Electrostatic interaction chromatography for separation of very similar proteins – effects of stationary and mobile phase properties

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Introduction

It is well known that very difficult separation of protein variants or isoforms can be achieved by electrostatic interaction chromatography (ion-exchange chromatography, IEC) although the mechanism has not yet been fully clarified. Separation behaviour of several protein variant systems was investigated. The number of binding sites B values were determined from linear (salt) gradient elution (LGE) experiments as a function of pH, The B-pH curves provided important information on the biorecognition mechanism of proteins. We also examined how mobile phase properties affect the separation behaviour by using different types of column materials such as membrane-based IEC, monolithic IEC disks as well as conventional porous packing IEC. In most cases proteins are retained near or at the isoelectric points (pI), and the resolution was better near the pI where the number of binding site B values are small, ca.2-3. We also examined which parts of proteins are responsible for the separation (or retention) based on protein-structure-charge distribution information.

Theoretical

Determination of Binding site by linear gradient elution experiments

The outline of our model [1-4] is briefly explained below. The peak retention volume is a function of gradient slope in LGE-IEC. The peak salt concentration I_R increases with increasing gradient slope, $g = (I_f - I_0)/V_g = (I_f - I_0)/(Ft_g)$ [M/mL] (I_f: final salt concentration, I_0:initial salt concentration, V_g: gradient volume, t_g: gradient time). The I_R values can be correlated with the following normalized gradient slope,

$$GH = (gV_o)[(V_t - V_o)/V_o] = g(V_t - V_o) \quad (1)$$

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

$$g = (I_F - I_o)/V_g \quad (2)$$

I_F is the final salt concentration, I_o is the initial salt concentration, and V_g is the gradient volume. 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 .

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

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

$$A = K_e \Lambda^{B} \qquad (4)$$

Here, B is the number of sites (charges) involved in protein adsorption, which is basically the same as the Z number [5] and the characteristic charge [6], 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 .

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

Here K is the protein distribution coefficient, K' is the distribution coefficient of salt, and I is the ionic strength (salt concentration). The SMA model equation [6] is reduced to Eq.(5) when the sample loading is low (Λ is not influenced by protein).

Results and Discussion

Figure 1 shows the binding site (B) values as a function of mobile phase pH for an acidic protein. β-lactoglobulin and a basic protein, ribonulease A [3]. For anion exchange chromatography AIEC and cation exchange chromatography CIEC, the B value – pH curve corresponds to the net charge-pH curve although the B value is not close to 0 at the pI of the protein. This is because the surface charge distribution is recognized by the ion-exchange group. On the other hand, the retention behavior for the HAC system is complicated, and difficult to understand. For the ribonuclease A (basic protein)- HAC system, the B-pH curve is similar to the ribonucleaseA-CIEC system. The low B value at pH 6 for the HA is due to the lowering of the dissociation of phosphate-site of the HA. It has been shown that HAC behaves like CIEC for basic proteins. Acidic protein- HA systems are more complex. Both the B and $I_R$values decrease with increasing pH although the net charge of acidic proteins increases. The interaction between acidic proteins and HA (calcium-site) might not due to electrostatic interaction but due to coordinate-bonding or ligand exchange. Furthermore the dissociation of calcium site is not constant but decreasing with pH, and the repulsion by the phosphate-site increases with pH.

Figure 2 shows the binding site (*B*) values as a function of mobile phase pH for β-lactoglobulin with different ion-exchange chromatography media. The B values are similar for different IEC media. This indicates the matrix properties or even the pore structure does not affect the binding site significantly.

Figures 3 shows schematic drawing of the relationship between *B* and pH where as the concept of binding site is shown in Figure 4.

We have also applied this method to cat-ion exchange chromatography (CIEC) of monoclonal antibodies by using 28 humanized monoclonal antibodies (hMab) model sample. By considering the structure of 28 hMab structure, we speculated that the surface positive charge distribution of the VH region is recognized by CIEC[4].

Finally, the retention of DNA was systematically studied with this method. It was found that the binding site *B* values of small oligo DNA (up to 12mer) correspond to the number of charges of DNA. So even such small molecules the *B* value is over 10 while medium-size protein show the B value in the range of 2 to 9 (see Figs 1 and 2).

We are now investigating how the conformational changes of proteins and DNAs affect IEC separations behavior by using the same method.

References

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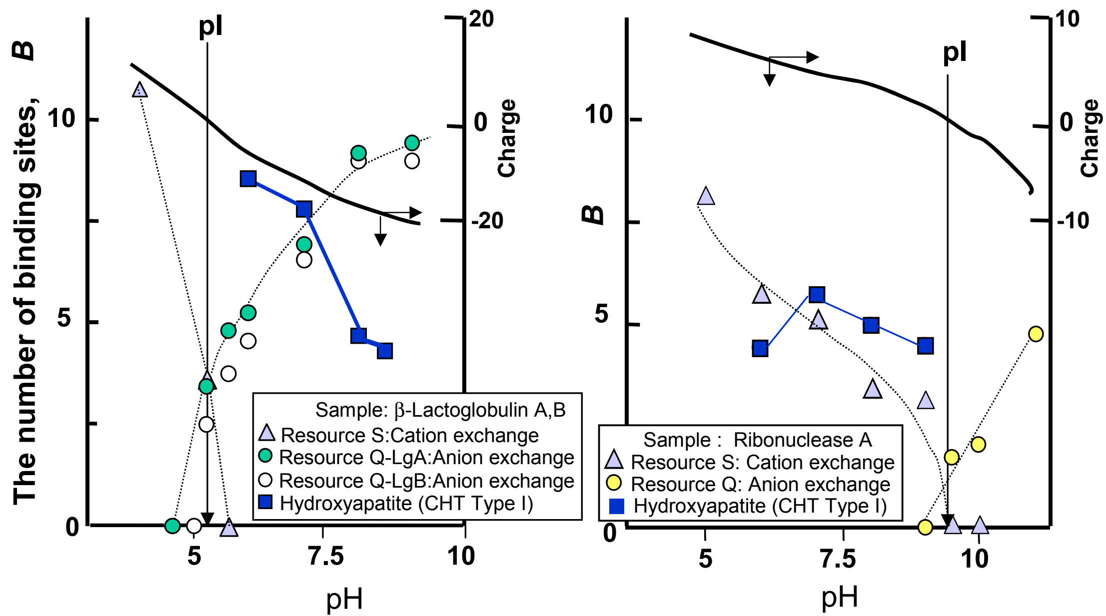

Figure 1 *B*-pH curves for ion exchange chromatography and hytdroxyapatite chromatography [3]

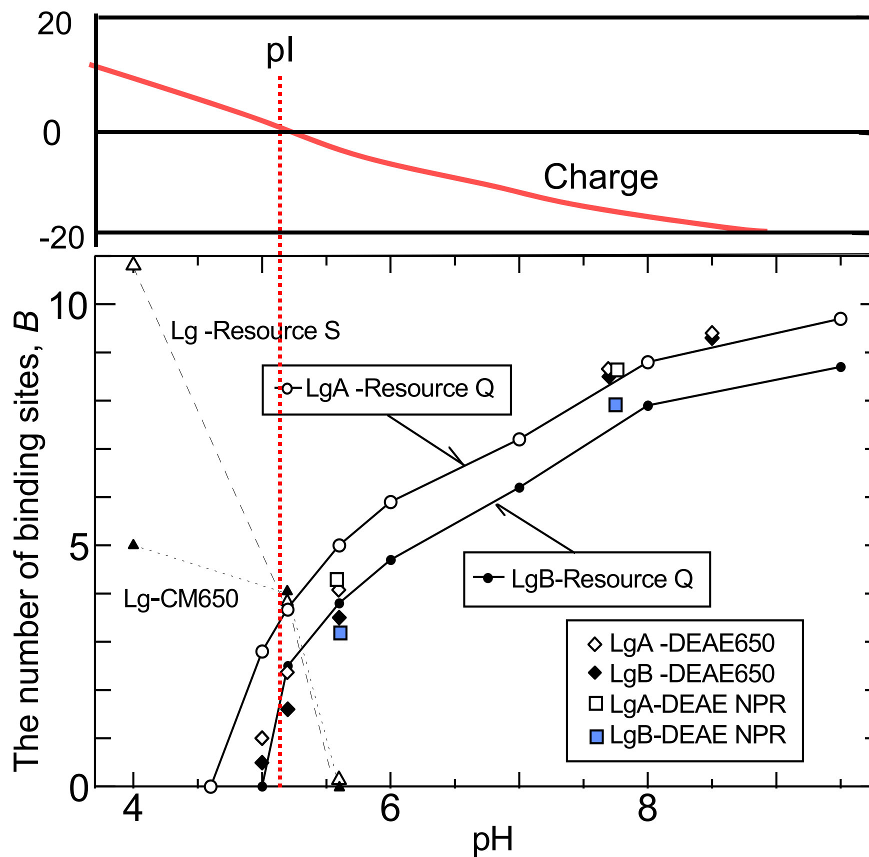

Figure 2 *B*-pH curves for ion exchange chromatography of different media [2].
650=Tosoh Toyopearl 650, DEAE-NPR=Tosoh DEAE NPR(non-porous)

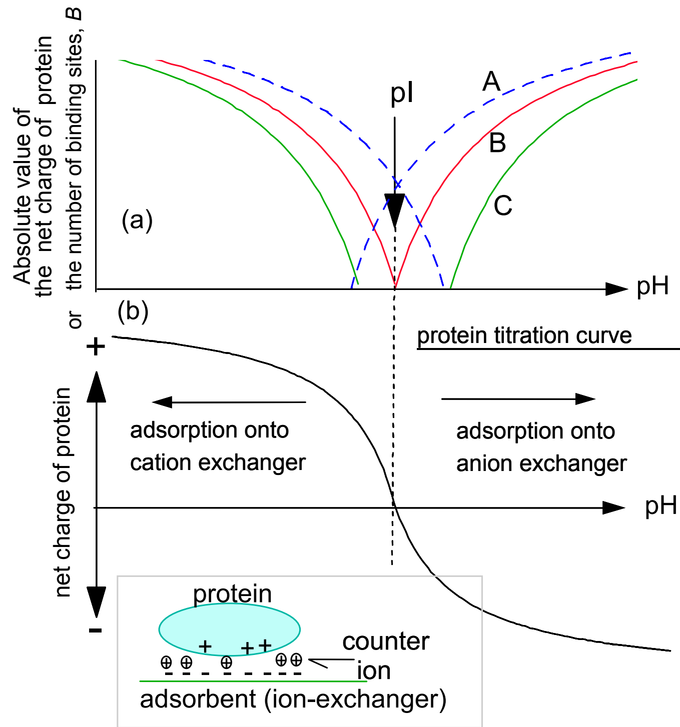

Figure 3 Schematical drawing on the net charge (or the binding site) and pH relationships [3]

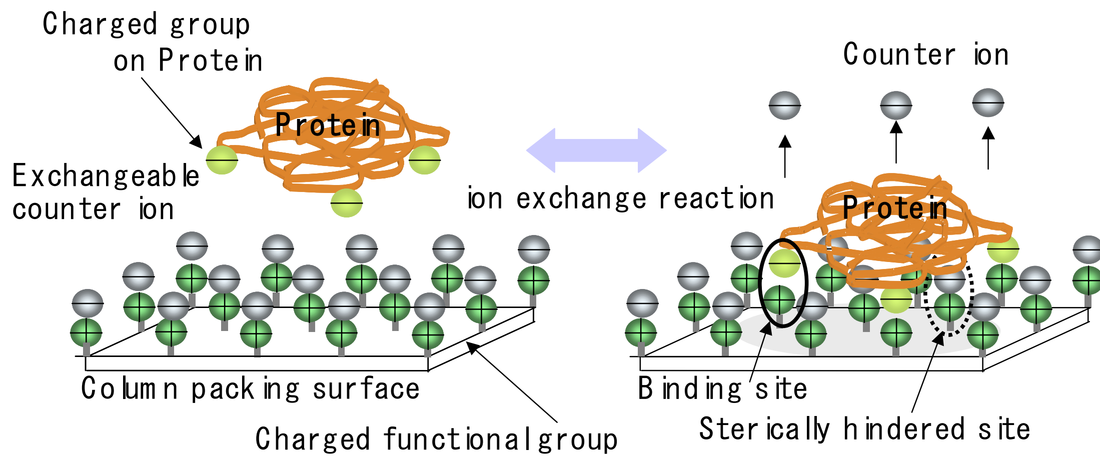

Figure 4 Electrostatic interaction (Ion-exchange reaction) between a protein and ion-exchange groups [3]