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Considerations of sample application and elution during size-exclusion chromatography-based protein refolding

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Abstract

A mechanism for size-exclusion chromatography-based protein refolding is described. The model considers the steps of loading the denatured protein onto a gel filtration column, and protein elution. The model predictions are compared with results of refolding lysozyme (10 and 20 mg/ml) using Superdex 75 HR. The main collapse in protein structure occurred immediately after loading, where the partition coefficient of unfolded lysozyme increased from 0.1 to 0.48 for the partially folded molecule. Use of a refolding buffer as the mobile phase resulted in complete refolding of lysozyme; this eluted at an elution volume of 15.6 ml with a final partition coefficient of 0.54. The model predicted the elution volume of refolded lysozyme at 19.3 ml. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

A new method for refolding denatured proteins, based on gel filtration chromatography, has been reported [1]. This method was shown to increase the refolding yields of denatured proteins at high initial concentrations. Size-exclusion chromatography (SEC) matrices were used to perform denaturant removal, promote the folding reaction and to minimise aggregate removal. The reduced diffusion of proteins in gel-filtration media and the obstruction effect of the gel matrix have been shown to suppress the non-specific interactions of partially folded molecules thus reducing aggregation. Refolding yields of two purified proteins, lysozyme and carbonic anhydrase, were significantly improved at high final protein concentrations using this method. Aggregation reaction was almost completely inhibited at the same time. The system also allowed renaturation of proteins at higher initial protein concentrations than typically used. This technique has also been applied to the refolding of recombinant hen egg white lysozyme [2] and ovine growth factor [3] from *E. coli* inclusion bodies. The refolding of secretory leukocyte protease inhibitor (SLPI) has been achieved using a rolled stationary phase of DEAE-cellulose in a chromatography column [4]. This report used a similar principle to the gel filtration-based method.

An initial explanation of protein refolding using size-exclusion media has been reported [1]. The mechanism was based on the initial assumption that

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protein size changes with a reduction in the denaturant concentration. This assumption was confirmed by Batas et al. [5] who also quantified the partition coefficient and Stokes radius of lysozyme at denaturant concentrations similar to those found during the refolding process. A preliminary theoretical consideration based on lysozyme equilibrium studies is presented here. The model considers the size changes of the folding protein during two key steps in the refolding process: loading of the sample onto the column; and elution of the protein with a refolding buffer. The earlier theoretical considerations did not account for such changes in protein size during column refolding.

2. Theoretical considerations of SEC refolding

The theoretical treatment of protein refolding using SEC is based on changes in protein size during its passage through the gel media. We consider here two key parts of the process. The first is the loading of the protein sample, in a denaturant solution, onto the chromatographic bed. At this time the protein is fully denatured, has no activity and is assumed to be significantly excluded from the gel pores. The denaturant may freely diffuse into the gel whilst the protein occupies the void volume. The second part considers the changes in the protein size as it renatures during elution with the refolding buffer. Both parts of this mechanism include the refolding reaction, which is represented in terms of the changing protein partition coefficient in the gel media (K_P). K_P is a function of protein Stokes radius (R_S) or hydrodynamic volume (V_h) [5].

The chromatographic-based refolding process is based on differential movement of solutes present in the original sample. The solutes to be separated are the target protein to be refolded and the denaturant species. The denaturant components, usually a low-molecular-mass chaotropic agent (urea or guanidine hydrochloride) with a reducing agent (dithiothreitol or β-mercaptoethanol), will partition freely into the gel media and will have the same partition coefficient ($K_d=1$). The chromatographic properties of the denaturant do not change during this process whereas the hydrodynamic properties of the protein change throughout the chromatographic process, as the denaturant diffuses away from the protein which then folds. These constant changes in protein structure, and K_P, as it refolds in the column distinguish size-exclusion-based refolding from conventional SEC.

2.1. Volumetric distribution of the gel bed

An understanding of the SEC gel bed is required for the development of the refolding mechanism. The SEC gel bed is divided in two distinct parts, the mobile and the stationary phases. The total gel volume (V_T) is made up of the mobile phase volume (i.e., the void volume, V_0), and the volume of the stationary phase (V_i), i.e., the volume of solvent inside the gel pores. It is assumed that the volume of the gel matrix itself is negligible. The ratio of the void volume-to-total volume (V_0/V_T) should theoretically occupy approximately one third of the bed as calculated from the space utilisation of tightly packed particles [6].

It is also important to consider the relationship between the solute elution volume (V_e) and the partition coefficient ($K_D=[V_e-V_0]/V_i$). Rearranging the definition of the partition coefficient gives

$$V_e = V_0 + K_d V_i \quad (1)$$

This equation shows that in the absence of reversible adsorption, which is assumed to minimal in a denaturant solution, the elution volume of a solute is the sum of the void volume of the gel bed plus a portion of the internal volume occupied by the solute. Using these basic principles of size-exclusion chromatography the mechanism of SEC refolding can derived as shown below.

2.2. Column loading

Initially, at $t=0$, unfolded protein in a high denaturant concentration (C_{Dapp}), is applied onto the column which is equilibrated with refolding buffer. It is important to note that the refolding buffer, and hence the void and gel volumes, may also contain moderate concentrations of denaturant [1]. During this step, the protein sample displaces its volume (V_{app}) of the refolding buffer in the void volume (V_0)

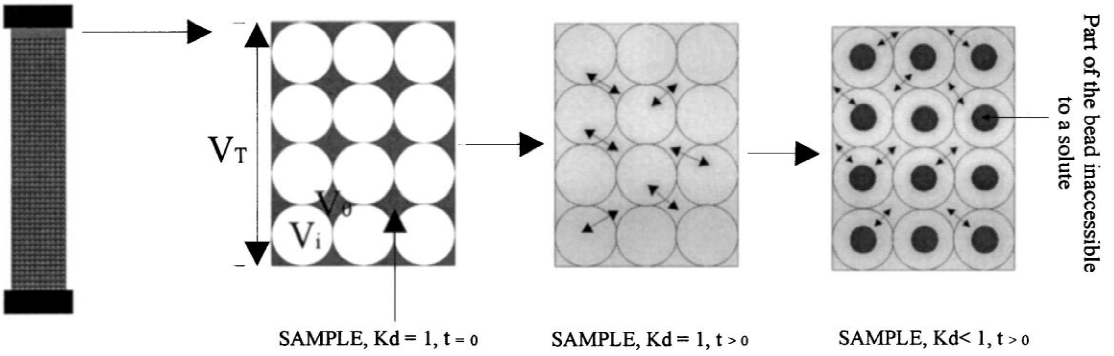

Fig. 1. Schematic illustration of sample application onto a gel filtration column.

of the bed (Fig. 1). It is assumed that there is no convective flow into the gel beads [6].

At $t=0+\delta t$ the sample starts to diffuse into the gel. The diffusion of the denaturant into the gel is assumed to be significantly higher than the diffusion of the protein. This is because of the higher diffusion coefficient of the denaturant, and the steric limitations to the unfolded protein partitioning. Sample diffusion results in a reduction of the denaturant concentration around the protein.

At $t>0$ the denaturant ($K_d=1$) diffuses rapidly into the gel and equilibrium is quickly established between the denaturant in the void and in the gel phase (Fig. 1). This equilibration will be faster for smaller beads because of the shorter diffusion lengths. The concentration of the denaturant in this initial equilibrium state can be calculated from a mass balance on the denaturant in the sample and that from the refolding buffer in the gel volume.

$$C_D V_T = C_{Dapp} V_{app} + C_{Di} V_i \quad (2)$$

where C_{Di} is the concentration of denaturant in the gel pores (i.e., the concentration of denaturant used to equilibrate the column), and C_D is the concentration of denaturant at the initial equilibrium. Thus,

$$C_D = \frac{C_{Dapp} V_{app} + C_{Di} V_i}{V_T} \quad (3)$$

In the reduced denaturant environment, the protein starts to fold and reaches a conformation with a size that is related to the denaturant concentration [5]. The rate of folding will depend on the individual protein and the denaturant used. Estimating the protein dilution after loading is more complex than for the denaturant. At $t=0$ the protein is not completely distributed between the void and gel phase, and its distribution is defined by the partition coefficient of the denatured protein (K_{PD}). At $t>0$ the protein distribution is defined by the protein equilibrium distribution coefficient (K_{PE}) (Fig. 1, $K_d<1$ and $t>0$). This is the protein partition coefficient that exists at the first equilibrium after sample loading. The distribution coefficient of protein in the initial equilibrium after loading depends on the denaturant concentration at that equilibrium which will be effected by the denaturant concentration in the mobile phase buffer [5]. The time necessary to reach this first equilibrium is dependent on the rate of folding under these conditions. Estimation of the protein partition coefficient at this first equilibrium state is required to calculate the elution volume of the refolded protein (see below).

2.3. Protein elution during size exclusion protein refolding system (SEPROS)

Protein elution starts after sample loading when the mobile phase buffer is running. At this point denaturant and protein are at equilibrium between the gel beads and the void fraction. The protein has a Stokes radius related to the denaturant concentration at this equilibrium. The principle of elution during refolding is the same as that for normal size-exclusion chromatography. Initially, before separation takes place the difference in distribution of the

protein and denaturant between the mobile and stationary phases is high. As the chaotrope concentration is reduced the protein partition coefficient between the mobile phase and the gel further increases as the protein folds and becomes more compact and native-like. When the protein is fully refolded its hydrodynamic radius is constant and the protein is eluted from the column as an active native protein. At the end when protein reaches its native state this difference becomes smaller. During separation the distribution of denaturant stays constant whereas the distribution of the protein changes as a function of denaturant concentration. This is a function of the separation rate between protein and denaturant as well as the folding kinetics.

The elution theory of SEC refolding is similar to that of standard SEC where the elution volume depends on the solute partition coefficient. For SEC refolding the refolding protein partition coefficient is initially small and increases with time, until it reaches a constant final value (K_{PF}). Thus the elution volume of the refolded protein is greater than that of the native protein [5]. As an first approximation we may consider an average partition coefficient, K_{PA}, which is defined as the mean of the partially folded protein after sample loading and the folded protein in its final state as eluted from the column:

$$K_{PA} = \frac{K_{PE} + K_{PF}}{2} \quad (4)$$

where K_{PF} is the partition coefficient of the final refolded protein and K_{PE} is first equilibrium partition coefficient. The value of K_{PA} can be calculated from experimental conditions, and then the value of the apparent elution volume of the refolded protein may be estimated.

The following equation can be used to approximate the average elution volume of the protein during SEC refolding, where there is no binding to the column matrix:

$$V_e = V_0 + K_{PA} \cdot V_i \quad (5)$$

Comparisons of the estimated and measured values of the elution volume for lysozyme refolding are described below.

3. Materials and methods

3.1. Chemicals

Crystalline hen egg white lysozyme (HEWL; EC 3.2.1.17; 52 000 units/mg), Blue dextran, *Micrococcus lysodeikticus* dried cells, reduced and oxidised glutathione (GSH, GSSG) and urea, were obtained from Sigma (Poole, UK). All other chemicals were analytical grade. Water used for the experimental work was ultrapure water obtained from reverse osmosis water system (Elgastat Prima).

3.2. Preparation of denatured lysozyme

Native hen egg white lysozyme (HEWL) was denatured by incubating the protein (10 or 20 mg/ml) in 0.1 *M* Tris–HCl, pH 8.6 containing 8 *M* urea and 0.15 *M* dithiothreitol (DTT) for at least 2 h at 20°C. The pH was subsequently adjusted using concentrated HCl.

3.3. Refolding lysozyme using SEC refolding

A 1.0-ml sample of denatured lysozyme was applied onto a Superdex 75 HR 10/30 column (Pharmacia Biotech, St. Albans, UK) previously equilibrated with refolding buffer (0.1 *M* Tris–HCl, pH 8.2, 1 m*M* EDTA, 3 m*M* reduced glutathione, 0.3 m*M* oxidised glutathione and 2 *M* urea). A Gilson high-performance liquid chromatography (HPLC) system (Middleton, USA) was used for all experiments as previously described [5]. The sample was eluted at a flow-rate of 0.5 ml/min (0.64 cm/min) at room temperature, using refolding buffer. The void and total volumes of the column were calibrated using Blue dextran and acetone [1].

3.4. Analytical methods

Lysozyme concentration was measured using $A_{280,}$ with absorbance values for 1 mg/ml of 2.63 (cell path length = 1 cm) for native lysozyme, and 2.37 for reduced, denatured lysozyme [7]. The activity of native and refolded lysozyme was determined at 25°C by following the decrease in absorbance at 450

nm of a 0.25 mg/ml *Micrococcus lysodeikticus* suspension in 0.06 *M* potassium phosphate, pH 6.2 [8].

4. Results and discussion

The gel filtration media and column used for refolding experiments here, were the same as those used to determine the changes in lysozyme partition coefficient as a function of urea concentration [5]. It was assumed, that the conformational changes of the protein during denaturant removal were instantaneous and that any changes in the lysozyme environment would result in an instant change in its size. This assumption is valid for lysozyme, which collapses instantly upon refolding with a half time for formation of a compact folded state of 50–200 ms [9]. This time is negligible in comparison to the time of SEC refolding.

The SEC refolding of lysozyme at 10 and 20 mg/ml is shown in Fig. 2. Two main peaks are observed. The first of these corresponds to refolded lysozyme that had 100% activity as compared to a native lysozyme control. The second peak refers to the elution of DTT which absorbs at 280 nm [10]. Urea also elutes at the same elution volume as DTT [1]. The elution volumes corresponding to the centre of the protein peaks are 15.6 ml and approximately 25.0 ml for the denaturant species. A greater peak absorbance was observed with the 20 mg/ml solution corresponding to the greater protein concentration. No protein aggregation was observed at these protein concentrations, which is in agreement with previous SEC refolding data for lysozyme [1].

Calibration of the gel filtration column gave a void volume of 8.1 ml and a total volume of 30.2 ml. Using Eq. (3) with these values, the denaturant concentration after sample loading was calculated, giving values of 3.6 *M* and 0.04 *M* for urea and DTT, respectively. The diffusion coefficients of urea and lysozyme were calculated to check the assumption that the denaturant will diffuse much faster than lysozyme into the gel media. Lysozyme diffusion coefficients were calculated using the following equation [11]:

$$D = \frac{1.69 \cdot 10^{-5}}{R_S} \tag{6}$$

where R_S is a solute (Stokes) radius. The Stokes radius for lysozyme in various concentrations of urea have been previously determined [5] and are plotted in Fig. 3. Thus the change in diffusion coefficient of lysozyme as a function of urea concentration may be calculated. The urea diffusion coefficient is also function of urea concentration and was calculated from [12]:

$$D_{(\text{urea})} = 1.380 - 0.0782[\text{U}] + 0.00464[\text{U}]^2 \tag{7}$$

where [U] is urea concentration in mol/l. Diffusion of the denaturant between the void and the beads is significantly faster then lysozyme as this enzyme has a diffusion coefficient approximately 14-times lower at all concentrations of denaturant. The value of the lysozyme partition coefficient is a function of the urea concentration. The partition coefficient values were previously determined [5] and are plotted in Fig. 4. The partition coefficient of lysozyme at the initial equilibrium ([urea]=3.6 *M*) is 0.48. This value of K_d is approaching the partition coefficient of native lysozyme in this system (K_d=0.59, [5]). The increasing value indicates that the folding reaction, as measured by the size of the protein molecule, is progressing at this early stage of the process. Comparing Figs. 3 and 4 it can be seen that the lysozyme partition coefficient increases with a decrease in urea concentration. This coincides with the decrease in Stokes radius that accompanies lysozyme refolding. This collapse in lysozyme structure, which is manifested as an increase in partition coefficient from 0.1 (unfolded protein, [5]) to 0.48 (partially folded protein), indicates that sample loading is particularly important because nearly all the conformational changes are complete after this initial step reaches equilibrium. The rate of this initial collapse was not measured but is assumed to be high for lysozyme because of its refolding characteristics [8].

After the initial equilibrium state, the lysozyme is then eluted with the refolding buffer and buffer exchange occurs slowly. The lysozyme eventually reaches equilibrium with the refolding buffer that has a urea concentration of 2 *M*. At this urea con-

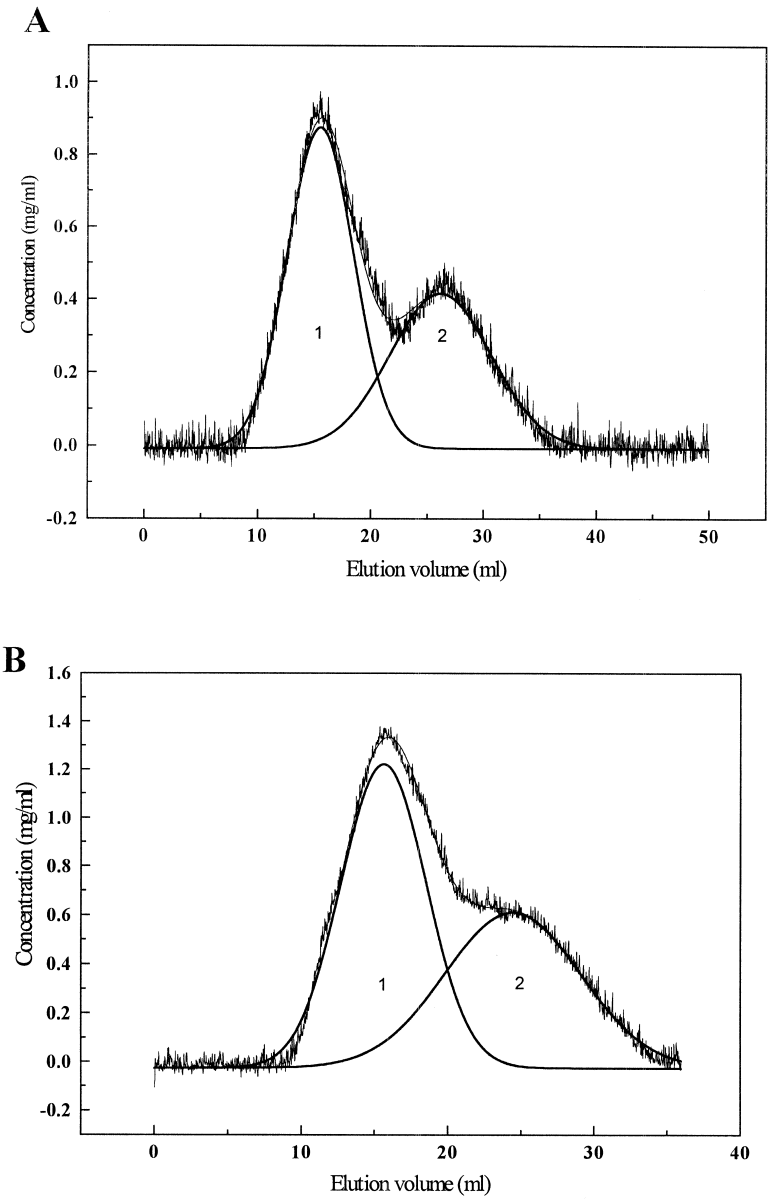

Fig. 2. Size-exclusion chromatography refolding of lysozyme. One ml of 10 mg/ml (A) and 20 mg/ml (B) denatured lysozyme was applied on a Superdex 75 HR 10/30 column. The protein was eluted with refolding buffer at 0.5 ml/min.

centration lysozyme has a partition coefficient of 0.54 (Fig. 4). As the denaturant is exchanged with the redox components in the buffer the protein is able to reform its disulphide bonds. This process of disulphide bond rearrangement and formation starts immediately after protein loading but is completely finished once the denaturant is exchanged for the refolding buffer. The reshuffling reaction in the protein when its structure is collapsed is highly effective because major conformational changes take place before the establishment of strong disulphide bonds. This reduces the possibility of formation of

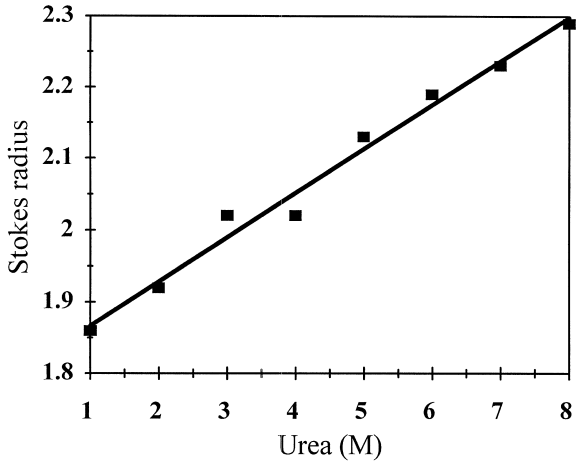

Fig. 3. Stokes radius of lysozyme as a function of urea concentration. (Data from Ref. [5].)

incorrect disulphide bonds because the partially folded conformation allows two complementary sulphydryl residues to come into contact.

It is possible to calculate the average K_{PA} using Eq. (4). The buffer exchange theoretically starts at a K_{PE} of 0.48 on initiation of flow and finishes at K_{PF} of 0.54 after sample elution, thus the calculated K_{PA} is 0.51. The apparent elution volume for refolded lysozyme may be calculated from Eq. (5), giving an value of 19.3 ml. The predicted elution volume occurs at about two thirds of the protein peak baseline (for both protein loadings). Thus the model slightly overestimates the elution volume of the refolding protein (Fig. 2). The calculation of denaturant concentration after sample loading inherently assumes there are no limitations to denaturant mass transfer into the gel pores. Size-exclusion gel media does not have uniform pores, and it is reasonable to assume that the denaturant cannot penetrate to the core of the gel beads as fast as is assumed here. Thus the loaded protein is not diluted as much as predicted and so the protein does not contract in size as much as the model suggests.

The proposed mechanism has its limitations, possibly because the rates of conformational changes between different conformers were not accounted for. Although it was assumed that these rates for lysozyme are high [9], it should also be noted that a low rate of exchange between folding conformers exists at constant concentrations of denaturant [5]. The discrepancies between the model and the experimental results may be attributed to slower rates of folding under a denaturant gradient.

This model of sample loading should be applicable to the refolding of other proteins by size-exclusion chromatography. For each protein it will be required to determine experimentally the changes in partition coefficient and Stokes radius with denaturant concentration. Measurement of the Stokes radius of the folded and unfolded proteins will be essential to determine the molecular mass fractionation range of

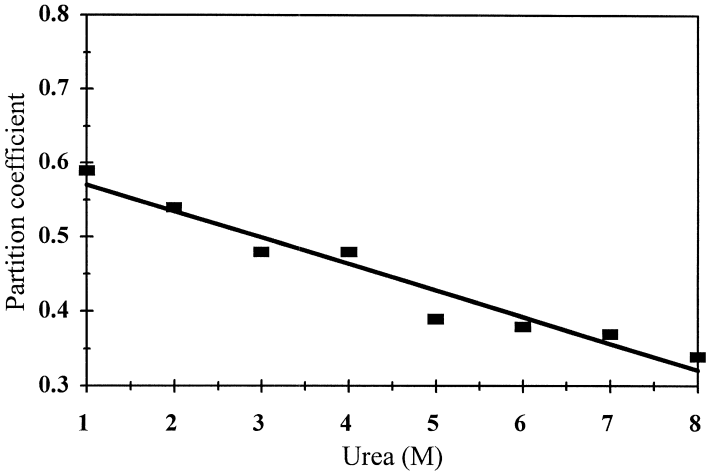

Fig. 4. Partition coefficient of lysozyme into Sephadex 75 HR as a function of urea concentration.

the gel media. There is a significant effect of gel fractionation range on protein refolding yield (Fahey and Chaudhuri, unpublished results). With this protein-specific data and the characteristics of the gel filtration column it will be possible to estimate the elution volume of the folded protein. The denaturant species will always elute at approximately one column volume and so the resolution between the folded protein and the denaturant can be predicted.

This is a preliminary model for SEC-based protein refolding. We have only considered the physical aspects and not the chemical requirements during refolding. It is clear from the consideration of sample loading that significant folding of the protein occurs during this initial diffusion of denaturant into the gel. The rates of protein refolding and aggregation in the column will therefore be very important. The concentration of denaturant directly affects the rate constants for both refolding and aggregation. Using guanidine hydrochloride it was shown that increasing the denaturant concentration reduced aggregation and slowed down the refolding rate of lyzozyme [13]. It is not unreasonable to assume that similar effects may be observed with other denaturants [14]. There is scope to improve this process through careful design of the sample loading step. For example, the use of a decreasing denaturant gradient in the refolding buffer may allow control of the changes in the protein Stokes radius and hence refolding rate and yield.

5. Symbols

C_D	Concentration of denaturant in the initial equilibrium (mol/l)
C_{Dapp}	Initial concentration of the denaturant in the sample (mol/l)
C_{Di}	Concentration of denaturant in the refolding buffer (mol/l)
D	Diffusion coefficient (cm^2/s)
K_d	Partition (distribution) coefficient
K_{DP}	Distribution coefficient of denatured protein
K_{PA}	Average partition coefficient during refolding
K_{PE}	Equilibrium distribution coefficient of protein after loading
K_{PF}	Partition coefficient of refolded protein
R_S	Stokes radius (nm)
t	Time (min)
V_0	Void volume (ml)
V_{app}	Volume of initial denatured protein sample (ml)
V_e	Solute elution volume (ml)
V_g	Matrix volume bound water (ml)
V_h	Hydrodynamic volume (nm^3)
V_i	Volume of gel media pores (ml)
V_T	Total volume of the gel bed (ml)

Acknowledgements

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