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Short communication

Ion-exchange chromatography of proteins near the isoelectric points

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Abstract

The retention and the resolution of β-lactoglobulin A and B (LgA, LgB) were investigated with various ion-exchange chromatography media. The number of sites involved in the retention (adsorption) decreased as the mobile phase pH approached the isoelectric points p*I* (=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 p*I* in anion-exchange chromatography columns where the number of adsorption site values are small (ca. 2–3). The two proteins were not separated on cation-exchange chromatography columns. Factors affecting the resolution and the retention near the p*I* were discussed.

Keywords: Isoelectric points; Lactoglobulins; Proteins

1. Introduction

Gradient elution ion-exchange chromatography (IEC) is a very powerful method for purifying proteins [1–6]. However, as the separation performance is governed by various operating and column variables in a complicated manner, it is not easy to optimize the separation. In addition, the mobile phase properties such 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 [1–4,7–9]. Among many standard proteins, two different forms of a milk protein, β-lactoglobulin A and B (hereafter LgA, LgB) [10] are known to show a very strange retention behavior near the isoelectric points (p*I*) [1–5,7–9]. LgA has one more negative charge in terms of the amino acid compositions, which causes the slightly lower p*I* value (ca. 5.1) than LgB (ca. 5.2) although the molecular weights are essentially the same (35 000) [7,8,11,12]. We have shown that LgA and LgB can be most efficiently separated on an anion-exchange column (Tosoh DEAE Toyopearl 650) at pH 5.2 which is almost close to their p*I* values [4,13].

In this paper, the retention and the resolution of LgA and LgB near the p*I* were investigated by using various types of ion-exchange chromatography media (Source, Sepharose, Toyopearl). These media have different base matrix gels and different ion-exchange groups. The salt concentration at the peak position I_R was determined from the gradient elution IEC for different gradient slopes at various pH's. Then, the normalized gradient slope GH and the I_R plots were constructed as a function of pH. The GH–I_R relationships were analyzed to examine the retention and separation mechanism of proteins near the p*I*. A simple ion-exchange model was applied to determine the number of sites involved in the retention (adsorption) of proteins, B from the GH–I_R curve. The B values for LgA and LgB as a function

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of pH were examined in order to extract possible explanations for the retention behavior and also good resolution with the anion-exchange columns near the p*I* (pH 5.2). A dimensionless variable for correlating the resolution in gradient elution chromatography reported previously was modified, and applied to the experimental results.

2. Experimental

Most experiments were performed on fully automated liquid chromatography systems, BioCad (Perseptive Biosystems, Boston, MA, USA) and Prosys workstation (Beckman, Fullerton, USA)]. Bovine milk β-lactoglobulin (Lg) was obtained from Sigma (product No. L0130, St. Louis, MO, USA), which contains both LgA and LgB. Other reagents were of analytical grade.

2.1. Ion-exchange chromatography media

Anion-exchange media: Q-Sepharose HP, ANX-Sepharose HP (nominal particle diameter d_p=34 μm), DEAE-Sepharose FF (d_p=90 μm), Resource Q (d_p=15 μm), DEAE-Toyopearl 650S (d_p=40 μm). Cation-exchange media: SP-Sepharose HP, CM-Sepharose HP (d_p=34 μm), Resource S (d_p=15 μm), CM-Toyopearl 650S (d_p=40 μm). Sepharose (agarose-based media) and Resource (stylene–divinylbenzene-based media) are products of Amersham Pharmacia Biotech (Uppsala, Sweden). Toyopearl (hydrophilic vinyl polymer) media were supplied from Tosoh (Tokyo, Japan). Sepharose gels were packed into a glass column (0.8 cm I.D. and bed height Z=15 cm) according to the recommended packing procedure [5]. Resource was supplied as a packed column (0.64 cm I.D. and Z=3 cm). Buffer solutions were 10 m*M* acetate buffer (pH 4.0–6.0) or 10 m*M* 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. Therefore, the g values for DEAE-Sepharose FF were much lower than those for the other media (gradient volume=4–32 column bed volume). The volumetric flow-rate was 0.5 ml/min for Sepharose FF, 8 ml/min for Resource and 1 ml/min for Sepharose HP. The sample (Lg) concentration was 1 mg/ml and the sample volume was 0.5 ml. The experiments were done at 298±1 K.

3. Results and discussion

Typical elution curves (chromatograms) in linear gradient elution are shown in Fig. 1. LgA and LgB were separated on anion-exchange chromatography (AIEC) columns at pH 5.2 although the degree of resolution varied from media to media (and with the operating conditions) as shown later. The resolution became poor 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.

The salt concentration at which the peak is eluted I_R was measured at various gradient slopes g, [M/ml] and plotted against the normalized gradient slope $GH=gV_s=g(V_t-V_0)$ where V_t=total bed volume and V_0=void volume. Usually the experimental GH–I_R data can be expressed by the following equation [4,9,10].

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

From the law of mass action (ion-exchange equilibrium) [1–4,7,9,15–17], the following relationship can be derived.

$$A = K_e \, \Lambda^B \quad (2)$$

where B is the number of sites (charges) involved in protein adsorption, which is basically the same as the 'Z' number in the literature [1–3,7,16]. K_e is the equilibrium association constant and Λ is the total ion-exchange capacity.

The GH–I_R curves shifted to larger I_R values and became steeper with increasing pH in the AIEC columns [4,9]. The slope on a log–log scale increases with increasing pH in the AIEC columns. This implies that the number of adsorption sites decreases when the pH approaches the p*I*, which is understandable in terms of the protein titration curve [2,3,8]. The slope increased with decreasing pH in the CIEC columns although the separation (resolu-

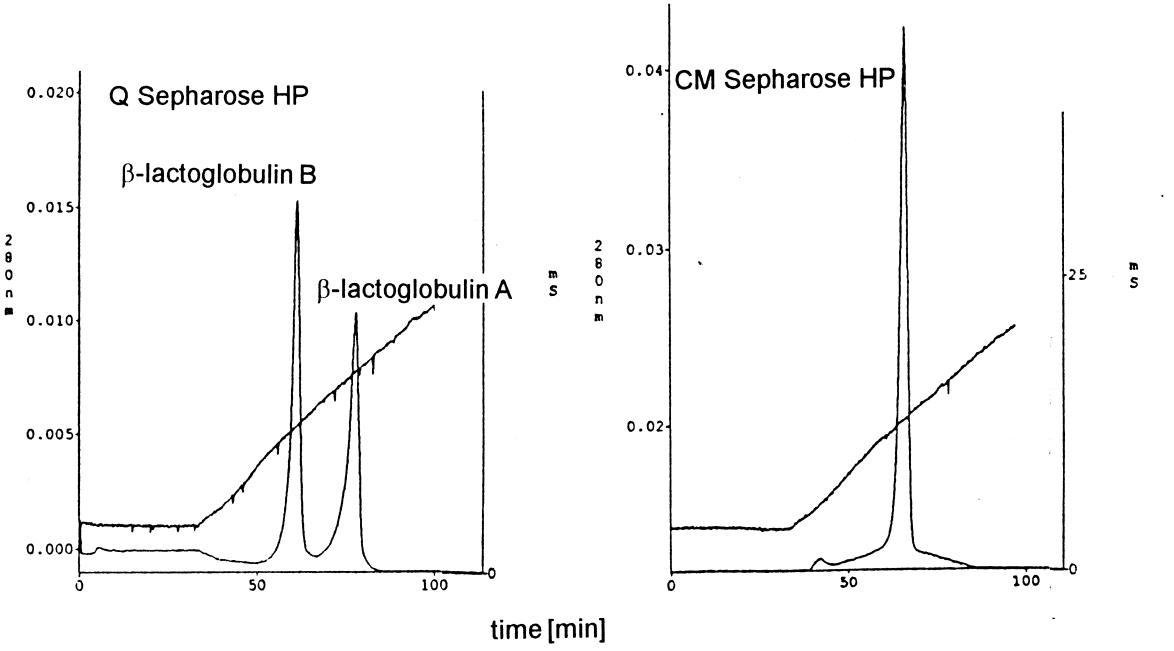

Fig. 1. Gradient elution curves of β-lactoglobulin at pH 5.2 with anion-exchange chromatography (AIEC), Q-Sepharose HP and with cation-exchange chromatography (CIEC), CM-Sepharose HP. The linear superficial velocity is 2 cm/min (=mobile phase velocity 5.7 cm/min). Note that β-lactoglobulin A and B (LgA, LgB) were completely separated with AIEC while they were not separated with CIEC.

tion) of LgA and LgB was not observed under the conditions employed here (even with very shallow gradient slopes at low flow velocities). The *B* values determined from the $GH–I_R$ curve as a function of pH are shown in Fig. 2. As is clear from Fig. 2, with increasing pH in the case of AIEC the *B* values increase and the difference between the *B* values of the two proteins decreases. Similarly, the *B* values in CIEC increases with decreasing pH from the p*I*. 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 as the effective ion-exchange capacity of CM650 decreases significantly when the pH is below 5.0 [4]. Even near the p*I* (pH 5.2) LgA and LgB were retained on both AIEC and CIEC columns. This is not expected on the basis of a simple protein net charge behavior. The resolution (separation) is highest around pH 5.2 (near the p*I*) with the AIEC columns. The $GH–I_R$ curves on a log–log scale for various AIEC columns at pH 5.2 are shown in Fig. 3. Although the I_R value at a certain *GH* is different from media to media, the slope on a log–log scale, namely, the number of adsorption sites is not much different. The slope for LgB was somewhat shallower than that for LgA. This implies less binding sites for LgB. The difference between the *B* values for LgA and those for LgB was ca. 1.0.

The resolution R_s values at pH 5.2 for various AIEC columns are shown in Fig. 4 as a function of a dimensionless parameter $[(ZD_aI_a)/(GH\ u\ d_p^2)]^{0.5}$ which was shown to be useful for optimizing the gradient elution performance [4,13–14]. However, as the peak salt concentration difference $\Delta I_R=(I_{R,LgA}-I_{R,LgB})$ was different from media to media (0.038–0.06 *M*), the R_s values were corrected with the ΔI_R of Resource Q ($\Delta I_{R,\text{Resource Q}}$). The corrected values $R_s'[=R_s\ (\Delta I_{R,\text{Resource Q}}/\Delta I_R)]$ were well correlated by a single curve. When ΔI_R values were taken into consideration, Q-HP is the most efficient media for the separation of LgA and LgB.

As described in Section 1, LgA and LgB are genetic variants, and known to be proteins having very asymmetrical charge distribution [2]. LgA has one more negative charge in terms of the amino acid compositions. It may be explained that because of

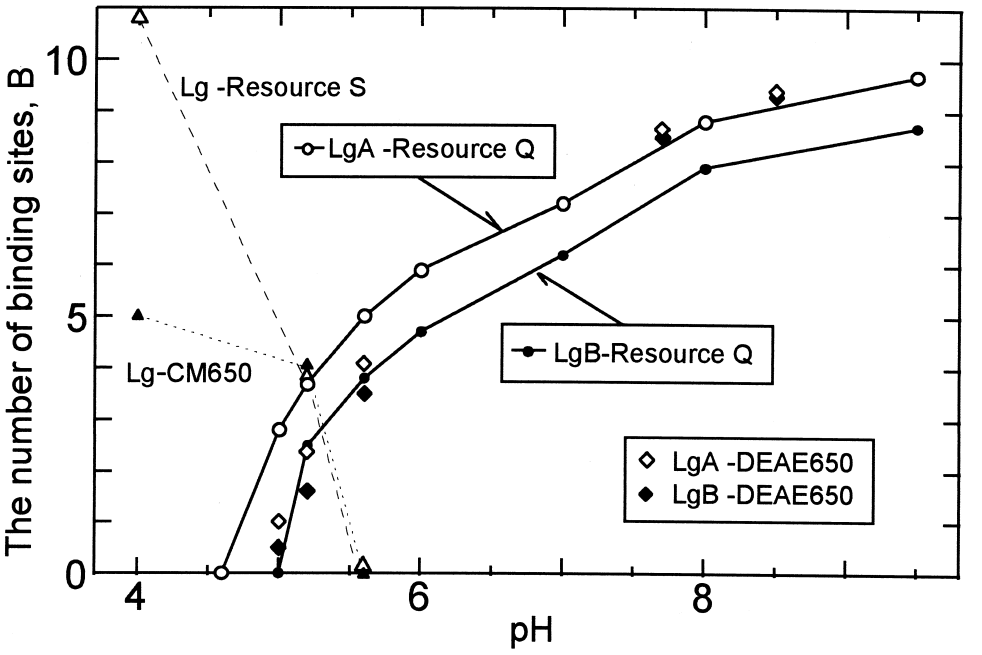

Fig. 2. The number of binding (adsorption) sites, *B* as a function of pH. The *B* values were determined from the GH–I_R curves such as those shown in Fig. 3. As β-lactoglobulin A(LgA) and β-lactoglobulin B(LgB) were not separated with CIEC (CM650 and Resource S), the *B* values are for LgA+LgB. CM650=CM Toyopearl 650S, DEAE650=DEAE Toyopearl 650S (data taken from Ref. [9]).

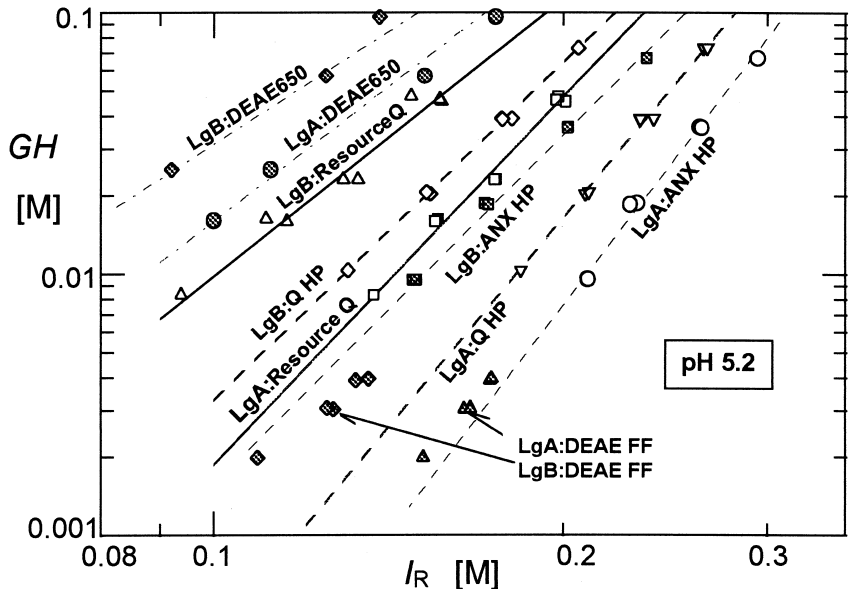

Fig. 3. GH–I_R plots for various IEC columns at pH 5.2. LgA=β-lactoglobulin A, LgB=β-lactoglobulin B, DEAE650=DEAE Toyopearl 650S, Res-Q=Resource Q, Q-HP=Q-Sepharose HP, ANX-HP=ANX-Sepharose HP, DEAE-FF=DEAE -Sepharose FF. Note that very shallow gradient slopes (small *GH* values) were chosen to attain the resolution for DEAE-FF.

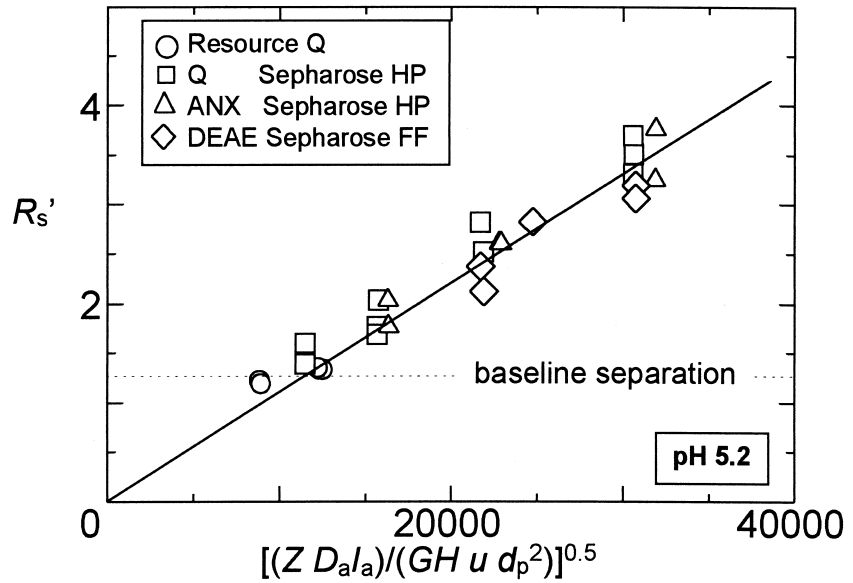

Fig. 4. Corrected Resolution R_s' as a function of $[(ZD_aI_a)/(GH\ u\ d_p^2)]^{0.5}$ at pH 5.2. D_aI_a having a value of 1 is the dummy variable so that $[(ZD_aI_a)/(GH\ u\ d_p^2)]$ becomes dimensionless. The R_s values were determined from the peak width of LgB, W_1 in time unit and the peak retention time t_R of the peak 1 (LgB) and 2 (LgA) as $R_s=(t_{R2}-t_{R1})/[(1/2)(W_1+W_2)]=(t_{R2}-t_{R1})/W_1$. This is because the LgA elution curves were skewed to lower elution volumes, especially at shallow gradient slopes. The R_s values were then corrected according to the following equation: $R_s'=R_s\ (\Delta I_{R,\text{Resource Q}}/\Delta I_R)$ where $\Delta I_R=(I_{R,LgA}-I_{R,LgB})$ is the difference in the salt concentration at which the peak is eluted. The ΔI_R value for Resource Q, $\Delta I_{R,\text{Resource Q}}$ is 0.053 *M* (average value for $GH=0.03$–0.09). The ΔI_R value for Q-Sepharose HP = 0.060 *M* (average value for $GH=0.04$–0.07).The ΔI_R value for ANX-Sepharose HP = 0.060 *M* (average value for $GH=0.04$–0.07). The ΔI_R value for DEAE-Sepharose FF = 0.040 *M* (average value for $GH=0.002$–0.004). The volumetric flow-rate = 8 ml/min for Resource Q, 0.94 ml/min for Q- and ANX-Sepharose HP and 0.66 ml/min for DEAE-Sepharose FF.

this additional negative charge, the separation of LgA and LgB is possible with AIEC columns. Although there might be some additional interaction such as hydrophobic interaction, high resolution near p*I* for anion-exchange chromatography is quite likely to be due to electrostatic interaction based molecular recognition.

The present experimental data showed that LgA and LgB can be easily separated at pH 5.2 (near the p*I* values) where the number of adsorption sites *B* values range between 2 and 4. Namely, multipoint attachment, a typical characteristic of protein adsorption in IEC is not attained. Increasing pH from the p*I* increased *B* values both for LgA and LgB although the resolution became worse. Blood plasma protein fractionation (albumin and γ-globulin) is also done at pH 5.2, which is near the p*I* of albumin (4.9) [2]. A weak interaction may be better for a fine separation of similar proteins. In order to establish a rule of sum (heuristics) for very fine IEC separation of similar proteins it is need to accumulate more experimental data with well characterized proteins having very similar physical and biochemical properties.

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