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Development of Novel Protein Refolding Using Simulated Moving Bed Chromatography

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Abstract–*In vitro* protein refolding is still one of the baffles in both structural biology and development of new biopharmaceuticals, especially for large-scale production of valuable proteins that are overexpressed as inclusion bodies in *Escherichia coli*. A new continuous refolding method using four zone simulated moving bed process based on size exclusion mechanism was developed to overcome difficulties of inclusion body refolding. Protein refolding using size exclusion SMB enables us to obtain refolded protein continuously with high productivity, low consumption of refolding buffer, and high efficiency of size exclusion medium. Thermodynamics and kinetic parameters for SMB operation were estimated from the best-fit values by comparing the simulation and experimental chromatography results. The SMB operation condition was obtained from the triangle theory, and experimental results were in good agreement with the simulation results.

Key words: Lysozyme, Continuous Protein Refolding, Size-exclusion Chromatography, Simulated Moving Bed Chromatography

INTRODUCTION

Recent advantages in recombinant DNA technology have opened a new era for protein production both for research and industrial application. Especially, recombinant protein expression in transformed microorganisms has brought an unlimited supply of rare, high-value proteins when their biological functions do not depend on post-translational modifications such as glycosylations. This technology is currently utilized in the cost-effective production of authentic human therapeutic proteins.

High expression levels of recombinant protein in *Escherichia coli* often result in the formation of micro-scale particles of aggregated protein, called "inclusion bodies (IBs)." IBs can be solubilized and refolded *in vitro*, most commonly by dilution in batch or fed-batch reactors. However, protein aggregation limits yield in these reactor systems [Middelberg, 1996], and separation of production from aggregates must be subsequently undertaken. Many attempts have been made to improve *in vitro* refolding [Altamirano et al., 1997], ranging from optimization of the physicochemical properties of the refolding environment [Rozema and Gellman, 1995] to optimization of reactor operation [Kotlarski et al., 1997], batch and fed-batch operation [Gao et al., 2002] and reactor design [Lee et al., 2001].

Developments in protein refolding have demonstrated that different types of chromatography columns can be useful for protein refolding purposes [Lanckriet and Middelberg, 2004]. In 1992, bovine serum albumin (BSA) and lysozyme were refolded with success using silica linked to polyethylene glycol having a hydrophobic endgroup [Geng and Chang, 1992]. In addition to matrices designed to interact with the protein molecules, size-exclusion chromatography (SEC) has been used for protein refolding [Warner et al., 1994]. This technique was used to refold recombinant human ETS (endothelins)-1 protein and bovine ribonuclease on Superdex 75 column. Batas and co-workers [Batas and Chaudhuri, 1996, 1999; Batas et al., 1997, 1999] undertook further studies in this field and proposed a mechanism for size-exclusion refolding. Refolding in a SEC packed bed is based on a buffer-exchange mechanism.

Chromatography processes have typical drawbacks of batch operation, in-effective solvent utilization, in-sufficient media utilization and high dilution. Therefore, to avoid them, a new continuous refolding method using four zone SMB process based on size exclusion mechanism was developed to overcome difficulties of batch IBs refolding process. Protein refolding using SMB enables one to obtain refolded protein continuously with high productivity, low consumption of refolding buffer and high efficiency of size exclusion medium.

SMB chromatography is a continuous chromatographic process developed by UOP (United Oil Products) in the 1960's [Broughton, 1968; Broughton et al., 1970]. SMB chromatography has novel advantages of higher productivity, lower solvent consumption, lower product dilution and therefore lower operating costs, and the disadvantage of higher fixed costs. For preparative and production scale separations, where the low operation cost overcomes the high fixed cost, the overall separation cost of SMB is lower than that of batch chromatography [Zhang et al., 2004]. It has been used in the petrochemical, sugar [Jacobson et al., 1987] industries for large-scale separation, and amino acids separation [Wu et al., 1998] and chiral separation [Pais et al., 2000] for fine chemical industries.

THEORY

1. Size-Exclusion Protein Refolding System (SEPROS)

SEPROS for the removal of denaturants and the separation of folding intermediates has been extensively used since the 1990's (Fig. 1) [Jungbauer et al., 2004]. It has been hypothesized that the gradual removal of the denaturant during buffer exchange while passing through the column and the different diffusion properties

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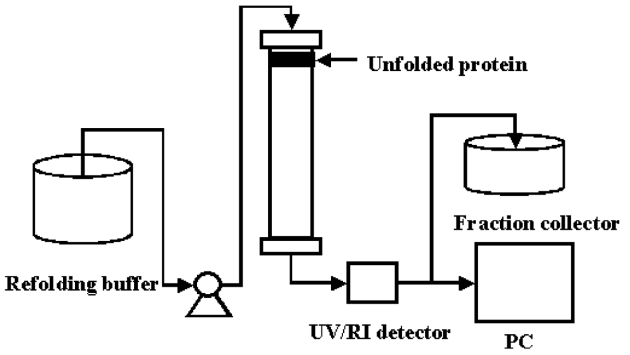

Fig. 1. Experimental system for protein refolding using size-exclusion chromatography.

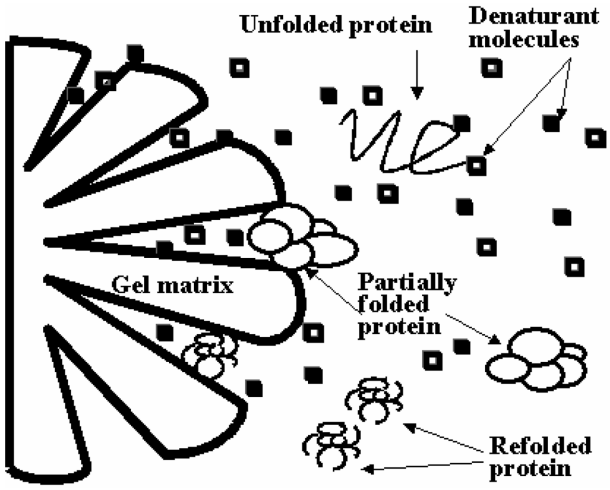

Fig. 2. Schematic representation of protein refolding within size-exclusion chromatography media.

of the unfolded protein, intermediates and aggregates in the stationary phase minimize the likelihood of aggregation [Batas and Chaudhuri, 1996]. Small molecules, like dithiotheritol (DTT) and urea, enter the pores of the resin and are separated from unfolded protein molecules. As the chaotrope concentration surrounding the unfolded species is reduced, the protein starts to fold yielding compact and partially folded protein molecules that are able to enter the resin pores (Fig. 2).

2. SMB Process

SMB unit consists of four zones and each zone has 1 column, which are connected in series. Between each of these columns, feed, desorbent, extract, and raffinate ports are placed. The port between the columns allows a path to open or close inlet streams (feed, desorbent) and outlet streams (raffinate, extract) at the switching time. The counter-current movement between the stationary and mobile phase is simulated by port movements as shown in Fig. 3. The feed is fed between zone II and III. The low affinity solute and high affinity solute are separated and collected in the raffinate and extract, respectively. Desorbent is fed between zone I and IV. In order to achieve a successful separation, the migration linear velocities of the solutes i in the zone, j, u_{si}^{j} must satisfy the following conditions [Ma and Wang, 1997]:

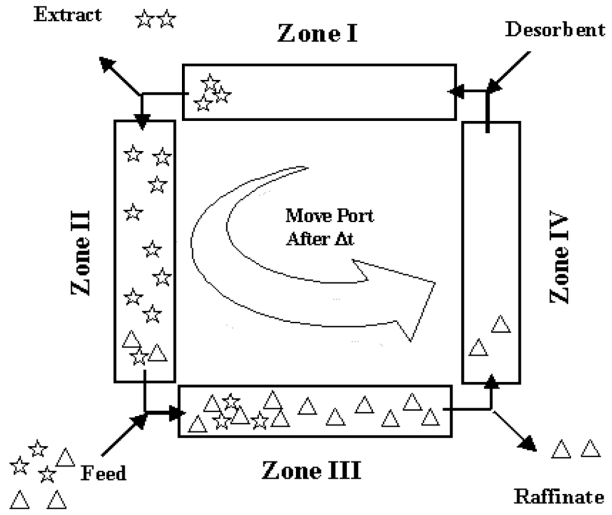

Fig. 3. Schematic diagram of a four-zone SMB system.

$$u_{s2}^{I} - v > 0,\ u_{s1}^{II} - v > 0,\ u_{s2}^{III} - v < 0,\ u_{s1}^{IV} - v < 0 \quad (1)$$

where v is the solid movement velocity, subscripts 1 and 2 are for the low (refolded protein) and the high (chaotropic and reducing agents) affinity solute, respectively.

3. Triangle Theory

The operation of SMB chromatography depends on the flow rate of the four zones, as well as the switching time. In order to find successful operating conditions, Morbidelli's group proposed the Triangle theory, based on the equilibrium theory. Although the equilibrium theory neglects the axial dispersion and mass transfer resistance, it provides a useful starting point for SMB experiments. The parameter m_j is the flow rate ratio, and is defined as the ratio of the net fluid flow rate over the solid phase in zone j. Based on the flow ratio m_j, the experimental conditions for SMB can be found:

$$m_j = \frac{Q_j^{SMB} t^* - V\varepsilon^*}{V(1-\varepsilon^*)} \quad (2)$$

where Q_j^{SMB} is the internal volumetric flow rate in zone j, t^* the switching time, ε^* the total porosity and V the volume of a single column. The complete separation region is defined by m_2 and m_3, which are in dependent of m_1, m_4. The detailed condition of constraints has previously been represented in the literature [Mazzotti et al., 1997].

EXPERIMENTAL

1. Materials

Crystalline hen egg white lysozyme (HEWL; EC 3.2.1.17; 52,000 units/mg), DTT, urea, reduced and oxidized glutathione (GSH, GSSH), *Micrococcus lysodeikticus* dried cells, ethylenediaminetetraacetic acid (EDTA) and Trizma base were obtained from Sigma (St. Louis, MO, USA). The Blue dextran and acetone, used for the column porosity measurements, were purchased from Sigma (St. Louis, MO, USA). All other chemicals were analytical grade. Water used for the experimental work was deionized water (DI) obtained from Milli-Q system (Bedford, USA), and filtrated through 0.22 μm filters. All solutions were degassed by using He.

2. Equipments

A fast protein liquid chromatography (FPLC) system (Pharmacia Biotech, Sweden) was used for the porosity and the isotherm measurements. The FPLC system consists of two Pharmacia P-920 pumps, Pharmacia INV-907 valve, UPC-900 Monitor and Trac-900. A UV-Vis spectrophotometer, Genesys 5 (Spectronic, UK), was used to analyze protein activity and concentration. The experiments were carried out in four XK 16/40 (Pharmacia Biotech, Sweden) columns packed with Sephacryl S-100 (HR) (Pharmacia Biotech, Sweden) according to the manufacturer's instructions. A laboratory four-zone SMB was used for the experiments. The SMB unit was composed of four columns, with each zone having one column. Two FPLC pumps controlled the feed and desorbent streams, and two HPLC pumps (LC-10 AD, Shimadzu) controlled extract and recycle streams. Four Rotary valves (VICI, Switzerland), with one inlet and four outlet ports, were used. Each column had one rotary valve connected to the four streams. Each valve changed the flow path at every switching time. The valves were controlled by the LabView program (National Instruments, TX, USA).

3. Preparation of Denatured Reduced Protein

This method is described fully in other study [Batas and Chaudhuri, 1996]. Native lysozyme (2-40 mg/mL) was denatured by incubating the protein in 0.1 M Tris-HCl, pH 8.6, containing 8 M urea and 0.15 M DTT for 2 h at room temperature. The protein activity was analyzed to confirm that the protein had been denatured completely. The denaturation buffer and all others used in all processes should be prepared and used within one day. Prior to experiment, all buffers were degassed and filtrated by 0.45 μm filters.

4. Lysozyme Refolding by Batch Chromatography

Fig. 1 shows the experimental arrangement for refolding in a size-exclusion column. Lysozyme denatured as described above was applied on a column and previously equilibrated with refolding buffer [0.1 M Tris-HCl, pH 8.2, 1 mM EDTA, 3 mM GSH and 0.3 mM GSSG; urea 2 M]. The sample was eluted at a flow rate from 0.34 to 0.9 mL/min using the refolding buffer. The sample volume applied was 200 μL. The experiments were carried out at room temperature. Fractions were collected and analyzed for enzyme concentration and activity. The activity of a sample was compared with the activity of native HEWL in an ideal buffer and expressed by refolded lysozyme concentration.

5. Analytical Methods

Protein concentrations were determined spectrophotometrically at 280 nm by using the absorption coefficients ε(1 mg/mL)=2.63 for native HEWL, ε(1 mg/mL)=2.37 for reduced, denatured HEWL protein after TCA precipitation and resuspension in 0.1 N NaOH to reduce interferential effect of detergents [Saxena and Wetlaufer, 1970; Bollag et al., 1996]. Ultrahydrogel 120 column (7.8×300 mm, Waters, USA) was used to determine DTT and urea concentration. The eluent was 0.5 M CH_3COOH/0.3 M Na_2SO_4. The flow rate was 0.8 mL/min and the injection volume was 20 μL. Urea was determined by refractive index, waters 410 (Waters, USA) [Pace, 1986]. The detection wavelength of DTT was 280 nm by using the Waters 486 detector. Prior to analysis, all solvents were degassed and filtrated by 0.45 μm filters.

6. Protein Activity Assay

Lysozyme activity was determined at 25 °C by following the decrease in absorbance at 450 nm of a 0.25 mg/mL *M. Lysodeikiticus* suspension in 0.06 M potassium phosphate, pH 6.2. The assay volume was 3 mL, and 50 μL of protein sample was added. One unit of activity corresponds to an absorbance decrease of 0.0026 per minute [Shugar, 1952]. The concentration of refolded lysozyme was determined by comparing the activity of the refolded lysozyme to the activity of standard solution of native lysozyme of different concentrations. Samples were stored at 4 °C until analysis, which was performed within 24 h.

7. Isotherm Determination

The equilibrium isotherm of urea playing the critical role in protein refolding was obtained from single frontal chromatography. The urea solution of a prepared concentration (0.4-8 M) was pumped into the column with an FPLC pump set at 0.8 mL/min and detected with a refractive index. The isotherm of DTT was calculated by using pulse tests at different concentrations (0.03-0.15 M), with a flow rate of 0.9 mL/min and detected 280 nm.

8. Isotherm Calculation

The Langmuir isotherm for a single component is:

$$q = \frac{aC}{1+bC} \tag{3}$$

where q and C are the concentration of the solute in the stationary and mobile phases at equilibrium, respectively [Mazzotti et al., 1997], and a and b are characteristic parameters of the solute in a given system. The parameters are estimated by using a single-component frontal analysis. The amount adsorbed onto the stationary phase can be calculated by the following equation [Yang et al., 2004]:

$$q_{i+1} = q_i + \frac{(C_{i+1} - C_i)\{V_{F,i+1} - V(V_O + V_D)\}}{V_{sp}} \tag{4}$$

where V_F is the retention volume of the inflection point of the breakthrough curve, and V_O and V_D the column void and system dead volumes, respectively. V_{sp} is the volume of adsorbed in the column. The subscript i relates to the number of step changes in the concentration.

The isotherm data of urea are shown in Fig. 4. The numerical values of the coefficients of the Langmuir isotherm were a=0.498, b=0.001.

RESULTS AND DISCUSSION

1. Effect of Chaotropic and Reducing Agents on Protein Unfolding

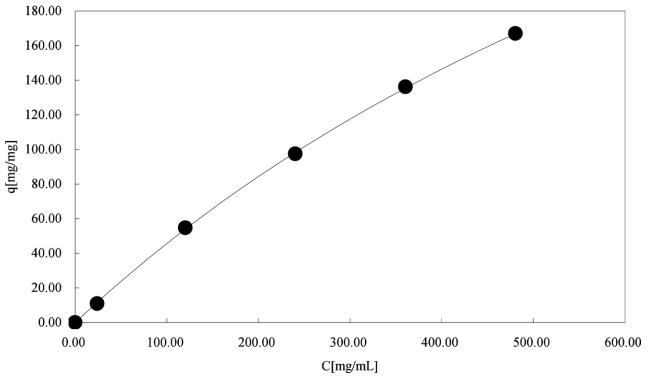

Fig. 4. Single component Langmuir isotherm of urea.

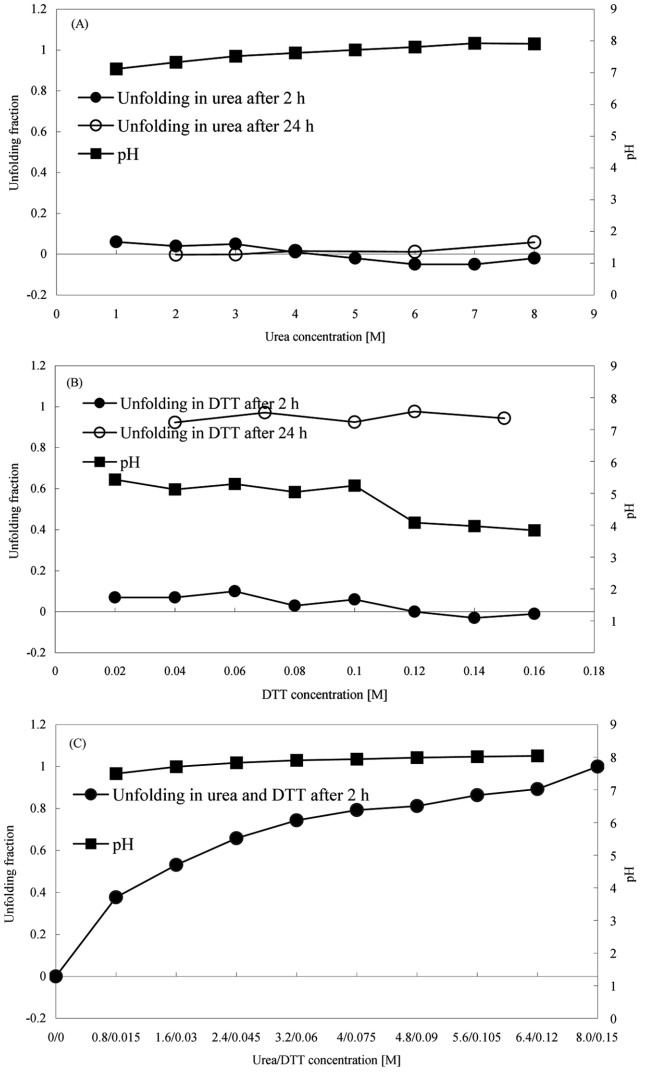

Fig. 5. Urea and DTT concentration dependence of lysozyme unfolding. Lysozyme unfolding in urea (A); unfolding in DTT (B); unfolding in urea and DTT molar ration of 8 : 0.15 (C).

The effect of urea on lysozyme stability was followed by activity transition experiments as a function of urea concentration by titration at a room temperature. The results are presented in Fig. 5. It is clear that at room temperature and around pH 7.0, addition of urea does not unfold the protein up to 8 M denaturant for 24 h. However, urea together with DTT molar ratio of 8 : 0.15 (denatured and reduced condition), unfolding sets in at all urea concentration and the transition reaches a fully unfolded state at 8 M urea and 0.15 M DTT. When the effect of DTT on lysozyme stability at room temperature was followed by activity experiments as a function of DTT concentration, just after 24 h the unfolding effect was confirmed for all concentrations. At each solvent composition the degree of conversion from the native to the unfolded state was estimated from the activity remaining, and the unfolded fraction (K) was calculated from:

$$K = \frac{(\Delta N - \Delta E)}{(\Delta N - \phi)} \quad (5)$$

where protein activity ΔN in native state, ϕ in unfolded state and ΔE in experimental state.

This phenomenon object to widely accepted fact in this field that a protein can be fully denatured at over 6 to 8 M urea. However, it can be explained by the critical role of DTT and the strong pH dependence of chemical protein unfolding [Elwell and Schellman, 1974]. Serge and Guifu showed that the interactions involved in the denaturation of lysozyme in the presence of urea were examined by thermal transition studies and measurements of preferential interactions of urea with the protein at pH 7.0, where it remains native up to 9.3 M urea, and at pH 2.0, where it undergoes transition between 2.5 and 5.0 M urea [Timasheff and Xie, 2003].

2. Effect of Chaotropic Agent on Protein Refolding

Gel filtration techniques offer an alternative approach to monitor folding-unfolding equilibria, including the large volume changes associated with these processes [Batas et al., 1997].

In the SMB refolding experiment, urea concentration varies from 2 to 8 M and lysozyme partition coefficient depends on urea concentration. So we studied the mobility changes of lysozyme with increasing urea concentration using a manual packed column. Before the experiment, the column was pre-equilibrated with different refolding buffers containing various urea concentrations, and then denatured and reduced lysozyme was applied onto the column with the same pre-equilibrated refolding buffer elution.

From 2 to 4 M urea concentration, the refolded protein peaks showed asymmetric shapes that can be considered as two different overlapped peaks. However, among them at 2 M urea concentration which is the best refolding condition, every part of the peak was almost fully active. Therefore, we assumed that the asymmetric shapes came from the different refolding pathways, and the mobility changes depend on urea concentration of refolding buffer. This fact can be inferred with other published studies about lysozyme refolding pathways that follow two different pathways (Fig. 6) [Anderson and Wetlaufer, 1976; Wildegger and Kiefhaber, 1997; Daniel et al., 1999]. It is that one follows a fast direct pathway and the other follows a slow intermediate prone pathway. For those reasons, we separated each chromatogram peak into two different peaks: the front peak (Lys_1) following the slow-folding pathway and the rear peak (Lys_2) following the fast direct pathway. We used multi-Gaus-

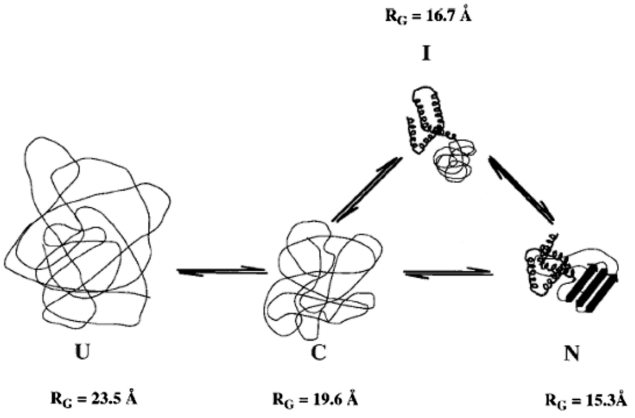

Fig. 6. Kinetic model for the molecular events occurring during lysozyme folding. U, C, I and N represent the completely unfolded protein, the collapsed state, the helical intermediate and the native protein, respectively.

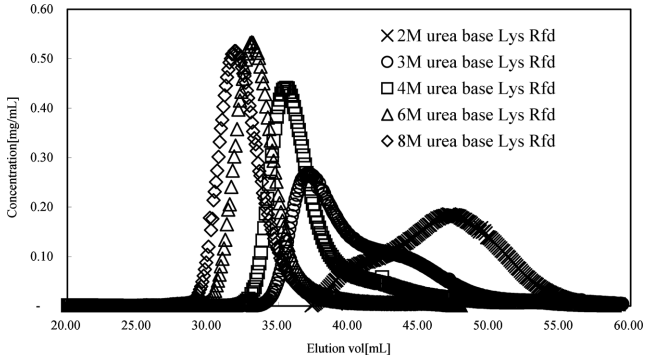

Fig. 7. Lysozyme elution in 8 M, 6 M, 4 M, 3 M and 2 M urea based refolding buffer. Denatured and reduced lysozyme loading: 200 μL of 10 mg/mL and 0.6 mL/min.

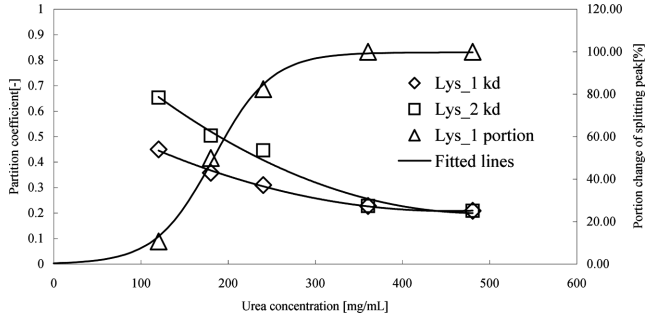

Fig. 8. Urea concentration effects on lysozyme partition coefficient and percentage change in the amount between front and rear portion of splitting peaks.

sian peaks fit (Microcal origin, version 6.0) so as to determine thermodynamics and kinetic parameters for SMB operation. As the urea concentration increased, the latter protein peak got smaller, and by 6 M urea only one peak was observed at this and upper concentration in that the reduced and denatured lysozyme might keep the denatured state (Fig. 7). Nevertheless, some activity of unfolded lysozyme was recovered, because after separation of DTT from feed sample, the redox system (GSH and GSSG) in refolding buffer containing 6 M urea presumably helped to reoxidize the reduced disulfide bonds of protein to oxidized state. As the concentration of urea decreased, the elution volume for the two peaks increased. In contrast to high urea concentrated environment, lower urea concentration leads unfolded protein to more compact native-like form. Thus, the elution volume increases as well as the peak broadened in terms of gradual urea concentration decreased. With the observed and corresponding results, we correlated the effect of urea concentration on protein refolding as a function of partition coefficient (k_d) and the tendency of percentage change in the amount between front and rear portion of splitting peaks depending on the urea concentration for the simulation parameters and SMB operation conditions (Fig. 8). We could verify our presumption about adequacy of peak separation with the good agreement between the experimental results and simulation (Fig. 9).

3. Urea in SMB Experimental and Simulation Results

Urea is not only a strong protein denaturant but also an effective aggregation inhibitor. It is found that urea at non-denaturing concentrations can improve the yield of correctly folded protein [Gu et

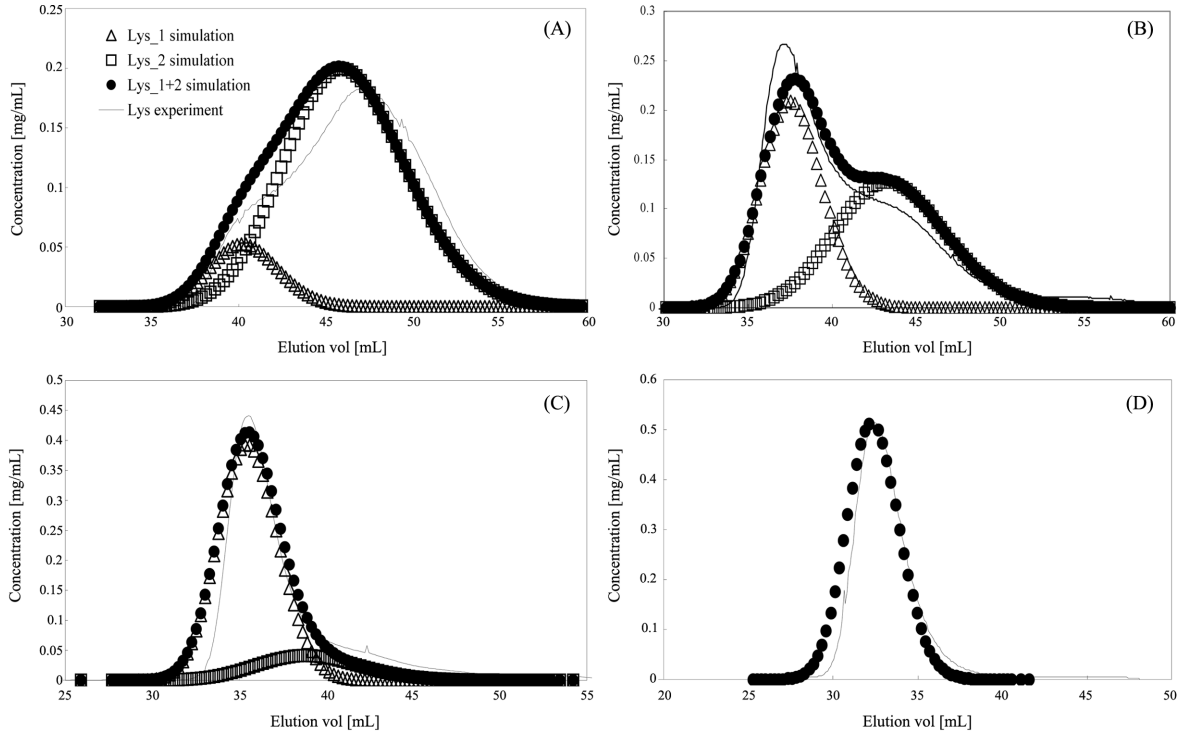

Fig. 9. Comparison of lysozyme refolding experimental with simulation results. Experimental results. Lysozyme elution in 8 M urea (D); 4 M (C); 3 M (B); and 2 M (A) urea based refolding buffer. Denatured, reduced lysozyme loading: 200 μL of 10 mg/mL and 0.6 mL/min.

Table 1. System parameters for SMB simulations

System parameters				
Column length [cm]		33.30	Mass transfer parameters [cm^2/min]	
Column internal diameter [cm]		1.60	Brownian diffusivity (D^{∞})	
Particle radius [μm]		47	Lys_1	7.0800d-5
Interparticle porosity (ε_i)		0.38	Lys_2	7.0800d-5
Intraparticle porosity (ε_p)		0.80	Urea	8.9933d-4
Isotherm parameters			DTT	4.3229d-4
	a	b	Intraparticle diffusivity [D_p]	
Lys_1	0.000	0.000	Lys_1	8.7406d-5
Lys_2	0.000	0.000	Lys_2	6.0512d-5
Urea	0.498	0.001	Urea	4.9962d-3
DTT	0.996	0.000	DTT	2.4016d-4
Partition coefficient (k_d)			Axial dispersion [E_b]	
Lys_1		0.45	Lys_1	0.0900
Lys_2		0.65	Lys_2	0.0360
Urea		1.00	Urea	0.0077
DTT		1.00	DTT	0.0108

al., 2001]. In this study, activity recovery of lysozyme decreased with the increase in the final urea concentration as the final urea concentration was higher than 3 M; this result disagreed with Gu et al. We contemplated that the concentration gradient of urea from 8 to 2 M in SMB would be very important to refold protein continuously. And urea mobility validation in SMB can be also a reliable boundary to optimize the operation conditions in terms of faster elution rate of urea than DTT and characteristics of unfolding and refolding agents. So we confirmed the single component mobility of urea in SMB operation and compared it with simulation results.

The configuration of the SMB was 1-1-1-1. The system parameters, which are the average values of the four columns, for simulation of the SMB operation are listed in Table 1. The numerical simulation was performed by using an algorithm. The operating conditions were obtained by using the Triangle theory. The composition of feed was 2 M urea and desorbent was 8 M urea. Table 2 shows the operation conditions, such as switching time of 92.2 min, zone flow rate and ports flow rate. Total running time was 1520.5 min; 16.5 step. Before the operation, columns were pre-equilibrated with 2 M urea. Comparison of experimental data and simulation showed a good agreement (Fig. 10). From this result, the operation condition was considered safe enough to separate detergents from feed sample for protein refolding without any contamination over urea concentration in refolding buffer at raffinate port.

Table 2. Operation conditions for urea and lysozyme refolding in SMB

Operation conditions		
Flow rate [mL/min]	Feed	0.1
	Zone I	0.8
	Zone II	0.5
	Zone III	0.6
	Zone IV	0.5
	Desorbent	0.3
	Extract	0.3
	Raffinate	0.1
Switching time [min]	92.15	

4. Protein Refolding in SMB Experimental and Simulation Results

The SMB experiment was performed with the isotherm values following the predicted parameters. Lysozyme follows the size-exclusion mechanism and two different mobilities with the different partition coefficients (k_d). The isotherm of urea is Langmuir and that of DTT is linear. And the average values of lysozyme and DTT were measured from the experiments and calculations. In this study, each inlet composition was changed as feed of 2 mg/mL denatured and reduced lysozyme and desorbent as refolding buffer. The operation condition was the same with the urea single component SMB and the total running time was 783.3 min; 8.5 step.

Despite the shortage of running time to reach steady state, Fig. 11 and Table 3 show the possibility of protein refolding by using SMB and better productivity in final active lysozyme concentration than batch operation in terms of low sample dilution and consumption of desorbent.

CONCLUSIONS

With the advantages of recombinant IBs, difficulty of refolding was the bottleneck for research and industrial applications. The urea gradient gel filtration refolding system is a new contribution to the SEPROS proposed by Batas and Chaudhuri. The observations of changes in the hydrodynamic and retention volume with urea concentration help to explain the process of refolding by using size-exclusion chromatography. And the two different mobilities in batch chromatography confirmed that lysozyme follows two different refolding pathways. When the 2 M urea based refolding buffer was used as the mobile phase, the average activity recovery of lysozyme in SEPROS for four columns was over 94% but the activity recovery decreased over 3 M urea concentration, respectively. With the observed and results, we correlated successfully the effect of urea

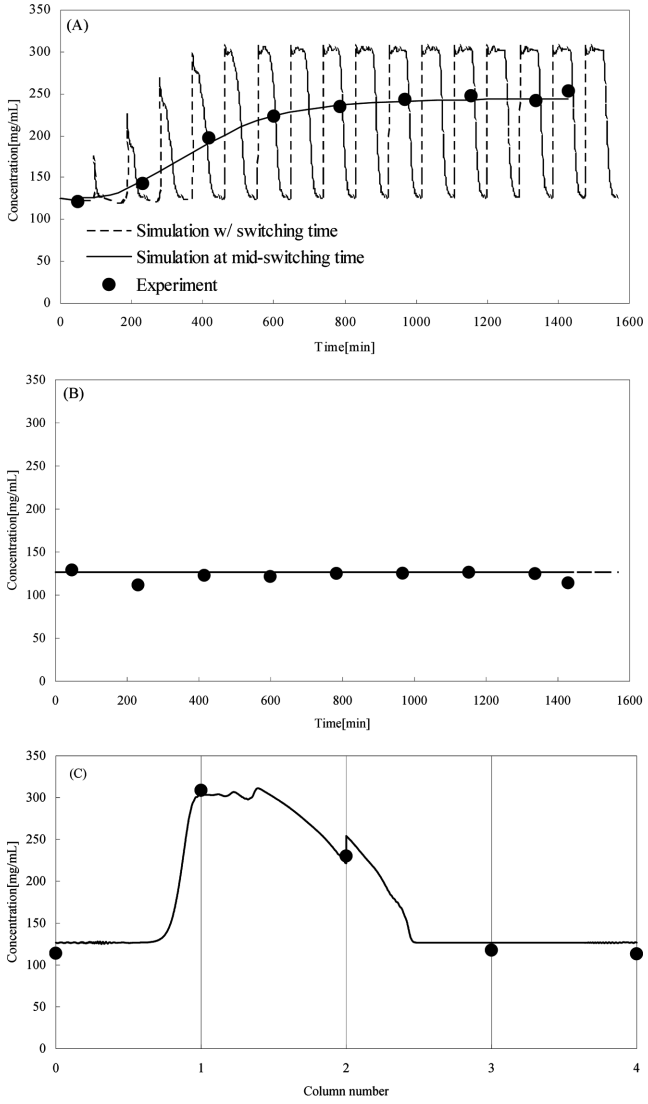

Fig. 10. Comparison of experimental with simulation results of urea in the effluent histories of extract (A); raffinate (B); and column profiles at 16.5 step (C).

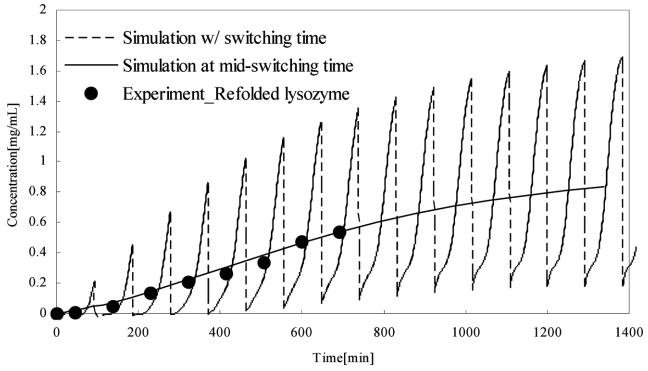

Fig. 11. Comparison of experimental with simulation results of refolded lysozyme in the effluent histories of raffinate.

concentration on protein refolding as a function of partition coefficient (k_d).

Table 3. Comparison of protein refolding using batch chromatography and SMB

	Batch	SMB
Refolding buffer consumption [mL]	221.16	221.16
Experiment number [-]	3	Continue
Starting concentration[a] [mg/mL]	10	2
Final concentration[b] [mg/mL]	0.08	0.25
Dilution [a/b]	121.41	7.93

[a]Denatured and reduced lysozyme concentration in feed
[b]Refolded lysozyme concentration at out port of batch and raffinate port of SMB

Thermodynamic and kinetic parameters for SMB operation were estimated from the best-fit values by comparing the simulation and experimental batch chromatography refolding result. The isotherm of urea playing the important role in protein refolding was obtained from single frontal chromatography and fitted as a Langmuir isotherm. In the case of DTT, the retention was constant over the concentration range of this work and the isotherm was assumed as linear. The SMB operation condition was obtained from the triangle theory and experimental results were in good agreement with the simulation results.

This work suggested the possibility of protein refolding by using SMB with its typical advantages: low consumption of refolding buffer, high productivity of refolding yield and higher efficiency of medium than SEPROS. The applications of protein refolding using SMB are not limited on SEPROS but also other chromatographic protein refolding methods.

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