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Theoretical background of short chromatographic layers Optimization of gradient elution in short columns

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Abstract

Although linear salt gradient elution ion-exchange chromatography (IEC) of proteins is commonly carried out with relatively short columns, it is still not clear how the column length affects the separation performance and the economics of the process. The separation performance can be adjusted by changing a combination of the column length, the gradient slope and the flow velocity. The same resolution can be obtained with a given column length with different combinations of the gradient slope and the flow velocity. This results in different separation time and elution volume at the same resolution. Based on our previous model, a method for determining the separation time and the elution volume relationship for the same resolution (iso-resolution curve) was developed. The effect of the column length and the mass transfer rate on the iso-resolution curve was examined. A long column and/or high mass transfer rate results in lesser elution volume. The resolution data with porous bead packed columns and monolithic columns were in good agreement with the calculated iso-resolution curves. Although the elution volume can be reduced with increasing column length, the pressure drop limits govern the optimum conditions.

Keywords: Short column; Ion-exchange chromatography; Iso-resolution curve; Gradient elution; Monolithic column; Optimization

1. Introduction

In many biotech companies recombinant protein drugs are purified by several chromatography processes. However, in many cases these processes are so complicated and delicate that the operation must be carefully carried out, and sometimes additional care must be taken in order to maintain very high purity and low variability of the product [1–6]. These difficulties arise mainly from the fact that a target recombinant protein drug must be purified from very similar protein variants or isoforms.

Chromatography using electrostatic interaction known as ion-exchange chromatography (IEC) is most commonly used for such protein drug separations. Although various elution methods are available for IEC, linear gradient elution (LGE), in which salt concentration is increased linearly, is the most efficient method for difficult protein separations [2,6,7]. However, since LGE has many variables such as gradient slope, flow velocity and column dimensions as well as mobile phase pH, it is difficult to optimize the separation.

It is well known that low mass transfer rates in the stationary phase govern the performance of protein chromatography [2,6,7]. Therefore, numerous efforts have been devoted to analyze mass transfer mechanism in protein chromatography and to develop chromatographic supports (media) that possess less mass transfer resistance. Even when porous supports having relatively large pores (ca. 100 nm) pore diffusion of large proteins is still very low. One of the methods that can enhance the mass transfer rate is to use a convection-aided support. Perfusion chromatography, membrane-based chromatography, fabric-support chromatography and monolithic-support chromatography are such convection-aided chromatography which can permit high separation performance at high flow velocities. Good review papers are available for the convection-aided chromatography [8–13].

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We have developed mathematical models for LGE-IEC [6,14–16]. These models can be applied to predict the separation performance and also to obtain important information on molecular recognition [17].

It is empirically well known that the impact of the column length on the separation performance in LGE-IEC is not so significant compared with the isocratic elution. We have shown that a very short column (1 cm) can be employed for fine separation of protein variants based on the dimensionless group derived from the mathematical model [6,15]. We have then demonstrated that the same resolution (separation performance) can be obtained with a given column when the gradient slope and the flow-velocity are properly adjusted [16]. However, it is also shown that the separation time and the elution volume (buffer consumption) change with the gradient slope and the flow velocity values at the same resolution. During our investigation on the resolution with short columns [15], we found that it was not easy to pack a very short column with conventional porous beads. However, very short (thin) columns are now commercially available as monolithic columns and membrane-based columns. Therefore, we have decided to re-investigate the separation performance of very short columns based on the extended version of our model [6,15,16].

In this paper, the effects of the column length and the mass transfer resistance on the separation performance in LGE-IEC were investigated on the basis of the model developed for LGE-IEC. The experimental data with monolithic stationary phase IEC as well as conventional porous bead IEC were analyzed with the model. The optimization strategies in terms of the separation time and the elution volume (buffer consumption) were developed.

2. Experimental

2.1. Chromatography column

SP Sepharose HP gels (6% cross-linked agarose, sulfopropyl group, particle diameter ca. 37 μm) were packed into a plastic column (50 mm × 9.0 mm i.d., total bed volume V_t = 3.18 mL,) according to the procedure recommended by the supplier (Amersham Biosciences, Uppsala, Sweden). Poly(glycidyl methacrylate-co-ethylene dimethacrylate) disks (3 mm × 12 mm i.d.) with a weak anion-exchange group were contained in a specially designed disk holder column from BIA Separations (Ljubljana, Slovenia). This disk is called hereafter CIM-QA.

2.2. Materials

Model proteins employed in this study are ribonuclease A (RNase A, product no.R4875) and Bovine milk β-lactoglobulin (Lg, product no.L0130) from Sigma (St. Louis, MO, 45A). β-Lg contains lactoglobulins A (LgA) and B (LgB). Other reagents were of analytical grade.

2.3. Chromatography apparatus

Column experiments were carried out on a fully automated liquid chromatography system ÄKTA Explorer 100 (Amersham Biosciences, Uppsala, Sweden).

2.4. Linear gradient elution experiment

The SP Sepharose HP column was equilibrated with a starting buffer (buffer A) containing 0.03 M NaCl. The same buffer solution containing 0.5–1.0 M NaCl was used as 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 NaCl concentration was increased with time at a fixed pH and buffer compositions. The gradient slope g is shown in M/mL. 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. The experiments were performed at 298 ± 1 K.

3. Theoretical

3.1. Peak retention volume as a function of gradient slope

The outline of our model [5,6,14–17] 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, $g = (I_f - I_0)/V_g = (I_f - I_0)/(F\,t_g)$ [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,

$$GH = (gV_o)\left[\frac{(V_t - V_o)}{V_o}\right] = g(V_t - V_o) \quad (1)$$

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

$$g = \frac{(I_f - I_0)}{V_g} \quad (2)$$

I_f is the final salt concentration, I_0 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_0