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PULSE RESPONSE TEST IN MEDIUM PRESSURE PROTEIN CHROMATOGRAPHY

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Abstract – An MPLC (Medium Pressure Liquid Chromatography) column was in-house packed with an anion exchange silica gel (PAE 1000, Amicon) in order to carry out pulse response experiments to investigate MPLC column efficiency. Bovine serum albumin was isocratically eluted from the column with varying ionic strength of NaCl, and the chromatograms obtained were analyzed by the moment method. Broadening of the elution band was caused mostly by a longitudinal dispersion in our system, and no significant numerical differences in HETP with the velocity change were observed at the high ionic strength.

Key words: Moment Analysis, Medium Pressure, Protein Chromatography

INTRODUCTION

Chromatography has been widely used to isolate and purify most protein samples as a high resolution step of a downstream process of biotechnology [Wheelwright, 1991; Subramanian, 1991; Ahn and Chung, 1985]. The chromatographic process was applied to produce recombinant proteins, which have been mainly purified in glass columns at a low pressure (under 1 atm). Although the so-called LPLC (low pressure liquid chromatography) requires low investments and simple handling, it usually provides low efficiency [Sisson et al., 1988].

HPLC (pressure over 100 atm) has been used since the late 1980's for the preparative production of recombinant proteins [Ladish and Kohlmann, 1992]. Unlike LPLC, HPLC is a powerful separation method with advantages in selectivity, sensitivity and rapidity. However, there are some limitations in operating an HPLC system. Preparative HPLC columns and packing media are very expensive, and pretreatment of sample is complicated and laborious. The packing materials are not sold separately and the selection of those media is limited for the proper use.

MPLC (medium pressure liquid chromatography, pressure range: 1-10 atm), which takes advantage of the merits of both LPLC and HPLC, has been introduced to achieve protein production economically [Sakamoto, 1987]. MPLC columns are easily packed in-house and operated with fewer precautions than that of HPLC. These columns could be made of pressure-resistant borosilicate glass and so be packed by watching the packing condition inside the column. The packing media has a rather large particle diameter of about 20 μm compared with the value of HPLC media (5-10 μm).

This work was initiated to study the efficiency of a protein MPLC column. The research on MPLC has mainly focused on the separation of low molecular weight organic chemicals by a reverse phase column. Until recently, the usage of MPLC in protein purification was not popular enough to make MPLC a promising application area for protein production [Kim et al., 1991]. An MPLC column has to be characterized to compare its efficiency with other chromatographies. Therefore, band broadening in an ion exchange MPLC column was studied by pulse response tests for various ionic strengths of NaCl, and the moment method was applied to analyze the contribution of rate constants. HETP (height equivalent to a theoretical plates) of the column was also calculated for various ionic strengths and mobile phase velocity.

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MATERIALS AND METHODS

1. Chemicals

PAE-1000 (20 μm D.), a weak anion exchanger, was obtained from Amicon, USA. Bovine serum albumin from Sigma, USA was used as a model protein. A sodium phosphate buffer solution of pH 7 with NaCl for desorption of BSA was used as a mobile phase.

2. Apparatus and Operation Procedure

A suspension of PAE-1000 in water was packed into a stainless steel column (250 mm L × 4.6 mm I.D.) from SEG, Australia by a high pressure pump. Fig. 1 shows a plot of flow rate versus pressure drop for one of our columns. Because Darcy's law applied well to this column, the packing condition of the column was good enough to perform a pulse response test.

In order to study the equilibrium and kinetic properties of the system, we applied a series of pulse response tests at 22 °C. A protein sample (4 mg/ml and 0.5 ml) was loaded into the column equilibrated with the buffer solution through an injector 7725i from Rheodyne, and the flow rate was kept constant by a solvent delivery pump (Model 910, Youngin, Korea). All buffer solutions were degassed and filtered by a micro filter of pore size 0.2 μm before being used. The protein concentration out of the column was determined by measuring

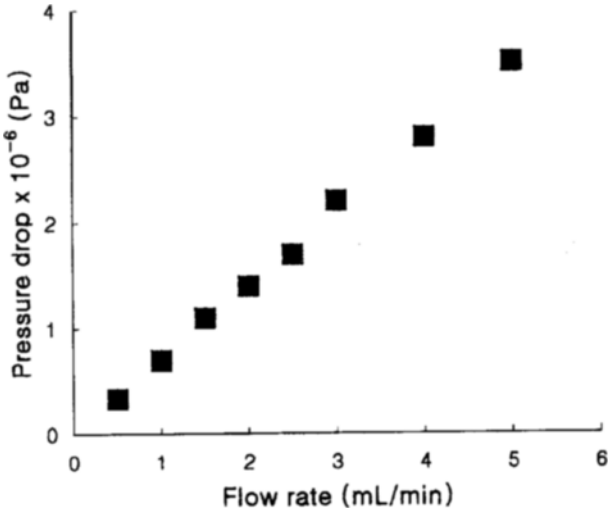


Fig. 1. Plot of mobile phase flow rate versus column pressure; column length=25 cm, column diameter=4.6 mm, packing material: PAE-1000 (average gel diameter 20 μm).

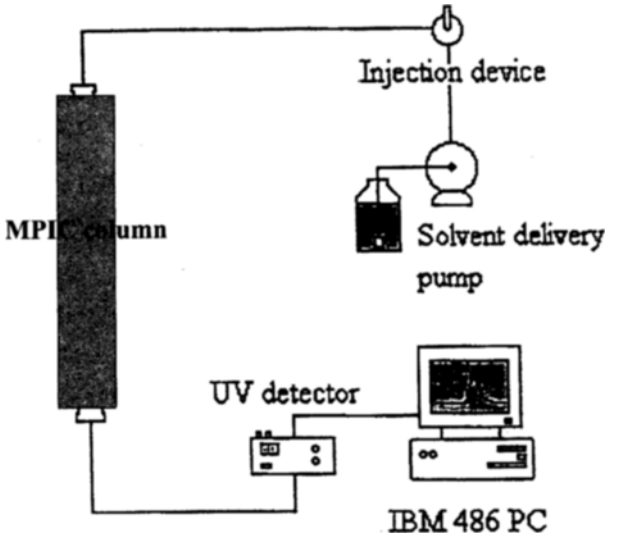


Fig. 2. Schematic diagram of the apparatus; pump: HPLC Model Youngin 910, injector: Reodyne Model 7725I, UV detector: HPLC Model Youngin M720.

the absorbance with a UV detector (M720, Youngin, Korea) at 280 nm. All data were filed automatically into an IBM 486 PC through a digital multimeter (Protek506, Hungchang, Korea), and the moments of elution bands were calculated directly. A schematic diagram of the apparatus is shown in Fig. 2.

3. Moment Analysis [Ghim, 1980; Park and Suh, 1984]

Moment analysis in chromatography theory has been used with the following considerations:

- Equilibrium isotherm is linear;
- Rate equations of chromatography process consider film and pore diffusion;
- Laplace transform method yields only moments of elution curve.

The mean residence time and peak variance, m_1' and m_2 were calculated according to the usual expression.

$$m_1' = \int_0^\infty c\,t\,dt \Big/ \int_0^\infty c\,dt \tag{1}$$

$$m_2' = \int_0^\infty c\,(t - m_1')^2 \Big/ \int_0^\infty c\,dt \tag{2}$$

The mean residence time is related to the distribution coefficient of protein (K). The expression for a linear system is derived as follows.

$$m_1' = \frac{L}{u_0} \cdot [\varepsilon + (1-\varepsilon)\cdot\beta\cdot(1+K)] \tag{3}$$

where, L : column length
u_0 : superficial velocity ($=u \times \varepsilon$)
β : porosity of particle
ε : void fraction of column

The second central moment, peak variance, including the numerical terms on mass transfer resistance, is expressed as follows.

$$m_2 = \frac{2LD_z}{u^3}\cdot[1+\phi\cdot(1+K)]^2 + \frac{2L}{u}\cdot\phi\cdot\left[\frac{K^2}{k_a} + \frac{R\cdot(1+K^2)}{3}\cdot\left(\frac{1}{k_f}+\frac{R}{5D_s}\right)\right] \tag{4}$$

where, D_z : longitudinal diffusion coefficient
D_s : gel phase diffusion coefficient
R : particle radius
$\phi = (1-\varepsilon)\times\beta/\varepsilon$
K : distribution coefficient of protein
u : interstitial velocity
k_a : adsorption rate constant
k_f : mass transfer coefficient

4. Simulation of Response Curve

The linear plate model [Yamamoto et al., 1988] was successfully applied to simulate the chromatograms of BSA at the outlet of the ion exchange column. Runge-Kutta 4th method was employed, and Microsoft FORTRAN was used on an IBM 486 PC [Kim et al., 1995].

RESULTS AND DISCUSSION

1. Confirmation of Linear Range

Fig. 3 shows the effect of loading protein concentrations on the mean residence time of the response curves. Since the first noncentral moment (mean residence time of peaks) was independent of the protein concentration, the chromatographic model for a linear system suggested here could reasonably be applied to our system [Kim et al., 1995].

2. Determination of K and ε

The values of K and ε were calculated from the plot of the first moment of response curve versus L/u_0 for various ionic strengths of NaCl (Fig. 4). As expressed in Eq. (3), the experimental data gave straight lines of which the slope decreased with an increase of ionic strength. The void fraction of the column, ε, was determined from the slope of the highest ionic

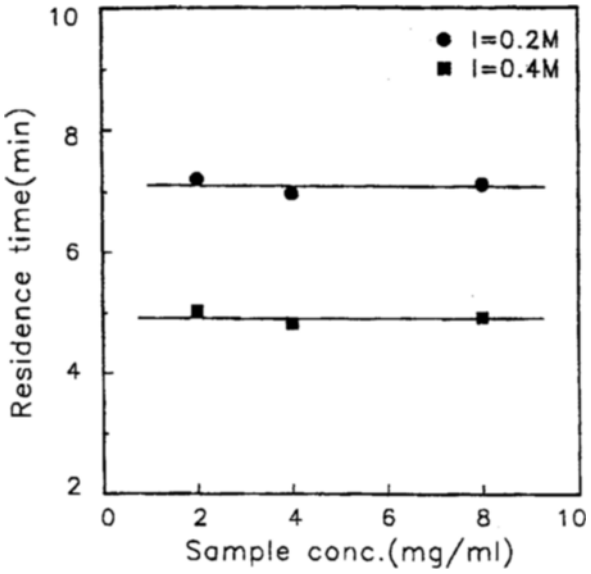


Fig. 3. Effect of loading protein concentration on the mean residence time of response curve; protein sample concentration: 4 mg/ml, protein sample volume: 0.5 mL.

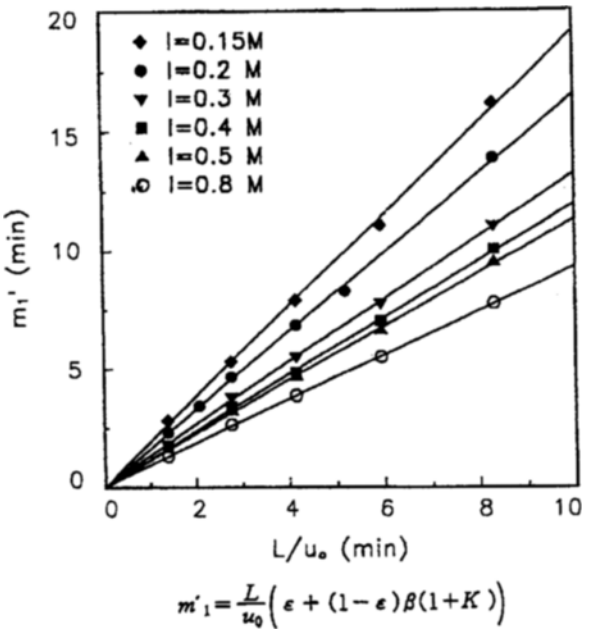


Fig. 4. Experimental relations between the first moment m_1' $[\{L/u_0[\varepsilon+(1-\varepsilon)\beta(1+K)\}]$ and L/u_0 for various ionic strengths of NaCl; NaCl concentration range: 0.15-0.8 M.

strength (I=0.8 mol/L), where electrostatic interaction between a protein and an ion exchanger is low (i.e., K=0.2). The distribution coefficients of BSA, K's, were calculated from the slopes of lines. Table 1 shows the values of K and ε. The K decreased by increasing the ionic strength of NaCl, and there was a linear relation in a log coordinate as in Fig. 5.

3. Determination of D_z/u

According to Eq. (4), the second central moment of the response curve was plotted as shown in Fig. 6. When the horizontal and vertical axes were remarked as $m_2/(2L/u)$ and 1/u, respectively, all experimental data gave a straight line.

Table 1. Parameter values obtained from pulse response test

I (M)	K	u^{-1} D_z
0.15	1.98	0.68
0.2	1.46	0.54
0.3	0.81	0.47
0.4	0.56	0.24
0.5	0.41	0.18
0.8	0.2	0.14

d_p=20 μm, ε=0.42, β=0.86

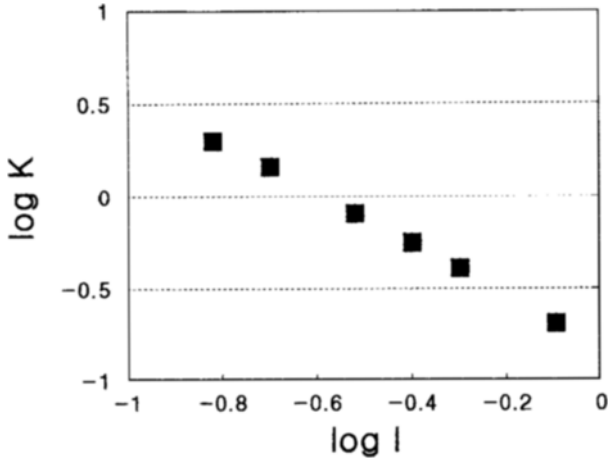
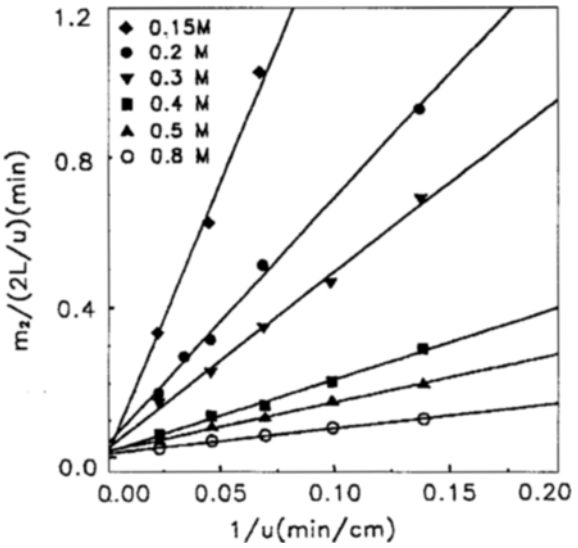


Fig. 5. Relationship between distribution coefficient K and ionic strength I; correlation reads to be log K=n log I+c where n=−1.3 and c=−0.78.



$$m_2=\frac{2LD_z}{u^3}\left[1+\phi(1+K)\right]^2+\frac{2L}{u}\phi\left[\frac{K^2}{k_a}+\frac{K(1+K)^2}{3}\left(\frac{1}{k_f}+\frac{R}{5D_s}\right)\right]$$

Fig. 6. Experimental relations between the second central moment m_2 and 1/u for various ionic strengths of NaCl; experimental conditions same as Fig. 4.

The values of D_z/u, which could be calculated from the slope of each line for various ionic strengths, had a tendency to decline as the ionic strength of NaCl increased. This is due to

Table 2. Order of magnitude calculation in Eq. (4)

Parameters	Typical value	Order of magnitude
φ	1.2	1
R	20 μm	10^{-5} m
D_s [Liu, 1993]	8×10^{-11} m^2/s	10^{-10} m^2/s
K	0.5	1-0.1
u	15 cm/min	10^{-3} m/s
$m_2/(2L/u)$	0.5 min	$1\text{-}10^2$ s
D_z/u	0.5 cm	$10^{-2}\text{-}10^{-3}$ m

$(D_z/u)[1+\phi(1+K)]^2(1/u)$ = Order of $10\text{-}10^2$ s

$\phi(K^2/k_a+R(1+K)^2[1/k_f+R/(5D_s)]/3)$=Order of 0.1-1 s

the fact that electrostatic interaction between BSA and the exchanger is diminished and the protein band travels through the column with less dispersion. Table 1 shows that the value of D_z/u becomes small with an increase of the ionic strength for a Reynolds number ($u\ \rho\ d_p/m$) of 0.024-0.143.

When a protein sample is distributed to the stationary phase in the case of a preparative column, mass transfer resistance existing between the stationary and mobile phase should be taken into consideration. Since the intercepts of each line in Fig. 6 have an order of 0.01, it could be thought that the second term on the right hand side of Eq. (4) has less effect on dispersion than the first term. Table 2 gives a sample calculation to compare the terms' magnitude in Eq. (4). Broadening of the response curve was mainly caused by longitudinal dispersion in our system.

4. HETP of MPLC Column

The efficiency of the MPLC column is often expressed by the height equivalent to a theoretical plate (HETP), which is defined as follows [Bungay and Belfort, 1987].

$$HETP = L/N_p = L(m_2'/m_1'^2) \quad (5)$$

The HETP versus superficial velocity, u_0, is shown in Fig. 7. As shown in the figure, HETP had a decreased value when the superficial velocity decreased and the ionic strength of NaCl increased. No significant numerical differences in HETP with the change of velocity were observed at the high ionic strength.

Table 3 shows the value of HETP calculated for several cases. HETP values in MPLC (order of 10^{-1} cm) were the same as those in LPLC [Kim et al., 1995], indicating that the efficiency of MPLC matches that of LPLC in the present investigation. However, the productivity of MPLC is greater since MPLC was operated at a higher mobile phase velocity with reduced time of operation.

5. Prediction of Elution Curve

The values of K and HETP obtained were used to simulate the plate model [Kim et al., 1995]. Fig. 8 shows the results of the predicted elution bands (solid line) compared with the experimentally obtained ones (points) for various ionic strengths. The predicted elution bands agreed fairly well with the experimental ones at low ionic strength (I=0.2 M, 0.3 M). In contrast, some deviations were observed at the high ionic strength (I=0.4 M, 0.5 M). These deviations could be due to the fact that the small value of peak variance, m_2, shown in Table 3 caused an large error in the calculation of HETP. The HETP causes a variation of sharpness in the chromatogram during simulation. The sharpness of the chromatogram was found to be sensitively affected by a small change in the value of HETP.

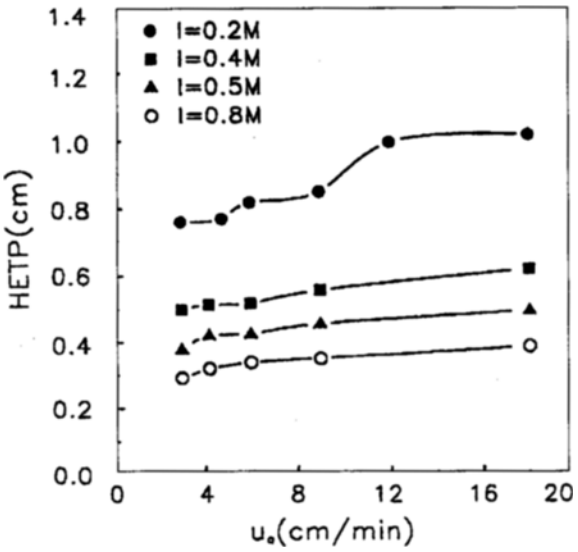


Fig. 7. Effect of ionic strength on HETP for mobile phase velocity; NaCl concentration range: 0.2-0.8 M.

Table 3. HETP calculation

I (M)	u_0 (cm/min)	First moment (min)	Second moment (min^2)	HETP (cm)
0.2	3.01	13.8	5.82	0.759
	18.1	2.31	0.216	1.01
0.4	3.01	10.0	2.01	0.499
	18.1	1.70	0.071	0.616
0.5	3.01	9.49	1.36	0.378
	18.1	1.60	0.051	0.493

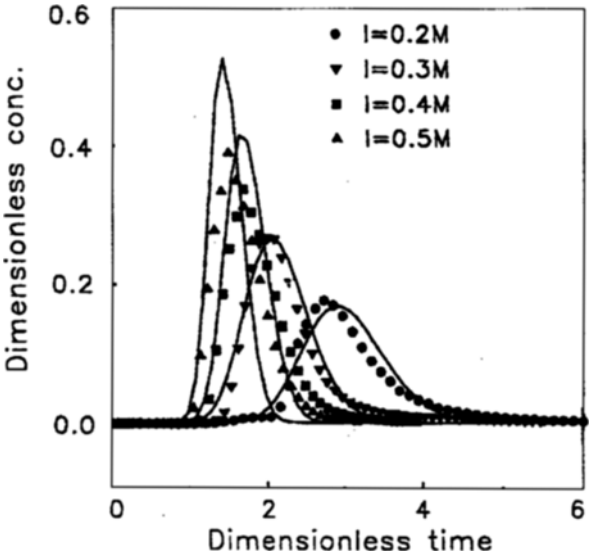


Fig. 8. Experimental data points and elution curves calculated by plate model for various ionic strengths of NaCl; Experimental condition: T=22 °C; pH=7.0; C_i=4 mg/ml; V_s=0.5 ml; horizontal dimensionless time (t)=t×u/L; vertical dimensionless concentration (C_o)=C/C_i.

CONCLUSIONS

A model ion exchange MPLC column was tested by a pulse response method to investigate the efficiency of the in-house packed MPLC column. The following conclusions could be drawn from the experiments.

1. The distribution coefficient K from the first moment was found to be inversely proportional to the ionic strength of NaCl on a log-log graph.
2. The chromatogram of the present column was mainly broadened by axial dispersion. The effect of mass transfer on chromatogram broadening was small compared to the axial dispersion.
3. The HETP of MPLC was comparable to that of LPLC, and so the main merit of MPLC came from the speed of operation.

NOMENCLATURE

C : protein concentration [mg/ml]
C_i : inlet concentration [mg/ml]
C_o : dimensionless concentration [$=C/C_i$]
D_s : gel phase diffusion coefficient [cm^2/min]
D_z : longitudinal diffusion coefficient [cm^2/min]
d_p : particle diameter [μm]
HETP : height equivalent a theoretical plate [cm]
I : ionic strength [M]
K : distribution coefficient of protein
ka : adsorption rate constant [min^{-1}]
kf : mass transfer coefficient [cm/min]
L : column length [cm]
m_1', m_2 : first noncentral moment and second central moment based on time
Np : number of plates
R : particle radius [cm]
t : time [min]
u : interstitial velocity [cm/min]
u_0 : superficial velocity ($=u \times \varepsilon$) [cm/min]
V_o : void volume of column [ml]
V_s : sample volume [ml]

Greek Letters

β : porosity of particle
ε : void fraction of column
ϕ : $(1-\varepsilon) \times \beta/\varepsilon$
μ : viscosity
ν : mobile phase flow rate [ml/min]
ρ : density
τ : dimensionless time [$= t \times u/L$]

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