*Korean J. Chem. Eng.*, 19(2), 314-317 (2002)

# Protein Separation by High-Performance Membrane Chromatography

**Seung Bum Hong and Kyung Ho Row†**

Center for Advanced Bioseparation Techology and Department of Chemical Engineering,
Inha University, 253 Yonghyun-Dong, Nam-Ku, Inchon 402-751, Korea
(*Received 29 August 2001 • accepted 3 December 2001*)

**Abstract**–High-performance membrane chromatography (HPMC) is a very effective chromatographic method that combines the advantages of both membrane technology and column chromatography. In this work, proteins (myoglobin, conalbumin and soybean trypsin inhibitor) were separated by HPMC. The separation mechanism involved anion-exchange, and the stationary phases were used anion CIM DEAE and QA disks (12×3 mm). Two types of mobile phase, buffer A (20 mM Tris-HCl, pH 7.4) and buffer B (buffer A+1 M NaCl) were used. As the amount of sodium chloride dissolved in buffer linearly increased, the retention time shortened and the resolution of the components was greatly improved. The optimum mobile phases and operating conditions were experimentally determined. From the experimental results, the proteins were separated within 2 min at a mobile phase flow rate of 4 ml/min.

Key words: High-Performance Membrane Chromatography, Ion Exchange, Buffer Concentration, Monolithic Column

## INTRODUCTION

The fast growth of biotechnology and the pharmaceutical industry requires development of new isolation and purification methods for biopolymers [Michael et al., 1998]. High-performance liquid chromatography (HPLC) has been widely used as a standard analytical instrument, and a number of stationary phases are commercially available. The separation is achieved using interactions among solutes, mobile phases and stationary phases, as well known from ion-exchange, hydrophobic interaction, reversed-phase, and affinity HPLC modes. HPLC columns are improved to increase the selectivity and the efficiency for the mixtures separated [Lee et al., 1996]. High-performance membrane chromatography (HPMC) combines the advantages of both membrane technology and column chromatography [Ales et al., 1999]. HPMC is based on the separation of proteins molecules on a very thin separation layer as a result of the interactions between the proteins molecules and the active groups on the surface of the pores [Wensheng and Fred, 1998]. Membranes and monoliths have been successfully applied in various chromatographic separations using gradient elution of large biomolecules in extremely short analysis times [Ales et al., 1999]. The main difference between monoliths and conventional HPLC columns lies in the structure of the support. Columns are commonly filled with highly porous particles with a diameter in the range of 3-15 μm. Most of the active groups are located within the pores which represent more than 90% of the total accessible surface area and provide a high specific surface area for interactions between molecules in the mobile and stationary phases. For separations under gradient flow conditions, multiple steps of the adsorption/desorption process should take place [Catherine, 1998].

In the case of HPMC, the length of the separation layer is much shorter (3 mm). Because of the short separation layer lengths and the resulting short residence times of the molecules within the separation layer, the multiple steps of the adsorption/desorption process were usually not considered as a possible mechanism for the separation [Joseph et al., 1992]. The separations of large biomolecules on short columns (membranes) are achieved by gradient elution [Laure et al., 2000].

The current market offers several designs of separation systems that involve chromatographic membranes in various shapes such as hollow fibers, stacked sheets, and individual disks [Keith Roper et al., 1995]. The majority of applications of monoliths are in the field of capillary electrochromatography [Svec, 2000], chromatography with microcolumns [Reginer, 2000] and capillaries [Gusev, 1999]. The concept of the ultra-short bed has been applied to a great extent for separation of bioploymers such as proteins and polynucleotides [Lian et al., 2001]. Recently, isocratic separation of plasmind DNA conformers under isocratic flow conditions on a 3 mm thick CIM (Convective Interaction Media) QA (Quaternary ammonium) monolithic disk was used [Michael et al., 1998]. However, no clear explanation of the phenomena governing the separation mechanism was provided [Vodopivec, 2000].

In this work, gradient separation of proteins (myoglobin, conalbumin, soybean trypsin inhibitor) in the anion-exchange mode was presented. The effects of the DEAE (diethylaminoethyl) disk and QA (quaternary ammonium) disk layer, the mobile phase composition of buffer B in sodium chloride concentration on the resolutions of the three peaks (myoglobin, conalbumin, soybean trypsin inhibitor) were discussed in terms of the plate theory on conventional HPLC columns.

## EXPERIMENTAL

### 1. Separation Unit and Chemicals

The separation of proteins (myoglobin, conalbumin, soybean trypsin inhibitor) was performed on commercially available CIM DEAE (diethylaminoethyl groups) disks, QA (quaternary amine) disks (BIA Separations, Slovenia). CIM disk monolithic columns bearing strong QA and weak DEAE anion groups were used throughout the ex-

†To whom correspondence should be addressed.
E-mail: rowkho@inha.ac.kr

perimental work. CIM disk monolithic columns consisted of a 3 mm length and 12 mm I.D. disk-shaped poly(glycidyl methacrylate-co-ethylenedimethacrylate), highly porous polymer matrix that was seated in a non-porous self sealing fitting ring. The bed volume of one disk was 0.34 ml, 60% of which resided in the highly interconnected flow through pores. The disk-shaped matrix (stationary phase) was inserted in commercially available PP or polyacetal housing (BIA Separations) and connected to a HPLC system. Standard chemicals of proteins in this experiment were purchased from Sigma-Aldrch Korea. The Tris [$NH_2C(CH_2OH)_3$] were purchased from J. T. Baker (Phillipsburg NJ, U.S.A.). Sodium Chloride was purchased from Oriental Chemical Industries (Korea). Hydrochloric acid was purchased from Junsei Chemical Co. (Japan). Water was distilled and deionized prior to use. The buffer A used a 20 mM Tris-HCl buffer, pH 7.4. and buffer B was used 20 mM Tris-HCl buffer A with 1 M sodium chloride.

**2. Equipment**

The HPMC system in this experiment was installed in Waters Model 600S liquid chromatography (Waters Associates, Milford, MA, U.S.A.) with the Waters 515 Multisolvent Delivery System and 486 Tunable Absorbance analytical Detector, and injector (5 ml sample loop) of Rheodyne. The data acquisition system was CHROMATE (Ver. 3.0, Interface Eng., Korea) installed in a PC. The wavelength was fixed at 280 nm and the experiment was performed at room temperature. The pH meter was purchased from EUTECH Instrument (Korea).

## RESULTS AND DISCUSSION

The set-up of the HPLC system is a crucial factor in achieving the optimal performance from CIM monolithic columns [Dubinina et al., 1996]. CIM monolith columns have been commonly used for gradient separations and ion exchange mode. Two different matrices bearing weak (DEAE) and strong (QA) anion exchange groups were tested for proteins (myoglobin, conalbumin, soybean trypsin inhibitor) separation with the effects of buffer concentration. To separate of proteins, mobile phases were used as buffer A (20 mM Tris-HCl) and buffer B (buffer A+1 M NaCl). In these experiments, the flow rate of mobile phase was set at 4 ml/min, and sample injection volume was 20 μl. As the retention times of the proteins were changed with the composition, the experimental variables were different sodium chloride concentrations ranging from 1 M to 0.1 M. The separated peaks eluted from the tube were analyzed on DEAE and QA disks again in a very short time (2 min). To investigate the effect of the mobile phase composition. DEAE and QA disks of thickness 3 mm were used.

Fig. 1 shows gradient HPMC separation of proteins (myoglobin, conalbumin, and soybean trypsin inhibitor) on a CIM DEAE disk. The high effective charge of the proteins molecules was taken into account to form the gradient conditions [Shuichi et al., 1999]. The first eluting components, that is to say myoglobin, was separated, but the other components, conalbumin and soybean trypsin inhibitor coeluted. Separation mechanism occurred to ion exchange mode between protein molecules and monolithic convective interaction media DAEA disk, QA disk [Xianfang, 1998]. The gradient condition was buffer A/buffer B=100/0-0/100 vol% during the linear gradient time 30 sec. The separation mechanism of HPMC is

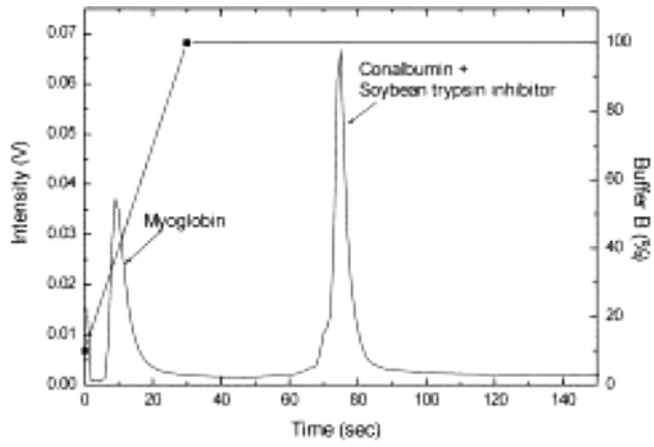


**Fig. 1. Chromatogram of myoglobin, conalbumin, and soy bean trypsin inhibitor in gradient mode (Buffer A of 20 mM Tris-HCl, Buffer B of 1 M NaCl in buffer A, Buffer A/Buffer B= 100/0-0/100 vol%, gradient time 30 sec, inj. volume=20 μl, column CIM DEAE monolithic weak anion disk, flow rate 4 ml/min, UV at 280 nm).**

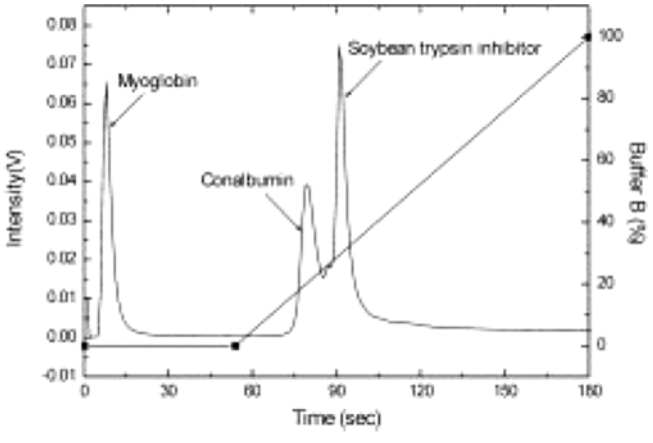


**Fig. 2. Gradient separation of myoglobin, conalbumin, and soy bean trypsin inhibitor using CIM DEAE monolithic weak anion disk (Buffer A/Buffer B=100/0-0/100 vol%, gradient time 50 sec).**

that competitive adsorption and desorption occurs in between activated membrane surface of anion exchanger and protein molecules. Anionic protein molecules are attracted on the cationic surface of membrane. If multiple adsorption/desorption steps occur in thin monoliths, the retention time of protein depends on the concentration of sodium chloride in the buffer B. As the concentration of buffer B increased, more sodium chloride is ionized to $Na^+$ and $Cl^-$. In Fig. 2, conalbumin and soybean trypsin inhibitor were separated by CIM DEAE disk. In this condition, for the first 50 sec, the concentration of sodium chloride was kept constant, and then gradually it increased to modify the differences in the retention times of conalbumin and soybean trypsin inhibitor.

Fig. 3 demonstrates gradient HPMC separation of proteins on a CIM QA disk, strong anion exchange group. The mobile phase composition was that buffer A/buffer B=100/0-40/60 vol% was changed during the first gradient time 10 sec, and then Buffer A/buffer B= 40/60-0/100 vol% during the second gradient time 40 sec. To im-

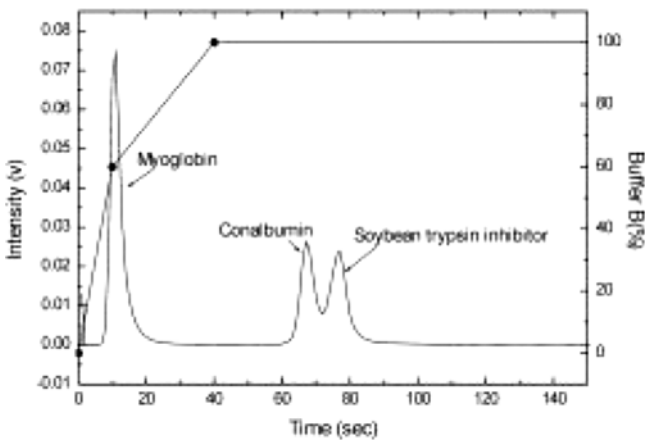


**Fig. 3. Gradient separation of myoglobin, conalbumin, and soy bean trypsin inhibitor using CIM QA monolithic strong anion disk. (Buffer A/Buffer B=100/0-40/60 vol%, gradient time 10 sec, Buffer A/Buffer B=40/60-0/100 vol%, gradient time 40 sec).**

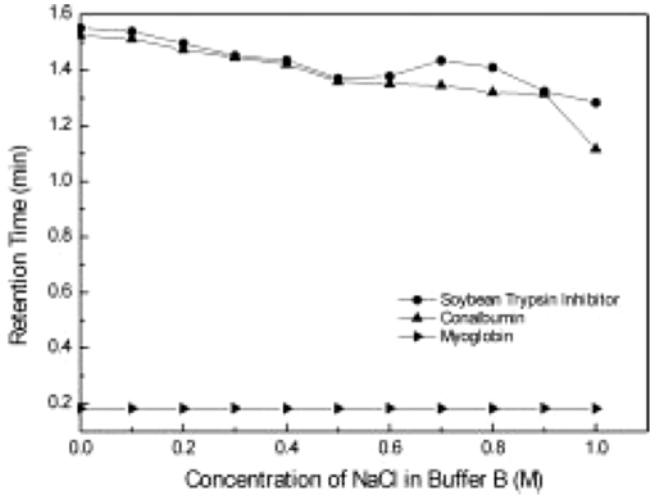


**Fig. 4. Effect of the concentration of NaCl on the retention times.**

prove the resolution of the two components, conalbumin and soybean trypsin inhibitor, a different type of stationary phase, the QA disk, was adopted. In this experimental run, the concentration of sodium chloride, was changed for 40 sec, but before elution of the last two components, the concentration of buffer B was constant. The composition of eluent for gradient chromatography approximately corresponded to the salt concentration. Column efficiency (N) and resolution (R) between components, A and B were calculated by Eqs. (1) and (2) for CIM DEAE (Fig. 2) and QA (Fig. 3), respectively.

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2 \quad (1)$$

Where $t_R$ denotes the retention time, and $W_{1/2}$ the peak width at the half height. As the number of theoretical plates are larger, the shape of the peak was sharper.

$$R = \frac{2\times(t_{R,B} - t_{R,A})}{W_A + W_B} \quad (2)$$

Where $t_{R,A}$ and $t_{R,B}$ are retention times of the eluted peaks, and $W_A$ and $W_B$ are the width of the peaks.

The calculated number of theoretical plates and resolutions with the difference type of disk DEAE and QA are listed in Table 1. The higher numbers of theoretical plate (N) means that the shape of peak becomes sharper. In terms of the numbers in weak DEAE and strong QA, it depends on the samples of protein, myoglobin, conalbumin, and soybean trypsin inhibitor. Moreover, it is greatly affected by the concentration of sodium chloride. In other words, the numbers of theoretical plates in the increasing concentration of sodium chloride in mobile phase is higher than that in high concentration.

**Table 1. Calculated number of theoretical plates and resolutions in DEAE and QA monolithic disks**

| | | DEAE (Fig. 2) | QA (Fig. 3) |
|---|---|---|---|
| $N_{Myoglobin}$ | | 35 | 70 |
| $N_{Conalbumin}$ | | 840 | 1400 |
| $N_{STI}^{*}$ | | 3900 | 1200 |
| R | myoglobin and conalbumin | 5.6 | 5.8 |
| | conalbumin and STI* | 0.8 | 1.1 |

STI*: Soybean Trypsin Inhibitor

Resolution depends on the two factors, the narrowness of the peaks and the distance between the highest points of two peaks. It was observed that the difference in the resolution of conalbumin and soybean trypsin inhibitor was small. Comparing the resolutions in the both cases, it was 0.8 in Fig. 2, while 1.1 in Fig. 3. The small molecules of myglobin were easily separated, but the complete separation of the other two large molecules was difficult to be performed.

The effect of buffer concentration on the gradient mode was studied in this work. In Fig. 4, the retention times of the proteins were plotted as the concentration of sodium chloride in buffer B. As the concentration of sodium chloride increased, the retention of myoglobin was not influenced. However, conalbumin and soybean trypsin inhibitor were eluted faster with increasing concentration of sodium chloride. It could be explained that competitive adsorption and desorption process on the anion exchange membrane arose between proteins and ions in buffer. Also in anion exchange membrane chromatography, samples were separated by the differences in isoelectric points (pI) [Elisabeth, 1999]. The pH of mobile phase equals the proteins pI. In the mobile phase of pH 7.4, the net charge of the protein changes from negative to neutral, leading to its desorption from the stationary phase of anion-exchanger. The protein separation and isolation was dependent on the pIs of samples, so the corresponding ion exchange membranes was selected. If pI value was below 7, anion exchange membrane was mainly used. And, cation exchange membrane was applied above pI of 7.

The dissolved anion proteins adsorbed on cation functional group. Also, proteins separation is a surface-dependent dynamic process, in which a portion of the protein molecule binds to the packing material through individual charge sites or via specific surfaces of the protein. There may be other explanations. Increased salt concentration in the mobile phase can lead to an increase in the hydrophobic

interaction of proteins with ion-exchange membrane disks. All proteins in the present work show a decreased retention time with increased buffer concentration throughout the concentration range studied.

## CONCLUSION

One of the unique features of DEAE and QA disk monolith columns is the fast and easy exchange of the stationary phase. Although much works still remain to be done in the study of thin monolithic separation layers for various proteins, this work confirms that outstanding separations of the proteins in a very short time were achieved by the optimization of the mobile phase and buffer with HPMC. In this work, HPMC was applied to investigate the feasibility of large molecular proteins and biopolymer, also characteristic of HPMC studied in purpose of separated. Our results show that proteins were well resolved by a gradient mode on anion exchange CIM DEAE and QA disks.

## ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial support of the Center for Advenced Bioseparation Technology and Inha University. This work was performed in the High-Purity Separation Laboratory of Inha University (Inchon, Korea).

## REFERENCES

Ales, P., Milos, B., Janez, J. and Ales, S., "High-Performance Membrane Chromatography of Small Molecules," *Anal. Chem.*, **71**, 2986 (1999).

Ales, P., Milos, B., Janez, J. and Ales, S., "Isocratic Separations on Thin Glycidyl Methacrylate-ethylenedimethacrylate Monoliths," *J. Chromatogr. A*, **848**, 51 (1998).

Boris, G., Belenkii, V. G. and Maltsev, "High-Performance Membrane Chromatography," *BioTechniques*, **18**, 288 (1995).

Catherine, C., "Purification of Proteins by Membrane Chromatography," *J. Chem. Technol Biotechnol.*, **71**, 95 (1998).

Dubinina, N. I., Kurenbin, O. I. and Tennikova, T. B., "Peculiarties of Gradient Ion-exchange High-performance Liquid Chromatography of Proteins," *J. Chromatogr. A*, **753**, 217 (1996).

Elisabeth, H., "Prediction of Protein Retention at Gradient Elution Conditions in Ion-exchange Chromatography," *J. Chromatogr. A*, **852**, 351 (1999).

Galina, A., Tishchenko, M. B., Jici, S. and Tomas, B., "Effect of Salt Concentration Gradient on Separation of Different Types of Specific Immunogobulins by Ion-exchange Chromatography on DEAE Cellulose," *J. Chromatogr. B*, **706**, 157 (1998).

Joseph, A., Gerstner, R. H. and Steven M. Cramer, "Membrane Chromatographic Systems for High-throughput Protein Separations," *J. Chromatogr.*, **596**, 173 (1992).

Keith Roper, D. and Edwin N. Lightfoot, "Separation of Biomolecules using Adsorptive Membranes," *J. Chromatogr. A*, **702**, 3 (1995).

Laure, G., Berreux, R. F. and Tatiana, B. T., "Comparison of Antibody Binding to Immobilized Group Specific Affinity Ligands in High Performance Monolith Affinity Chromatography," *J. Pharm. Biomed. Anal.*, **24**, 95 (2000).

Lee, Y. W., So, M. S., Lee, J. W., Chung, S. T. and Row, K. H., "Retention Models of Capacity Factor with Different Compositions of Organic Modifier in RE-HPLC," *J. of Chem. Eng.*, **13**, 578 (1996).

Lian, S. and David, J. Anderson, "Effect of Buffer Concentration on Gradient Chromatofocusing Performance Separating Proteins on a High-performance DEAE Column," *J. Chromatogr. A*, **909**, 191 (2001).

Michael, B., Tennikov, N., Gazdina, V., Tatiana, B. and Tennikova, F. S., "Effect of Porous Structure of Macroporous Polymer Supports on Resolution in High-performance Membrane Chromatography of Proteins," *J. Chromatogr. A*, **798**, 55 (1998).

Peter, R., Levison, A. K. and Hopkins, P. H., "Influence of Column Design on Process-scale Ion-exchange Chromatography," *J. Chromatogr. A*, **865**, 3 (1999).

Peter, R., Levison, S. E., Badger, P. H., Martin, J., Davies, I. J. and Bruce, V. G., "New Approaches to the Isolation of DNA by Ion-exchange Chromatography," *J. Chromatogr. A*, **827**, 337 (1998).

Shuichi, Y. and Eiji, M., "Retention Behavior of Very Large Biomolecules in Ion-exchange Chromatography," *J. Chromatogr. A*, **852**, 25 (1999).

Shuichi, Y., Peter, K., Watler, D. F. and Oliver, K., "Characterization of Unstable Ion-exchange Chromatographic Separation of Proteins," *J. Chromatogr. A*, **852**, 37 (1999).

Sridhar, P., "Design of Affinity Membrane Bioseparations," *Chem. Eng. Technol.*, **19**, 398 (1996).

Vodopivec, M., Berovic, M., Jancar, J., Podgornik, A. and Strancar, A., "Application of Convective Interaction Media disks with Immobilized Glucose Oxidase for On-line Glucose Measurements," *Analytica Chimica Acta*, **407**, 105 (2000).

Wensheng, X. and Fred, E. R., "Protein-protein Interactions on Weak-cation-exchange Sorbent Surfaces During Chromatographic Separations," *J. Chromatogr. A*, **828**, 357 (1998).

Xianfang, Z. and Eli, R., "Cross-linked Macroporous Chitosan Anion-exchange Membrane for Protein Separations," *Journal of Membrane Science*, **148**, 195 (1998).