplier method (Biosepra).

2.2. Materials

Oligonucelotides (3- to 95-mer poly A and poly T) were purchased from Espec (Tokyo, Japan). Prior to use for chromatography oligonucletotide samples were thermally pretreated according to the following temperature program: from 298K to 368K (20 minutes), 368K (3 minutes), from 368K to 313K (20 minutes) and from 313K to 298K (45 minutes).

Most standard proteins were purchased from Sigma-Aldrich. Other reagents were of analytical grade.

2.3. Chromatography setup

Chromatography experiments were carried out on a fully automated liquid chromatography system ÄKTA explorer 100 (GE Health Care, Uppsala, Sweden).

2.4. Linear gradient elution experiment

The column was equilibrated with a starting buffer (buffer A) containing a specified concentration of NaCl for IEC. Although M (=mol/dm^3) is a non-SI unit symbol, it is used in this paper as it is still common for many chromatography researchers. The same buffer solution containing 0.5-2.0 M NaCl was used as a final elution buffer (buffer B). The linear gradient elution was performed by changing the buffer composition linearly from buffer A to buffer B with time. Namely, the salt concentration was increased with time at a fixed pH and buffer compositions. In this study 14 mM Tris-HCl (pH7.7) was chosen as buffer A for IEC.

For HAC the standard buffer solutions are buffer A 10mM phosphate buffer and buffer B 400mM phosphate buffer.

The linear mobile phase velocity u was calculated with the cross-sectional area A_c and the column bed void fraction ε as $u = F/(A_c \varepsilon)$ where F is the volumetric flow rate. The column bed void fraction ε was determined from the peak retention volume of Dextran T 2000 pulses. For the QA-CIM disk, the void faction was assumed to be 0.60. The experiments were performed at 298±1K. Experiments were carried out for at least four different gradient slopes. Each run was repeated twice. The gradient slope g is shown in M/mL.

3. Theoretical

The outline of our model for linear gradient elution (LGE) is briefly explained below. The peak retention volume is a function of gradient slope in linear gradient elution IEC. The peak salt concentration I_R increases with increasing gradient slope, g. The I_R values can be correlated with the following normalized gradient slope,

$$GH = (gV\mathrm{o})[(V_t - V\mathrm{o})/V\mathrm{o}] = g(V_t - V\mathrm{o}) \quad (1)$$

V_t is the total bed volume, V_o is the column void volume, and g is the gradient slope of the salt. $H = (V_t - V\mathrm{o})/V\mathrm{o} = (1-\varepsilon)/\varepsilon$ is the phase ratio. $\varepsilon = V\mathrm{o}/V_t$ is the bed void fraction (interstitial volume of the bed). The gradient slope g is defined by the following equation.

$$g = (I_F - I_o)/V_g = (I_f - I_0)/(F_v t_g) \quad (2)$$

I_f is the final salt concentration, I_o is the initial salt concentration, V_g is the gradient volume, F_v is the volumetric flow rate, and t_g is the gradient time. Linear gradient elution experiments are performed at different gradient slopes (*GH* values) at a fixed pH. The salt concentration at the peak position I_R is determined as a function of *GH*. The *GH*- I_R curves thus constructed do not depend on the flow velocity, the column dimension, the sample loading (if the overloading condition is not used), or the initial salt concentration I_o. The experimental *GH*- I_R data can commonly be expressed by the following equation.

$$GH = I_R^{(B+1)}/[A\,(B+1)] \quad (3)$$

From the law of mass action (ion exchange equilibrium), the following relationship can be derived.

$$A = K_e \Lambda^B \quad (4)$$

Here, *B* is the number of sites (charges) involved in electrostatic interaction, which is basically the same as the *Z* number and the characteristic charge, K_e is the equilibrium association constant, and Λ is the total ion exchange capacity. From the ion-exchange equilibrium model and Eq. (4), the following equation is derived .

$$K - K' = K_e \Lambda^B I^{-B} \quad (5)$$

K is the distribution coefficient of charged biomolecule, *K'* is the distribution coefficient of salt, and *I* is the ionic strength (salt concentration). The SMA model equation is reduced to Eq.(5) when the sample loading is low (Λ is not influenced by sample adsorption).

4. Results and discussion

The binding site for proteins is a function of pH, which is not as simple as expected from the net charge concept.

There is a good correlation between the number of charges of DNA and the binding site for IEC as shown in Fig.1 Even a small single strand DNA (10-20 mers) is retained with the binding site value greater than 10, and NaCl concentration of 0.6 M or higher is needed for the elution (Fig.2) . Although it is quite difficult to separate large DNAs, we were able to separate DNAs (50 mer poly A-poly T and 95 mer poly A-poly T) by using a monolithic convection-aided IEC disk at high flow velocity and with a shallow gradient.

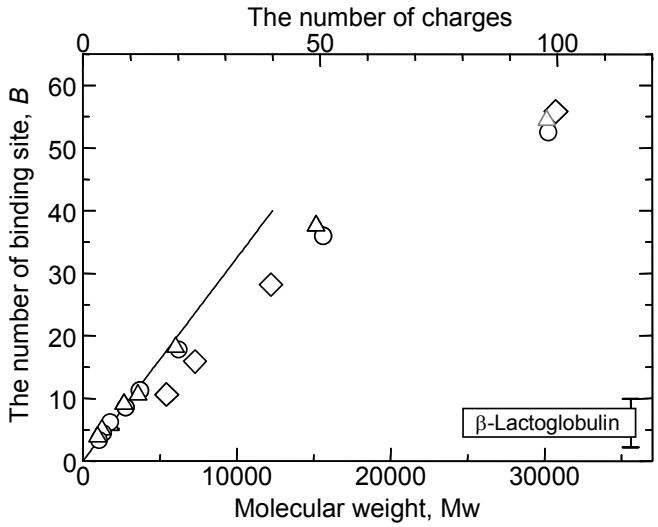

Fig.1 Relationships between the number of binding sites B and the molecular weight or the number of charges of poly A and poly T DNAs. The straight curve in the figure shows B =the number of charges..Note that the range of B values for β-lactoglobulin is shown in the figure for comparison. The B value for the double-stranded 95mer-polyA and poly T was ca.97 although it was not shown in the figure.Circle:poly A, triangle: poly T, diamond: ds-poly A-polyT

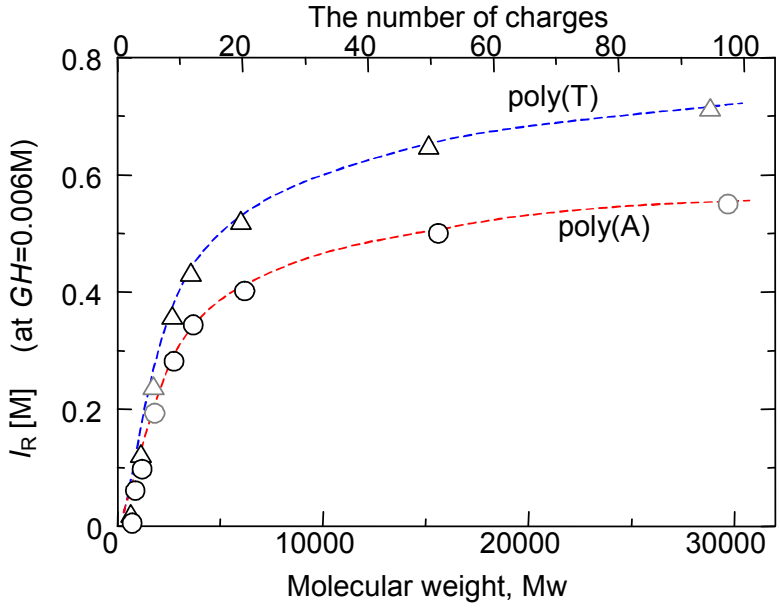

Fig.2 Relationships between the peak salt concentration I_R at GH=0.006 and the molecular weight or the number of charges for single-strand poly A and poly T DNAs.

On the other hand, single strand DNAs were very weakly retained on HAC whereas double strand DNAs were retained. Consequently, separation of single-strand and double strand DNAs are very efficiently performed by HAC(Fig.3). This is due to repulsion of phospate-site of HAC.

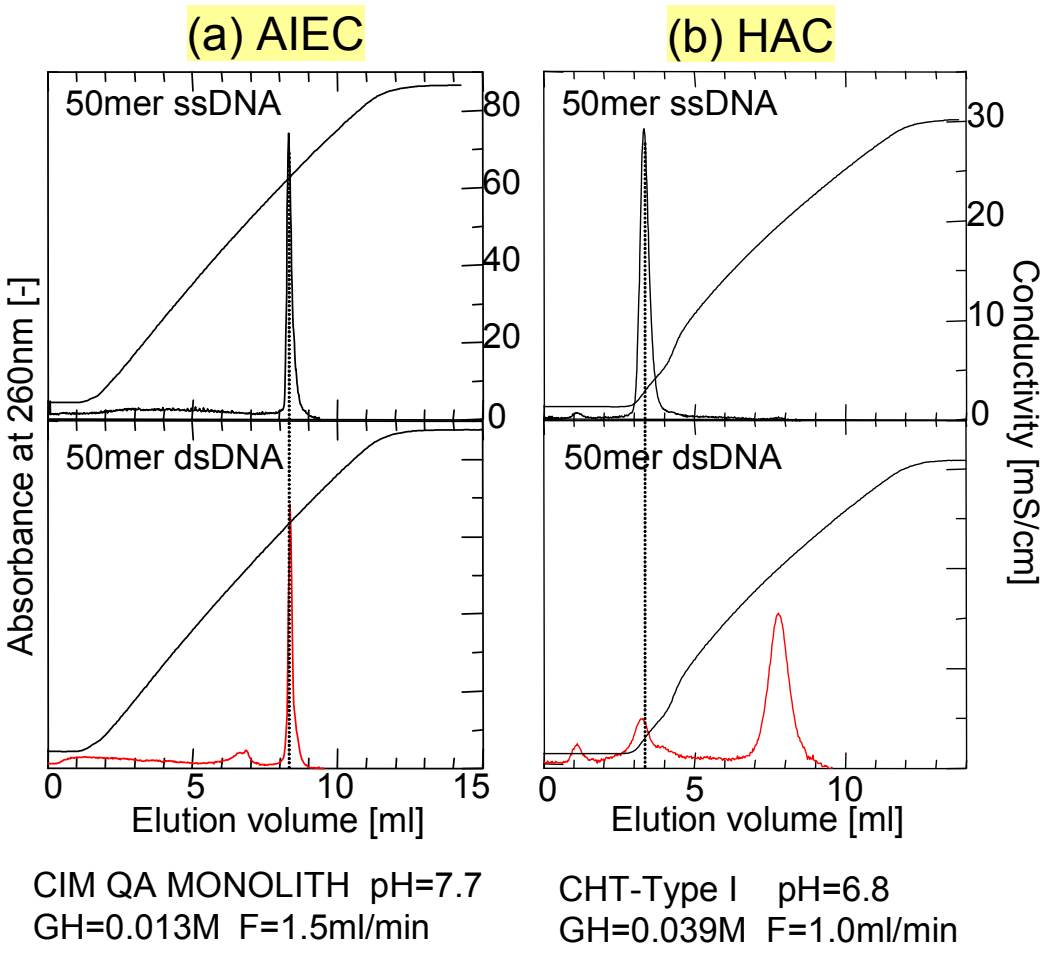

Fig.3 Linear gradient elution curves of ss- and ds-DNA,

Nomenclature

A	$= K_e\Lambda^B$	
B	the number of binding sites	[-]
d_p	particle diameter	[cm]
F_v	volumetric flow rate	[mL/min]
G	gradient slope	[M/mL]
GH	normalized gradient slope = $(gV_o)H = g(V_t\text{-}V_o)$	[M]
H	phase ratio $(=(V_t\text{-}V_o)/V_o)$	[-]
I	ionic strength (salt concentration)	[M]
I_f	final salt concentration	[M]
I_0	initial salt concentration	[M]
I_R	peak salt concentration	[M]
K	distribution coefficient	[-]
K'	distribution coefficient of salt	[-]
K_e	equilibrium association constant	
t_g	gradient time	[min]
t_R	retention time	[min]
U	linear mobile phase velocity	[cm/min]
V	elution volume	[mL]
V'	elution volume for the salt	[mL]
V_g	gradient volume	[mL]
V_o	column void volume(interstitial volume)	[mL]
V_R	retention volume	[mL]
V_t	column volume	[mL]
ε	void fraction of column $=V_o/V_t$	[-]
Λ	total ion exchange capacity	[mequiv/mL]

Although M (=mol/dm^3) is not a SI unit, it is used in this paper for the sake of convenience.

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