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Resolution and Retention of Proteins near Isoelectric Points in Ion-Exchange Chromatography. Molecular Recognition in Electrostatic Interaction Chromatography

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ABSTRACT

Both molecular recognition and transport phenomena play important roles in very high resolution ion-exchange (electrostatic interaction) chromatography (IEC) of proteins. A fast and simple method for obtaining important information on molecular recognition, peak retention (and the number of binding sites), and peak resolution of proteins from linear gradient elution experiments (salt concentration is increased linearly at a fixed mobile phase pH) is described. The proposed method was verified with β-lactoglobulin A and B (LgA, LgB) as model proteins. The gradient elution experimental data were obtained over a wide range of mobile phase pH with various types of IEC media. The number of binding sites involved in the retention (adsorption) decreased as the mobile phase pH approached the isoelectric points pI (= 5.1–5.2). However, even at pH 5.2 both LgA and LgB were retained on anion- and cation-exchange chromatography columns. The separation (resolution) of LgA and LgB became better when the pH approached the pI in anion-exchange chromatography columns where the number of adsorption site values are small (ca. 2–3). The two proteins were not separated even with efficient cation-exchange chromatography columns. The resolution and the retention near the pI (pH 5.2) did not significantly depend on sample loading compared with those at pH 6.0.

Key Words. Gradient elution ion-exchange chromatography; Molecular recognition; Resolution in gradient elution; Number of adsorption sites

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INTRODUCTION

Ion-exchange chromatography (IEC) is an efficient separation method of proteins where electrostatic interaction is the principle of separation (1–7). Although various types of chromatographic operations are possible (4, 7–9), linear gradient elution (LGE) IEC is most efficient for purifying a target protein from similar contaminant proteins. LGE-IEC is a method in which a linear increase of salt concentration is applied to an IEC column at a fixed pH to elute proteins which are initially loaded on the column. This method is best suited for recombinant protein-based pharmaceutical purification processes as many isoforms must be removed completely (7). However, since IEC has many operating and column variables, it is not easy to optimize the separation. In addition, such mobile phase properties as pH and salt concentration very often affect the retention and the resolution of proteins significantly. It is known that the surface charge distribution rather than the total net charge is responsible for the binding (adsorption or interaction) in IEC of proteins (1–4, 10–13). For example, we have reported that two different forms of a milk protein, β-lactoglobulins A and B (hereafter LgA and LgB) (14), are retained even near their isoelectric points (pI = 5.1–5.2) (15, 16) on both anion- and cation-exchange columns, and can be separated only on anion-exchange columns under certain mobile phase and operating conditions (4, 12, 13). Molecular recognition plays an important role in high resolution IEC although transport phenomena governs the separation performance mainly due to very low diffusion in pores of porous media (particles). Both molecular recognition and transport phenomena must be analyzed simultaneously to establish very high resolution, high performance IEC of proteins.

In this paper we first show how to obtain important information on molecular recognition such as the number of binding sites in IEC from linear gradient elution (LGE) experiments. Then, by using this method we investigated the retention and the resolution of proteins on various types of IEC media as a function of pH. The model proteins used were LgA and LgB. The resolution and retention near the pI were carefully examined because many proteins behave very differently. Effects of sample loadings near the pI were investigated. The solubilities of the model proteins near the pI were also measured and compared with other standard proteins.

EXPERIMENTAL

Most experiments were performed on fully automated liquid chromatography systems [BIOCAD (Perseptive Biosystems, Boston, USA) and Prosys workstation (Beckman, Fullerton, USA)]. Bovine milk β-lactoglobulin (Lg) was obtained from Sigma (product no. L0130, St. Louis, MO), which contains both β-lactoglobulin A (LgA) and B (LgB). Bovine serum albumin (BSA) and

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ovalbumin (OVA) were also purchased from Sigma. Other reagents were of analytical grade.

Ion-Exchange Chromatography Media

Anion-exchange media: Q-Sepharose HP, ANX-Sepharose HP (nominal particle diameter d_p = 34 μm), Resource Q (d_p = 15 μm), TSK gel DEAE-NPR (d_p = 2.5 μm). Cation exchange media: SP-Sepharose HP, CM-Sepharose HP (d_p = 34 μm), Resource S (d_p = 15 μm), TSK gel SP-NPR (d_p = 2.5 μm). Sepharose (agarose-based media) and Resource (styrene-divinylbenzene-based media) are products of Amersham Pharmacia Biotech (Uppsala, Sweden). TSK-gel NPR (nonporous polymer particle) media were supplied from Tosoh (Tokyo, Japan). Sepharose gels were packed into a glass column (0.8 cm ID and bed height Z = 10 cm) according to the recommended packing procedure (5). Resource (0.64 cm ID and Z = 3 cm) and NPR (0.46 cm ID and Z = 3.5 cm) columns were supplied as packed columns. Buffer solutions were 10 mM acetate buffer (pH 4.0–6.0) or 10 mM Tris-HCl buffer (pH 7.0–9.5). The initial mobile phase solution (Solution A) was the buffer containing 0.03 M NaCl, and the final solution (Solution B) was the same buffer containing 0.5 M NaCl. The gradient slopes g [M/mL = (mol/dm^3)/cm^3] were chosen so that baseline separation of LgA and LgB was attained (gradient volume = 4–32 column bed volume). Unless otherwise noted, the following experimental conditions were employed: The volumetric flow rate F was 0.5 mL/min for NPR and 1 mL/min for Resource and Sepharose HP. The linear mobile phase velocity u was calculated with the column cross-sectional area A_c and the column bed void fraction ε as $u = F/(A_c\varepsilon)$. The column bed void fraction ε was determined from the peak retention volume of Dextran T2000 pulses. The sample (Lg) concentration was 1 mg/mL, and the sample volume was 0.5 mL. The experiments were done at 298 ± 1 K.

RESULTS

Shortcut Methods for Obtaining the Distribution Coefficient by Linear Gradient Elution Experiments

Linear gradient elution (LGE)-IEC is a method in which a linear increase of salt concentration is introduced to an IEC column inlet at a fixed pH after a sample protein is charged to the column. The charged protein is first strongly retained (adsorbed) to the column, and then gradually moves down the column after the introduction of the linear increase of salt concentration, I. This is because the distribution coefficient K decreases sharply with increasing I. It is important to know $K(I)$ for predicting the peak retention time in LGE (4, 8, 9, 12, 13).

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Important information on the electrostatic interaction between proteins and IEC media can be extracted from $K(I)$ as follows: If we assume that the law of mass action (ion-exchange equilibrium) is valid (1–4, 10–13, 18–21) and K does not depend on the protein concentration C_P (low C_P or linear adsorption equilibrium), the following equation is derived:

$$K = K_e \Lambda^B I^{-B} \tag{1}$$

where K_e is the equilibrium association constant, Λ is the (effective) total ion-exchange capacity, and B is the number of sites (charges) (per one protein molecule) involved in protein adsorption, which is basically the same as the "Z" number in the literature (1–3, 10, 20). Basically $K(I)$ can be obtained either by isocratic elution or by a batch experiment. However, there are several difficulties in obtaining $K(I)$ by these methods. First, there is no a priori information on $K(I)$ so that it is not easy to choose I arbitrarily. For example, if I is too low, the corresponding K value may be so large (say $K \gg 10$) that the peak can not be detected. $K(I)$ should be determined in the range of $K = 1$ to 10. Second, even in the isocratic elution method it is not easy to detect a target protein peak if contaminants are included. In many cases the resolution of the target protein in isocratic elution is not good enough. On the contrary, in LGE-IEC most proteins are eluted in the range of $I = 0.03$ to 0.5 M ($=$ mol/dm^3). The resolution can be easily improved by decreasing the flow velocity as well as by decreasing the gradient slope (4, 17, 18).

Our method for obtaining $K(I)$ or predicting the peak retention volume is explained briefly (4, 12, 13, 17, 18). LGE-IEC experiments are performed at different gradient slopes g ($= I_f - I_0)/V_G$, [M/mL]) at a fixed pH ($I_f =$ final salt concentration, $I_0 =$ initial salt concentration, $V_G =$ gradient volume). The salt concentration at the peak position I_R (see Fig. 1) is determined as a function of the normalized gradient slope $GH = gV_s = g(V_t - V_0)$, where $V_t =$ total bed volume and $V_0 =$ void volume. The GH–I_R curve thus constructed does not depend on the flow velocity, the column dimension, the sample loading (if it is not in overloading conditions), or the initial salt concentration I_0 (4, 12, 17, 18). It is recommended that GH values are in the range of 0.001 to 0.05. The experimental GH–I_R data can usually be expressed by the following equation (4, 12, 13, 18):

$$GH = I_R^{(B+1)}/[A(B+1)] \tag{2}$$

From the law of mass action (ion-exchange equilibrium) (1–4, 10–13, 18–21), the following relationship can be derived:

$$A = K_e \Lambda^B \tag{3}$$

If we are only interested in predicting the peak retention in LGE-IEC, Eq. (2) can be used with A and B as experimental values (4, 12, 18). However, if

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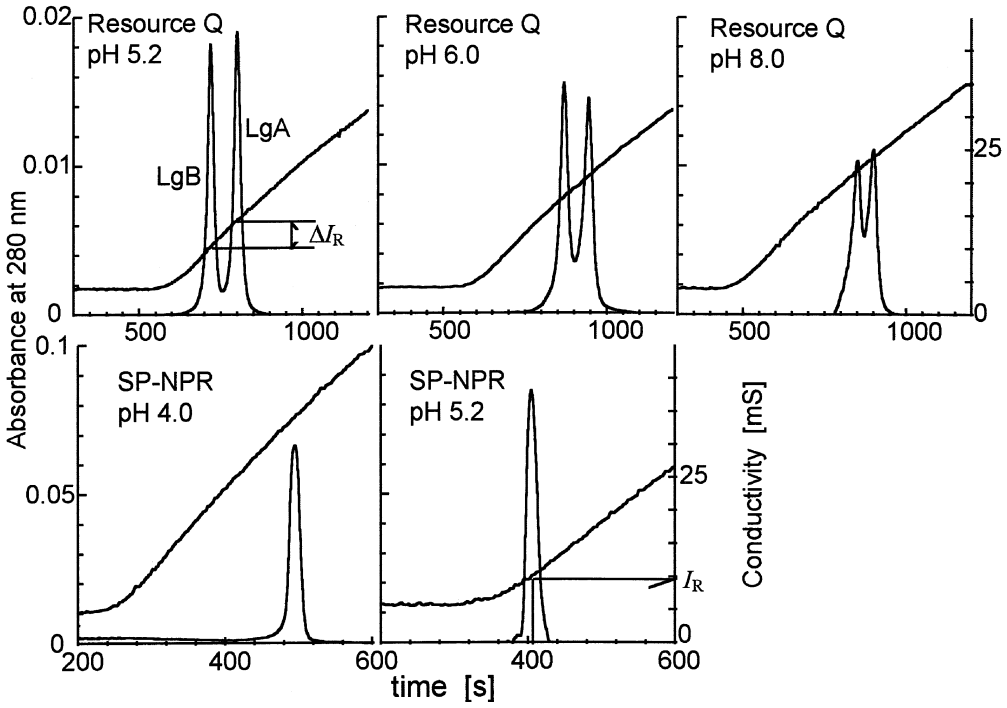

FIG. 1 Typical elution curves of β-lactoglobulin A (LgA) and B (LgB). The same gradient slope and the same flow rate were employed for Resource Q. LgA and LgB were not separated (resolved) for SP-NPR even at a very shallow gradient slope (pH 5.2).

GH–I_R curves as a function of pH are prepared and the B values determined from the GH–I_R curves are plotted against the mobile phase pH, quite important information can be obtained on the retention (or molecular recognition) and the resolution of proteins as a function of pH. When the B–pH relationships are compared with the protein titration curve and/or the protein surface charge distribution, information on the molecular recognition (binding mechanism) can be obtained. This is especially useful near the protein isoelectric point pI as many proteins behave very differently near the pI.

Application of the Proposed Method for the Separation of β-Lactoglobulin A and B Forms

Typical elution curves (chromatograms) in LGE-IEC are shown in Fig. 1. β-Lactoglobulin A (LgA) and β-lactoglobulin (LgB) were separated on anion-exchange chromatography (AIEC) columns at pH 5.2 although the degree of resolution varied from media to media (and depended on the operating conditions) as shown later. The resolution became poorer when the pH was increased from 5.2. The two proteins were not separated on any cation-exchange chromatography (CIEC) columns used in this study at pH 4–5.6, not even on

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the most efficient column (nonporous HPLC, SP-NPR) at shallow gradient slopes as shown in Fig. 1.

The GH–I_R curves shifted to larger I_R values and became steeper with increasing pH in the AIEC columns (4, 12, 13) as shown in Fig. 2. The slope on a log-log scale increases with increasing pH. This implies that the number of adsorption sites B decreases when the pH approaches the pI, which is understandable in terms of the protein titration curve (1–6). The slope increased with decreasing pH in the CIEC columns although the separation (resolution) of LgA and LgB was not observed under the conditions employed here, as stated previously.

The B values determined from the GH–I_R curve as a function of pH are shown in Fig. 3. When the pH for AIEC was increased, the B values increased. Similarly, the B values in CIEC increase with decreasing pH from the pI. The reason for a much lower B value for CM Toyopearl 650 at pH 4 than that for Resource S is quite likely ascribable to its weak cation-exchange functionality because the effective ion-exchange capacity of CM650 decreases significantly when the pH is below 5.0 (4). Even near the pI (pH 5.2), LgA and LgB were retained on both AIEC and CIEC columns. This can not be explained on the basis of a simple protein net charge concept as already pointed out by Regnier and coworkers (1, 10). The GH–I_R curves on a log-log scale for various AIEC columns at pH 5.2 were examined. The peak salt concentration I_R value at a certain GH was different from media to media. However, the slope on a log-log scale, namely the B value, was similar for Resource Q, DEAE 650, and DEAE NPR (nonporous particle) although some scatter was observed. This suggests that the pore structure, the base matrix properties, or the ion-ex-

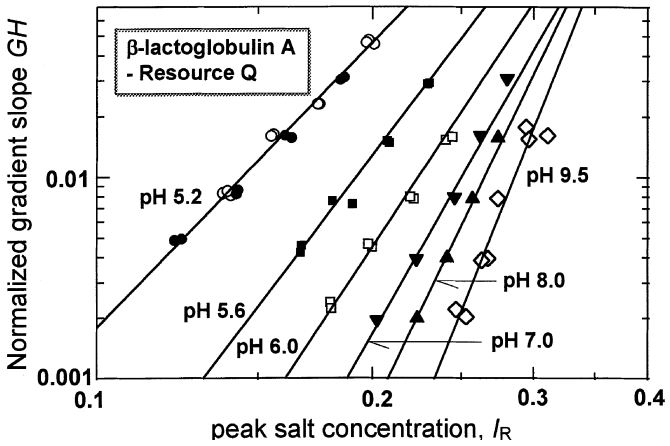

FIG. 2 GH vs I_R of β-lactoglobulin A on Resource Q as a function of mobile phase pH. Note that GH–I_R curves do not depend on the flow velocity (3–25 cm/min). For the sake of clarity, only the data points at pH 5.2 are shown. (●) = 3 cm/min, (○) = 25 cm/min.

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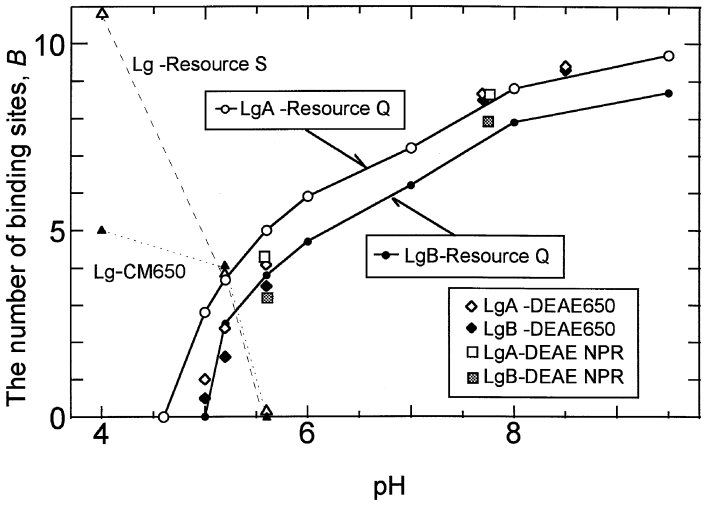

FIG. 3 The number of binding sites, B, as a function of mobile phase pH. LgA = β-lactoglobulin A, LgB = β-lactoglobulin B, DEAE650 = DEAE Toyopearl 650, CM650 = CM Toyopearl 650 (data taken from Ref. 12), and DEAE NPR = TSK gel DEAE NPR.

change group properties do not affect significantly the protein–IEC interaction (binding or adsorption). The B value (LgA) minus the B value (LgB) for AIEC was ca. 1.0 for pH 5.2 to 9.5.

The resolution (separation) R_s values were highest around pH 5.2 (near the pI) with the AIEC columns as shown in Fig. 4 [$R_s = 2(t_{R,LgA} - t_{R,LgB})/(W_{LgB}$

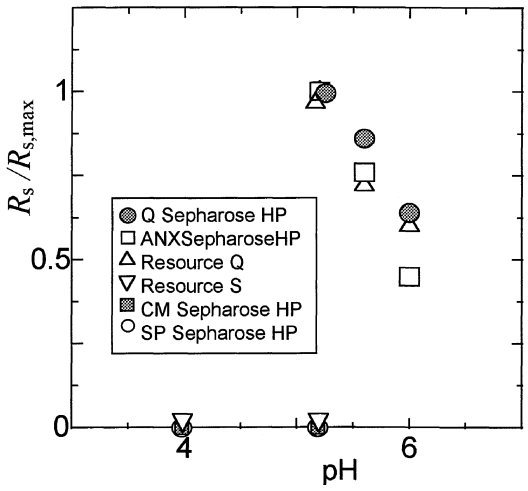

FIG. 4 Effect of pH on the resolution R_s of LgA and LgB. $R_{s,max} = R_s$ at pH 5.2; $R_s = 0$ for CIEC columns as LgA and LgB were not resolved. Note that the experimental data at $GH = 0.02$ and $u = 7$ cm/min for Sepharose and $GH = 0.008$ and $u = 8.5$ cm/min for Resource were used for Figs. 4 and 5.

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$+ W_{LgA}$), t_R = retention time, W = peak width at baseline in time unit]. The R_s decreased very sharply with increasing pH for AIEC from pH 5.2 to 7.0. We have already proposed a dimensionless parameter $Y = [(ZD_aI_a)/(GHud_p^2)]$ and shown that the resolution R_s can be tuned on the basis of this parameter (4, 17). D_aI_a is a dummy variable having a numerical value of 1 so that Y becomes dimensionless. Further, when R_s values at pH 5.2 for various AIEC columns are corrected as R_s' [$= R_s\ (\Delta I_{R,\text{Resource Q}}/\Delta I_R)$], R_s' were well correlated with Y [$\Delta I_R = (I_{R,LgA} - I_{R,LgB})$ = peak salt concentration difference, $\Delta I_{R,\text{Resource Q}} = \Delta I_R$ for Resource Q column] (13).

Figure 5 shows the relationships between $\Delta I_R/\Delta I_{R,max}$ and the mobile phase pH. The values at pH 5.2 were taken as reference values. A sharp decrease in ΔI_R with increasing pH is responsible for a sharp decrease in R_s as shown in Fig. 4. Because of their weak anion-exchange functionality, LgA and LgB did not separate on ANX HP at pH 9.5.

One of the concerns on IEC of proteins near the pI is that the solubility of proteins becomes low (precipitation may occur) and the resolution may be quite sensitive to sample loading. Figure 6 shows the absorbance at 600 nm (turbidity) of Lg, bovine serum albumin (BSA), and ovalbumin (OVA). BSA showed a sharp increase in turbidity when the concentration was in the 1 to 10 mg/mL range. The turbidity of OVA at pH 4.8 also increased when the concentration was higher than 10 mg/mL. Lg was quite stable and did not show a remarkable increase in turbidity even when the concentration was higher than 10 mg/mL.

The effects of sample loading on the resolution R_s and the peak salt concentration I_R are shown in Figs 7 and 8. Both R_s and I_R values were constant up to

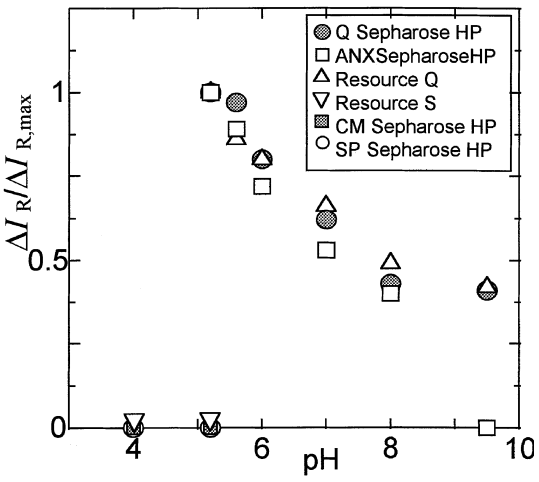

FIG. 5 Effect of pH on the peak salt concentration difference. ΔI_R = (I_R for LgA) − (I_R for LgB), $\Delta I_{R,max} = \Delta I_R$ at pH 5.2. $\Delta I_R = 0$ for CIEC columns as LgA and LgB were not resolved.

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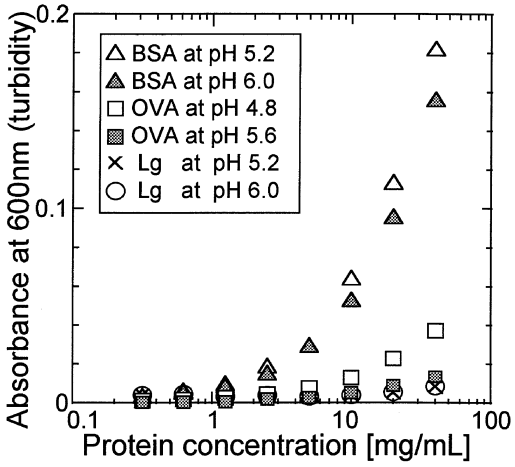

FIG. 6 Turbidity (absorbance at 600 nm) as a function of protein concentration. BSA = bovine serum albumin, OVA = ovalbumin, Lg = β-latctoglobulin.

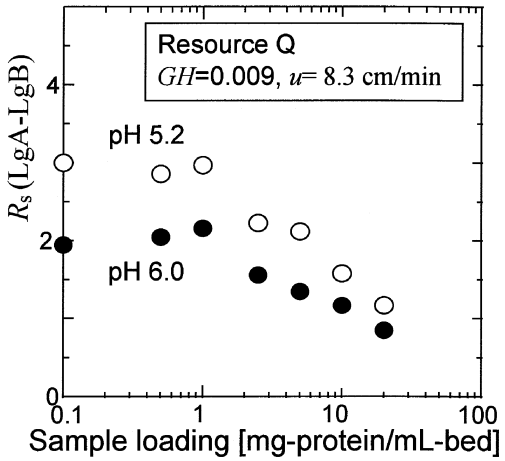

FIG. 7 Effect of sample loading on the resolution R_s of β-latctoglobulins A (LgA) and B (LgB).

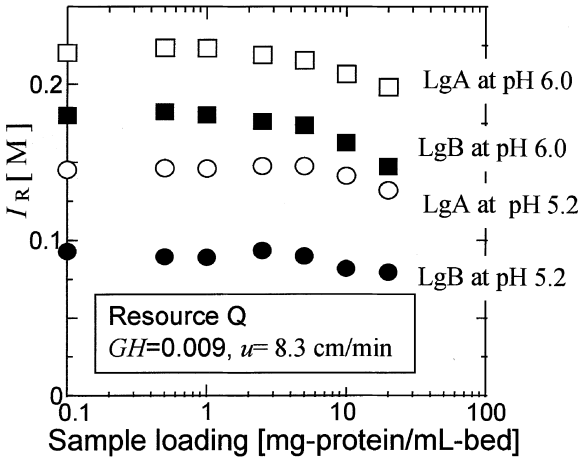

FIG. 8 Effect of sample loading on the peak salt concentration (salt concentration at the peak retention time) I_R of β-latctoglobulins A (LgA) and B (LgB).

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1 mg-protein/mL-bed. The R_s values at pH 5.2 were higher than those at pH 6.0 even when the sample loading was 20 mg/mL. The dependence of I_R on the sample loading at pH 5.2 was similar to or slightly weaker than that at pH 6.0.

DISCUSSION

β-Lactoglobulin A and B (LgA, LgB) are genetic variants (14) and known to be proteins having very asymmetrical charge distributions (2). LgA has one more negative charge in terms of their amino acid compositions. The 3D-structure examined by computer (RasMol viewing system with data from The Protein Data Bank) indicates that this negative charge is located on the surface of the protein. It may be explained that because of this additional negative charge, the separation of LgA and LgB is possible only with AIEC columns. In other words, electrostatic interaction-based molecular recognition governs high resolution near p1 for anion-exchange chromatography although there might be some additional interaction such as hydrophobic interaction.

The present study showed that LgA and LgB can be most efficiently separated at pH 5.2 (near the pI values) where the number of adsorption site *B* values range between 2 and 4. Multipoint attachment, a typical characteristic of protein adsorption in IEC, is not involved in this high resolution IEC of LgA and LgB. Increasing pH from the pI increased B values both for LgA and LgB although the resolution became worse as shown in Fig. 4. There are some applications where the IEC of proteins are performed near the pI. For example, blood plasma protein fractionation (albumin and γ-globulin) is carried out at pH 5.2, which is near the pI of albumin (4.9) (2). A weak interaction may be better for the fine separation of similar proteins. In order to establish a rule of thumb (heuristics) for very fine IEC separation of similar proteins, further research should be done on the basis of various experimental data with well-characterized proteins having very similar physical and biochemical properties. For that purpose, we are investigating other model protein separation systems.

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