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Application of a chromatography model with linear gradient elution experimental data to the rapid scale-up in ion-exchange process chromatography of proteins

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Abstract

We applied the model described in our previous paper to the rapid scale-up in the ion exchange chromatography of proteins, in which linear flow velocity, column length and gradient slope were changed. We carried out linear gradient elution experiments, and obtained data for the peak salt concentration and peak width. From these data, the plate height (HETP) was calculated as a function of the mobile phase velocity and iso-resolution curve (the separation time and elution volume relationship for the same resolution) was calculated. The scale-up chromatography conditions were determined by the iso-resolution curve. The scale-up of the linear gradient elution from 5 to 100 mL and 2.5 L column sizes was performed both by the separation of β-lactoglobulin A and β-lactoglobulin B with anion-exchange chromatography and by the purification of a recombinant protein with cation-exchange chromatography. Resolution, recovery and purity were examined in order to verify the proposed method.

Keywords: Proteins; Ion-exchange chromatography; Separation; Chromatography models

1. Introduction

Ion-exchange chromatography (IEC) is one of the major unit operations in the protein drug purification process [1–5]. However, chromatographic process development for protein purification has been performed using a trial-and-error approach based on the heuristic knowledge, expertise and experience of the researchers involved; therefore, the process development is labor intensive and time consuming.

Scale-up of chromatography, an important process development item, is generally performed by keeping the linear flow velocity, gradient slope and column length constant while increasing the column diameter. However, these three parameters can be altered by limitations such as column compression, allowable process time and desired buffer consumption at scale-up manufacturing. In such cases, scale-up is difficult, and a trial-and-error approach is generally needed to choose the right conditions, which provide a resolution comparable to that achieved on a small scale, because in linear gradient elution IEC the gradient slope and the flow rate as well as the column length affect the separation behavior in a complicated way [3].

The chromatography model is considered to be a useful tool for the purpose of reducing traditional process development efforts. While researchers in academia have made extensive contributions to the modeling preparative chromatography of proteins [6,7], there have been relatively few attempts to use models for purification process development. Johnson and Frenz [8] have used a multi-component Temkin isotherm to model the fractionation of a heterogeneous mixture of a variable glycosylated protein. Shukla et al. [9] have used selective displacement chromatography for the purification of an antigenic protein from an industrial process stream. Staby et al. [10–14] have made a comparison of chromatographic ion exchange resins using models. We have developed a model to optimize linear gradient elution IEC, in which the same resolution can be obtained with a given column when the gradient slope, flow velocity and column length are adjusted (iso-resolution curve concept) [15–17].

In this study, we applied the iso-resolution curve concept for determining the right scale-up conditions rapidly and systematically, in which linear flow velocity, column length and

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gradient slope were changed. In the scale-up studies, we investigated the separation of β-lactoglobulin A and β-lactoglobulin B in anion-exchange chromatography (AEX) and the purification of a biopharmaceutical recombinant protein in cation-exchange chromatography (CEX).

2. Experimental

2.1. Chromatography media and column

SP Sepharose HP columns (6% cross-linked agarose, sulfopropyl group, particle diameter *ca.* 34 μm, column size 10 cm × 8 mm I.D., total bed volume V_t = 5 mL and 10 cm × 180 mm I.D., total bed volume V_t = 2.5 L) and Q Sepharose HP columns (6% cross-linked agarose, quaternary ammonium group, particle diameter *ca.* 34 μm, column size 10 cm × 8 mm I.D., total bed volume V_t = 5 mL, 3.5 cm × 6 cm I.D., total bed volume V_t = 100 mL and 10 cm × 180 mm I.D., total bed volume V_t = 2.5 L) were used as CEX and AEX media, respectively. These media are products of GE Healthcare (Uppsala, Sweden).

2.2. Materials

A standard model protein on Q Sepharose HP chromatography, Bovine milk β-lactoglobulin (Lg) was obtained from Sigma (product no. L0130, St. Louis, MO, USA), which contains both β-lactoglobulin A (LgA) and β-lactoglobulin B (LgB). LgA has one more negative charge in terms of the amino acid compositions, which causes a slightly lower isoelectric points (pI) value (ca. 5.1) than in LgB (ca. 5.2), although the molecular weights (Mr) are essentially the same (ca. 35,000). A recombinant protein (recP, pI ca. 9, Mr ca. 36,000) was produced at Kirin (Takasaki, Japan) by fermentation with *Escherichia coli* (*E. coli*). The recP is poly (ethylene glycol)-conjugated (or PEGylated) cytokine without sugar chains. Partially purified recP containing impurities was used for the load sample in SP Sepharose HP chromatography. Other reagents used in these studies were of analytical grade.

2.3. Chromatography apparatus

Most of the experiments with 5 and 100 mL columns were performed on a fully automated liquid chromatography system, the ÄKTAexplorer 100 (GE Healthcare). A scale-up experiment with 2.5 L columns was performed with the chromatography control system, K-prime (Millipore, Bedford, MA, USA).

2.4. Linear gradient elution experiment for model analysis

For Q Sepharose HP chromatography, 10 mM sodium acetate pH 5.6 containing 0.03 M NaCl and 10 mM sodium acetate pH 5.6 containing 0.5 M NaCl were used as the initial mobile phase and final mobile phase, respectively. For SP Sepharose HP chromatography, 10 mM sodium phosphate pH 8.0 and 10 mM sodium phosphate pH 8.0 containing 0.15 M NaCl were used as the initial mobile phase and final mobile phase, respectively. A 5 mL column was equilibrated with the initial mobile phase. The protein sample loading was fixed at 1 mg/mL-gel bed. Elution was performed with a linear gradient of NaCl by mixing the initial and final mobile phases. Namely, the NaCl concentration was increased with time at a fixed pH and fixed buffer compositions. The gradient slopes g (M/mL) were chosen so that gradient volume was 4, 8, 16, 32 or 48 columns per bed volume (CV). The volumetric flow rate (F) was 0.5, 0.75, 1.0, 1.5 or 2.0 mL/min. The linear mobile phase velocity (u) was calculated with the cross-sectional area (A_c) and the column bed void fraction (ε) as $u = F/(A_c\varepsilon)$. ε was determined from the peak retention volume of Dextran T 2000 pulses. The experiments were performed at room temperature.

2.5. Experiment for the scale-up study

The 100 mL and 2.5 L columns for the scale-up study were equilibrated with the same buffer in Section 2.4. The protein sample was loaded on the column with a 1 mg/mL-gel bed. After loading, the column was washed with 5 CV of the initial mobile phase. The linear gradient elution was performed with an increasing final mobile phase concentration from the initial mobile phase, and the gradient volume was set at a determined column volume. F was also set at determined values. The peak collection criteria of SP Sepharose HP chromatography were as follows: The fractions in the range of the UV output that is over 30% of the recP peak height were collected as the pooled fraction.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was conducted on 10–20% polyacrylamide gels commercially available from Daiichi Pure Chemicals, Japan. A protein sample was loaded (2 μg/lane). SDS-PAGE gels were stained with silver for detection.

3. Results and discussion

3.1. Gradient elution data analysis

Linear gradient elution experiments of Lg with Q Sepharose HP were performed as a function of flow velocity at different gradient slopes (Fig. 1). As described in a previous paper [15], the elution peak becomes wider as the flow velocity increases, while the peak position (elution volume) remains constant. When the gradient slope g (M/mL) becomes steeper, the peak retention volume decreases, whereas the peak salt concentration I_R increases (data not shown). The I_R was measured at various g and plotted against the normalized gradient slope $GH = gV_s = g(V_t - V_0)$, where V_t = total bead volume and V_0 = void volume. The GH–I_R curves did not depend on the flow velocity (Fig. 1), as mentioned in previous papers [15–23]. This indicates the applicability of the model to the present experimental system. From the following

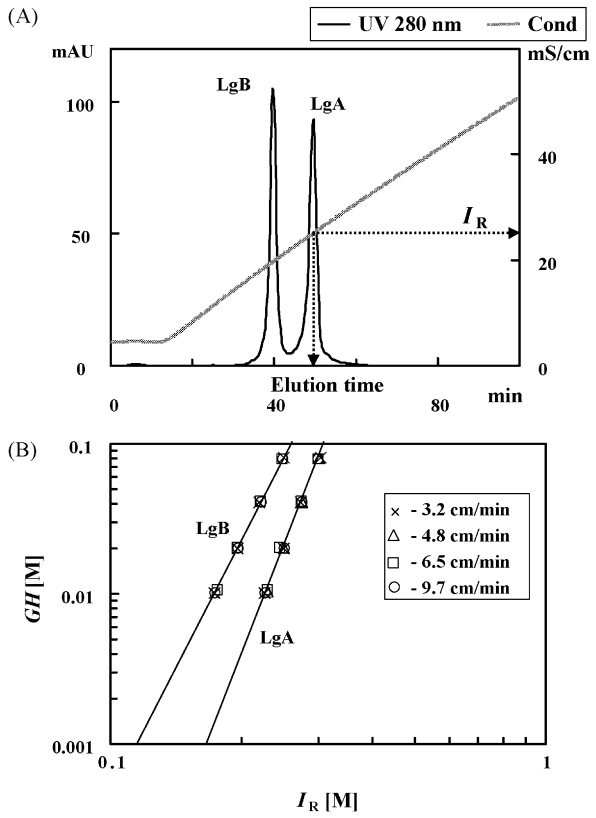

Fig. 1. Typical elution curves of linear gradient elution and GH–I_R curves with anion-exchange gel column. (A): Elution curves of β-lactoglobulin A (LgA) and β-lactoglobulin B (LgB), $u = 9.53$ cm/min, $GH = 0.0126$, $Z = 10$ cm, I_R: peak salt concentration. (B): GH–I_R curves of LgA and LgB. Column: Q Sepharose HP (10 cm × 0.8 cm I.D., $V_t = 5$ mL), mobile phase pH 5.6, sample: 5 mL β-lactoglobulin (1 mg/mL) solution.

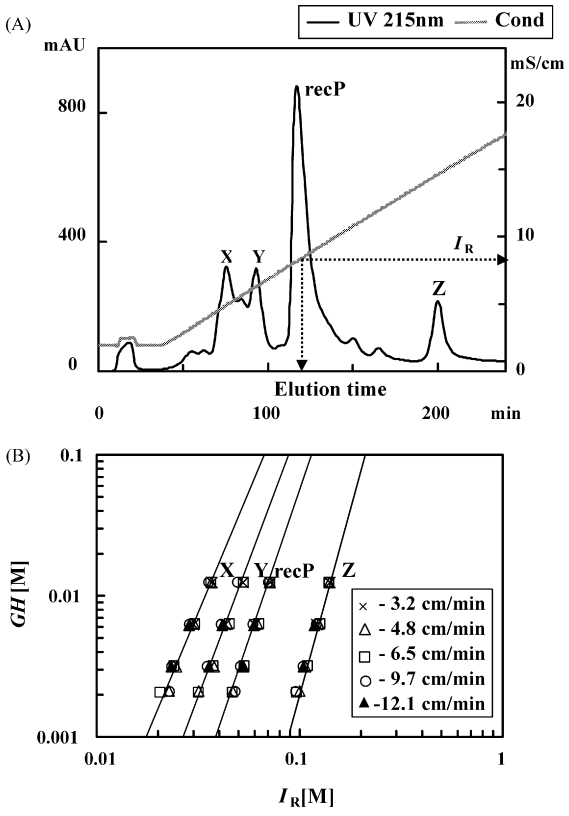

Fig. 2. Typical elution curves of linear gradient elution and GH–I_R curves with cation-exchange gel column. (A): Elution curves of recombinant protein (recP) and impurities (X, Y, Z), $u = 3.03$ cm/min, $GH = 0.00472$, $Z = 10$ cm, I_R: peak salt concentration. (B): GH–I_R curves of recP and X, Y, Z. Column: SP Sepharose HP (10 cm × 0.8 cm I.D., $V_t = 5$ mL), mobile phase pH 8.0, sample: 5 mL SP load solution (1 mg/mL).

equations:

$$GH = \frac{I^{(B+1)}}{[A\,(B+1)]} \tag{1}$$

$$A = K_e \Lambda^B \tag{2}$$

and the GH–I_R curves, the values of A and B for LgA and LgB were obtained (Table 1). A can be derived from the law of mass action (ion-exchange equilibrium) [1–3,24–27]. Here, B is the number of sites (charges) involved in protein adsorption, K_e is the equilibrium association constant, and Λ is the total ion exchange capacity. The same analysis was performed for CEX of recP and impurities. The results are shown in Fig. 2 and Table 1.

Table 1
Parameter values obtained by linear gradient elution data analysis

	A	B
LgA	3.03×10^{-4}	6.2
LgB	6.99×10^{-4}	4.8
recP	1.80×10^{-4}	3.4
Impurity X	3.57×10^{-4}	2.4
Impurity Y	4.09×10^{-4}	2.7
Impurity Z	1.25×10^{-3}	3.8

A: $K_e \Lambda^B$, K_e: equilibrium association constant, Λ: total ion exchange capacity, B: number of sites (charges) involved in protein adsorption, LgA: β-lactoglobulin A, LgB: β-lactoglobulin B, recP: recombinant protein.

3.2. Scale-up study of AEX of Lg

With the following procedure, scale-up chromatography conditions were determined systematically when the linear flow velocity, gradient slope or column length are changed in the scale-up.

Fig. 3A shows the HETP–u plots of LgB. The experimental data can be expressed by a simplified van Deemeter equation, $A^o + C^o u$, where A^o, the intercept, is the contribution due to axial dispersion, and the slope of the curve C^o is the stationary phase diffusion resistance. The purpose of this plot is just to show how the peak width changes with the flow rate and can be incorporated in the separation time-separation volume relationships.

As a small-scale base case, the chromatography condition was chosen for LgB (Table 2). In the linear gradient elution of a protein, the same resolution can be obtained with various combinations of gradient slope, column length and flow rate based on the dimensionless variable O [16,17]. The O value for the base-case condition was calculated to be 15528 using

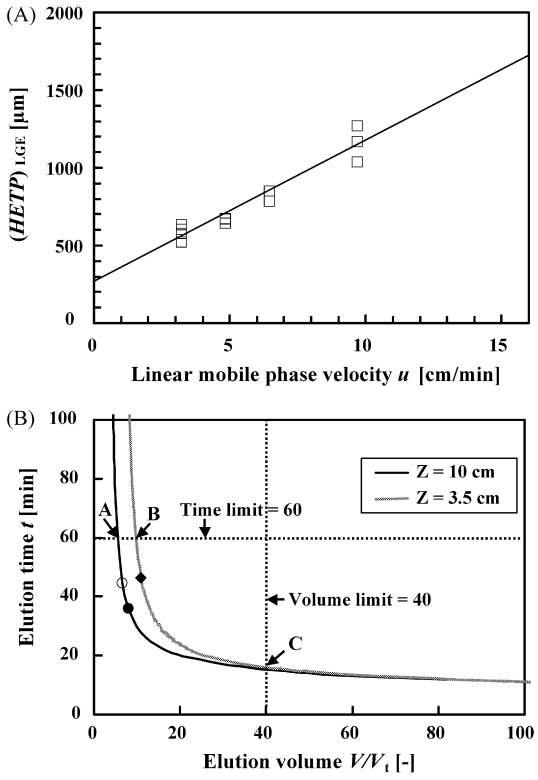

Fig. 3. Scale-up studies for the separation of β-lactoglobulin A (LgA) and β-lactoglobulin B (LgB) with dimensionless parameter O. (A): $(HETP)_{LGE}$ and u relationships of LgB. (B): Iso-resolution curves of LgB. Column: Q Sepharose HP, mobile phase pH 5.6. The open circle represents the data for the base-case condition. The closed rhombic point represents one of the scale-up chromatography conditions chosen at $Z = 3.5$ cm. The closed circle represents one of the scale-up chromatography conditions chosen at $Z = 10$ cm.

Fig. 3A, Eq. (1) and the equation:

$$O = \frac{(ZI_a)}{[G(\mathrm{HETP})_{LEG}]} \tag{3}$$

where Z is column length, I_a is dimensional constant 1, G is the gradient slope normalized with respect to column void volume, and $(HETP)_{LEG}$ is the plate height in the linear gradient elution and is calculated with the elution curves (retention time and peak width) from the linear gradient elution experiments.

When the u is increased, the g must become shallower in order to obtain the same O value. Similarly, when u is decreased, g must be increased. With the aid of the HETP–u curve this calculation can be done. The method for determining the $(HETP)_{LEG}$–u relationship was described in a previous paper [16]. Once the u and the g values are determined, the separation time t_S and the buffer consumption BC are calculated as follows:

$$t_S \approx t_R \approx \left[\frac{(I_R - I_o)}{g}\right] + V' \tag{4}$$

$$BC \approx \frac{V_R}{V_t} = \frac{Ft_R}{V_t} = \frac{t_R(u\varepsilon)}{Z} \tag{5}$$

where t_R is elution time, I_o is initial salt concentration, V' is the elution volume for the salt. We call the $t_R - V_R/V_t$ curve at a constant O value the "iso-resolution curve," as the same resolution with a different separation time and buffer consumption can be obtained on this curve.

The calculated iso-resolution curves for LgB are shown in Fig. 3B. The open circle represents the data for the base-case condition. In Fig. 3B, the separation time-elution volume relationship at a constant O value when changing the column length Z is also shown. The same resolution can be obtained on the two curves at $Z = 10$ and 3.5 cm. As shown in the figure, the separation time becomes longer as the elution volume decreases. However, a large elution volume is needed for rapid separation. It is especially important to know where the separation conditions are located. For example, as the base-case condition is located at the open circle in Fig. 3B, it is not appropriate to decrease the flow velocity for reducing the buffer consumption.

In the case of the separation of LgA and LgB, we tried to change Z from 10 to 3.5 cm in 100 mL scale-up, and in 2.5 L scale-up (setting the same bead height, $Z = 10$ cm), we tried to reduce the separation time to lower than that of the base-case. We also set constraints of both the elution volume less than 40 V_t and the elution time less than 60 min. At the 100 mL scale-up, scale-up chromatography conditions were arbitrary points between point B and point C on iso-resolution curves at $Z = 3.5$ cm (Fig. 3B). So the closed rhombic point was chosen as one of the scale-up chromatography conditions (Table 2). At the 2.5 L scale-up, objective chromatography conditions were arbitrary points between point A and point C of iso-resolution curves at $Z = 10$ cm, where the elution time t was lower than that of the base-case (open circle in Fig. 3B). So, the point of closed circle was chosen as one of the scale-up chromatography conditions (Table 2).

To verify the scale-up chromatography conditions determined above, the elution curves and resolution were evaluated. Fig. 4 shows the elution curves on Q Sepharose HP under base-case and scale-up chromatography conditions. Both the elution curves and the resolution Rs in 100 mL and 2.5 L scale-up elutions were comparable to those of the base-case. The elution time of the 2.5 L scale-up elution was shortened compared with those of the base-case condition, as expected. These results indicate that the scale-up chromatography conditions were acceptable compared to the small-scale base-case conditions in terms of the resolution as well as the reduced process time.

Table 2
Base-case and scale-up chromatography conditions for Q Sepharose HP chromatography

	u (cm/min)	Gradient volume (columns per bed volume)	Z (cm)
Base-case			
LgB for 5 mL	4.85	16	10
Scale-up			
LgB for 100 mL	3.07	35	3.5
LgB for 2.5 L	7.24	21	10

LgB: β-lactoglobulin B, u: linear mobile phase velocity, Z: column length velocity.

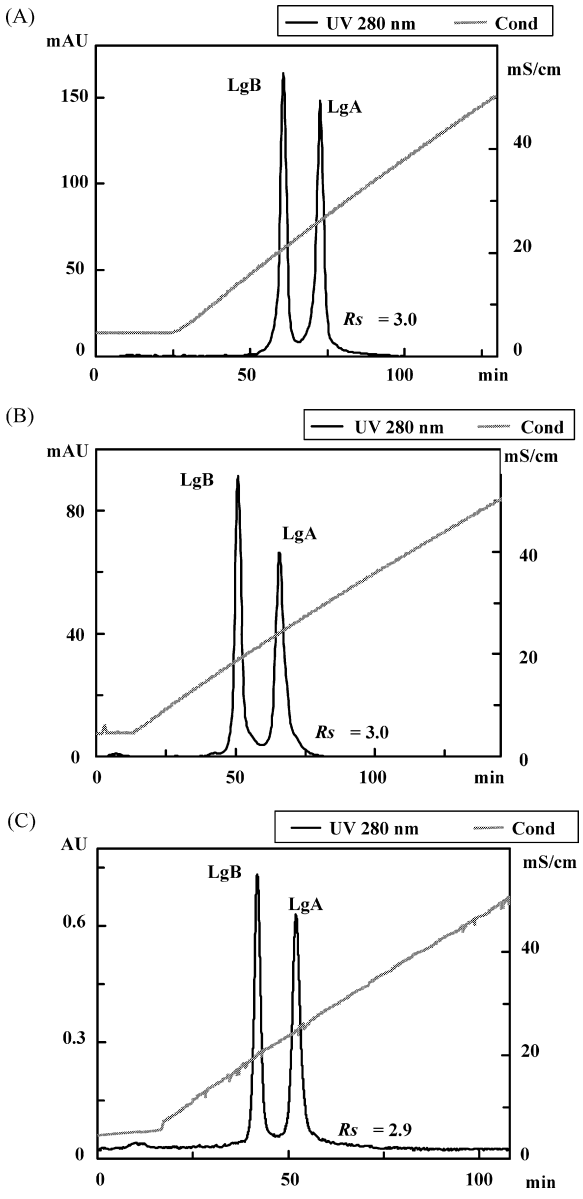

Fig. 4. Elution curves of β-lactoglobulin A (LgA) and β-lactoglobulin B (LgB) with anion-exchange chromatography in a scale-up study with dimensionless parameter O. (A) Base-case conditions: $V_t = 5$ mL, $u = 4.85$ cm/min, $GH = 0.0204$, $Z = 10$ cm, (B) 100 mL scale-up conditions: $V_t = 100$ mL, $u = 3.07$ cm/min, $GH = 0.0100$, $Z = 3.5$ cm, (C) 2.5 L scale-up conditions: $V_t = 2.5$ L, $u = 7.24$ cm/min, $GH = 0.0155$, $Z = 10$ cm. Column: Q Sepharose HP, mobile phase pH 5.6, sample load: 1 mg protein/resin mL. The O values in (A), (B) and (C) were set to be equal. Note that because HETP is not proportional to u ($u \cdot GH$), its values are not equal in (A), (B) and (C).

3.3. Scale-up study of CEX of recP

With the same approach as described in Section 3.2, the scale-up chromatography conditions for recP purification with CEX were systematically determined when the linear flow velocity and gradient slope were changed in the scale-up.

Fig. 5A shows the HETP–u plots of recP. As a base-case on a small scale, the chromatography condition was chosen for recP purification (Table 3). The O value for the condition was calculated as 19060 with Eq. (1), Eq. (3) and Fig. 5A. The calculated

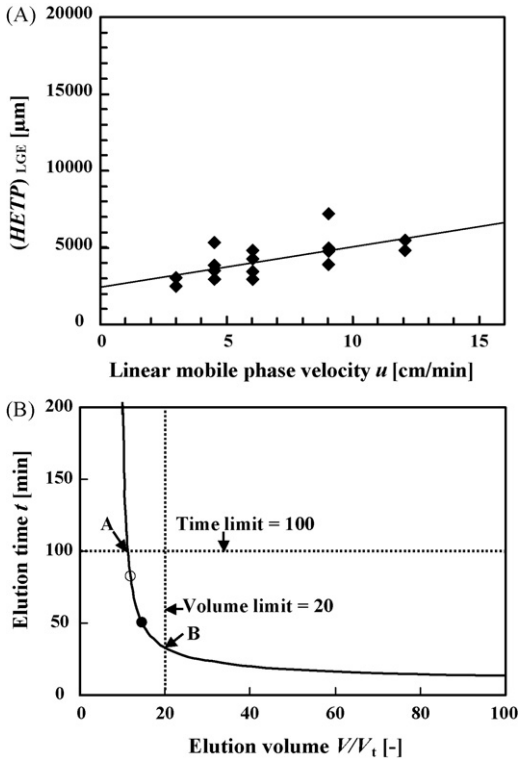

Fig. 5. Scale-up studies for the purification of recombinant protein (recP) with dimensionless parameter O. (A): $(HETP)_{LGE}$ and u relationships of recP. (B): Iso-resolution curves of recP. Column: SP Sepharose HP, mobile phase pH 8.0. The open circle represents the data for the base-case conditions. The closed circle represents one of the scale-up chromatography conditions chosen.

iso-resolution curves for recP are shown in Fig. 5B. The open circle represents the data for the base-case condition.

In the case of recP purification for the 2.5 L scale-up, we also tried to reduce the separation time to lower than that of the base-case. We also set constraints of both the elution volume less than 20 V_t and the elution time less than 100 min. The objective chromatography conditions were arbitrary points between point A and point B of iso-resolution curves, where t was lower than that of the base-case (open circle in Fig. 5B). The point of the closed circle was therefore chosen as one of the scale-up chromatography conditions (Table 3).

To verify the scale-up chromatography conditions determined above, the elution curves, the recovery and the purity were evaluated. Fig. 6A and B show the elution curves on SP Sepharose HP under base-case and scale-up chromatography

Table 3
Base-case and scale-up chromatography conditions for SP Sepharose HP chromatography

	u (cm/min)	pcGradient volume (columns per bed volume)	Z (cm)
Base-case recP for 5 mL	4.53	32	10
Scale-up recP for 2.5 L	7.58	41	10

recP: recombinant protein, u: linear mobile phase velocity, Z: column length.

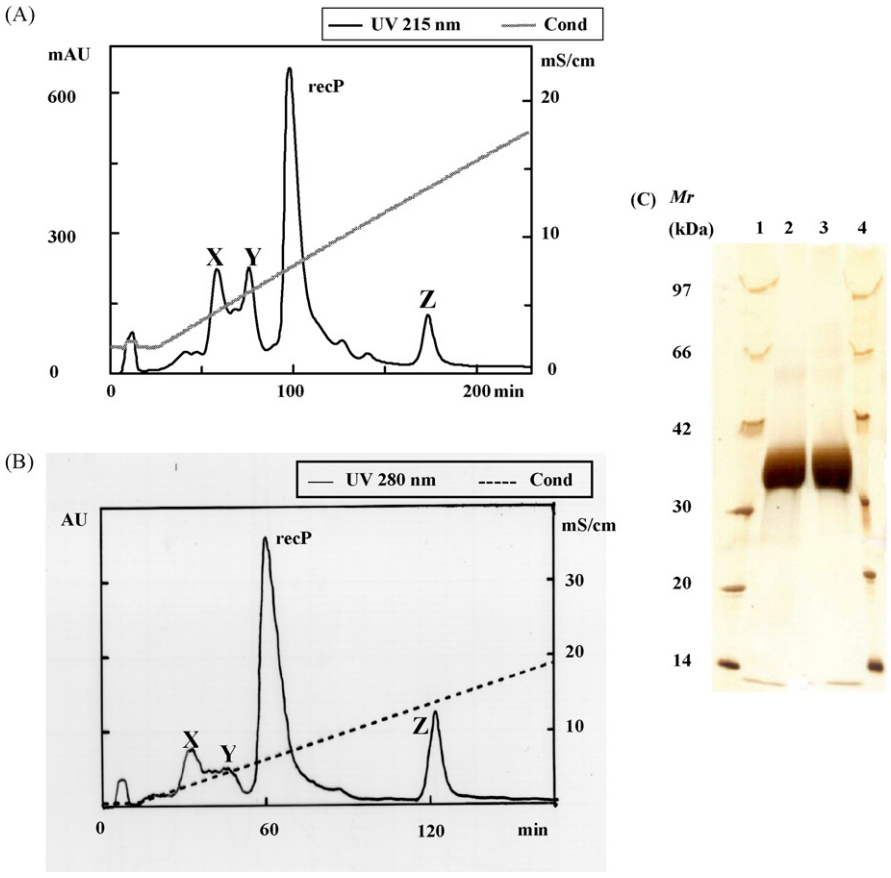

Fig. 6. Elution curves of recombinant protein (recP) with cation-exchange chromatography in a scale-up study with dimensionless parameter O and silver-stained SDS-PAGE analysis of purified recP under non-reducing conditions. (A) Base-case conditions: $V_t = 5$ mL, $u = 4.53$ cm/min, $GH = 0.00315$, $Z = 10$ cm, (B) 2.5 L scale-up conditions: $V_t = 2.5$ L, $u = 7.58$ cm/min, $GH = 0.0024$, $Z = 10$ cm. Column: SP Sepharose HP, mobile phase pH 8.0, sample load: 1 mg protein/resin mL. X, Y, Z: impurity X, impurity Y, impurity Z. The O values in (A) and (B) were set to be equal. Note that because HETP is not proportional to u (u·GH), its values are not equal in (A) and (B). (C) Lanes 1 and 4: molecular weight standards. Lane 2: base-case ($V_t = 5$ mL). Lane 3: 2.5 L scale-up ($V_t = 2.5$ L). *Note*: 2 μg/lane except molecular weight standard.

conditions. The elution curve of the 2.5 L scale-up was similar to that of the base-case. The elution time of the scale-up elution was also shortened compared with those of the base-case condition, as expected. The recovery ratio of the scale-up (30%) is quite comparable to that of the base-case (26%). As shown in Fig. 6C, the SDS-PAGE data also showed comparable purity of the recovered fraction from these chromatography runs. These results also indicate that the scale-up chromatography condition was acceptable compared to the small-scale base-case condition in terms of the purity and the recovery as well as the reduced process time.

For the rapid and systematic design and optimization of scale-up chromatography conditions in linear gradient elution, when linear flow velocity, column length or gradient slope were altered, the method presented is useful and can shorten the period of the process development of protein purification compared to the conventional trial-and-error approach. For example, with this method we can design and optimize the scale-up chromatography conditions within 2 days (1 day of linear gradient experiments, 1 day of data analysis). In contrast, the trial-and-error approach will require more than 1 month for designing and optimization.

4. Conclusion

Our developed chromatography model was applied to the separation of LgA and LgB with AEX, and to recP purification with CEX in order to scale-up chromatography conditions rapidly and systematically. Both chromatographies were first analyzed by our simple method that uses linear gradient elution experimental data. By using the iso-resolution curve concept, the scale-up chromatography conditions, in which linear flow velocity, gradient slope and column length were altered, were determined for linear gradient elution. The scale-up elution was successfully carried out in terms of the peak shape, the resolution, the recovery and the purity value. The method presented here is a useful tool for the rapid scale-up of protein purification.

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