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Continuous chromatographic protein refolding

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Abstract

Column-based protein refolding requires a continuous processing capability if reasonable quantities of protein are to be produced. A popular column-based method, size-exclusion chromatography (SEC) refolding, employs size-exclusion matrices to separate unfolded protein from denaturant, thus refolding the protein. In this work, we conduct a comparison of SEC refolding with refolding by batch dilution, using lysozyme as a model protein. Lysozyme refolding yield was found to be extremely sensitive to the chemical composition of the refolding buffer and particularly the concentration of dithiothreitol (DTT) introduced from the denatured protein mixture. SEC refolding was not adversely affected by DTT carry-over as small contaminants in the denatured solution are separated from protein during the refolding operation. We also find that, contrary to previous reports, size-exclusion refolding on batch columns leads to refolding yields slightly better than batch dilution refolding yields at low protein concentrations but this advantage disappears at higher protein concentrations. As batch-mode chromatography would be the limiting step in a column based refolding downstream process, the batch column refolding method was translated to a continuously operating chromatography system (preparative continuous annular chromatography, P-CAC). It was shown that the P-CAC elution profile is similar to that of a stationary column, making scale-up and translation to P-CAC relatively simple. Moreover, it was shown that high refolding yields (72%) at high protein concentration (>1 mg ml^{-1}) could be obtained.

Keywords: Annular chromatography; Protein folding; Proteins

1. Introduction

High expression levels of recombinant protein in *Escherichia coli* often result in the formation of micron-scale particles of aggregated protein, called inclusion bodies (IBs) [1]. IBs can be solubilised and refolded in vitro, most commonly by dilution in batch or fed-batch reactors. However, protein aggregation limits yield in these reactor systems [2], and separation of product from aggregates must be subsequently undertaken. Many attempts have been made to improve in vitro refolding, ranging from optimisation of the physicochemical properties of the refolding environment [3–5] to optimisation of reactor operation [6] and design [7].

Developments in protein folding have demonstrated that different types of chromatography columns can be useful for protein folding purposes. In 1986, Creighton reported that horse cytochrome c and bovine pancreatic trypsin inhibitor could be successfully refolded when bound on a weak ion exchange matrix [8]. Suttnar et al. showed that recombinant protein from *E. coli* inclusion bodies could be refolded using a strong anion exchanger [9]. Hydrophobic interaction chromatography has also proved a useful tool for protein renaturation. In 1992, Geng and Chang [10] refolded bovine serum albumin (BSA) and lysozyme with success using silica linked to polyethylene glycol having a hydrophobic end-group. In addition to matrices designed to interact with the protein molecules, size-exclusion chromatography (SEC) has been used for protein refolding. Werner et al. (1994) used this technique to refold recombinant human ETS-1 protein and bovine ribonuclease on a Superdex 75 column. Batas and co-workers [11–15] 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. Small molecules, like dithiothreitol (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 able to enter the resin pores. Chaudhuri and co-workers proposed that folding can be completed within the pores with reduced likelihood of aggregation. Despite numerous studies

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focussing on size-exclusion refolding, a convincing comparison between batch dilution refolding and size-exclusion refolding has yet to be established and explained.

A drawback of chromatography is that it typically operates in batch mode. Its processing capacity is moreover limited by the available interfacial surface area for surface-dependent processes. Hence, a continuous chromatography system will be needed to avoid bottlenecks in a chromatography-based downstream process chain. Preparative continuous annular chromatography (P-CAC) is a continuous chromatography system that overcomes some of the disadvantages of batch systems [16]. Successful implementation of a continuous high-yielding refolding technique will enable inclusion body processing, traditionally a batch-mode process [17], to be executed in a continuous fashion. A fully continuous flowsheet would then be enabled by combining continuous refolding with the chemical extraction technology developed by Falconer and co-workers [18,19], which can also be operated continuously. In any case, the success of P-CAC in the biotechnology industry will be largely dependant on its operational flexibility and the speed of process development. As manufacturers of biopharmaceuticals aim to minimise time-to-market, it is critical that protein refolding procedures developed on laboratory-scale batch chromatography columns can easily be translated to large-scale manufacturing processes.

This current work presents a detailed study of size-exclusion refolding through comparison with batch dilution refolding, using lysozyme as a model protein. A continuous SEC refolding route using P-CAC is developed, and the ease of protocol transfer between stationary, batch column refolding and continuous P-CAC refolding is demonstrated.

2. Experimental

2.1. Chemicals

Urea and tris(hydroxymethyl)aminomethane (Tris) were purchased from Fisher Scientific (Loughborough, UK). Hen egg white lysozyme, monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (Dorset, UK). L-Cysteine, L-cystine and acetonitrile (HPLC grade) were purchased from Fluka (Dorset, UK). Sephacryl S200 HR size-exclusion resin (allyl dextran cross-linked with *N*,*N*′-methylene bisacrylamide, d_p = 25–75 μm) was obtained from Amersham Biosciences, UK (Bucks., UK). Coomassie Plus Protein Assay Reagent was purchased from Pierce (Rockford, IL, USA).

2.2. Analytical methods

2.2.1. UV absorption

Concentrations of both native and denatured lysozyme were determined using ultraviolet (UV) absorption spectroscopy (Shimadzu, UV-160A Spectrophotometer, Duisburg, Germany) at 280 nm (A_{280}). Protein concentrations were calculated using the Beer–Lambert law with extinction coefficients of 2.63 and 2.37 ml mg^{-1} cm^{-1} for native and denatured lysozyme, respectively [20].

2.2.2. Lysozyme activity assays

For batch dilution and batch SEC experiments, lysozyme enzymatic activity was measured at ambient temperature by following the decrease in absorbance at 450 nm (Shimadzu, UV-160A spectrophotometer, Duisburg, Germany) of a cell suspension (0.15 mg ml^{-1} *Micrococcus lysodeikticus*, 0.067 M sodium phosphate, pH 6.2). Twenty microliters of the refolded lysozyme samples was added to 980 μl of cell suspension and rapidly mixed. After 5 s, the absorbance was monitored for 40 s. A linear decrease in absorbance was observed. The concentration of refolded lysozyme was determined by comparing the activity of the refolded lysozyme to the activity of standard solutions of native lysozyme of different concentrations. Samples were stored at 4 °C until analysis, which was performed within 24 h.

For P-CAC experiments, lysozyme enzymatic activity was measured at ambient temperature by following the decrease in absorbance at 450 nm (Bio-Tek, EL340 Biokinetics Reader, Vermont, USA) of a cell suspension (0.60 mg ml^{-1} *M. lysodeikticus*, 0.067 M sodium phosphate, pH 6.2). Three hundred microliters of cell suspension was added to 25 μl of the refolded lysozyme sample. The solutions were mixed rapidly using the plate reader's shake mode and the absorbance was monitored for 40 s. A linear decrease in absorbance was observed. The concentration of refolded lysozyme was determined by comparing the activity of the refolded lysozyme to the activity of standard solutions of native lysozyme of different concentrations.

2.2.3. HPLC analysis

About 10 μg protein was injected onto a C_5 reversed-phase column (Phenomenex UK, Cheshire, UK, Jupiter 5 μm, C_5, 300 Å, 150 mm × 4.60 mm, $d_p = 5.15 \pm 0.3$ μm, $d_{pore} = 320 \pm 40$ Å). The HPLC system consists of two LKB Bromma 2150 HPLC pumps and a LKB Bromma 2151 variable-wavelength detector set at 280 nm. Samples were eluted at 25 °C with an acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA), starting at 34% (v/v) acetonitrile increasing at 1.3% (v/v) acetonitrile/min over 10 min.

2.3. Lysozyme denaturation

2.3.1. Standard denatured lysozyme

A standard denatured and reduced lysozyme was prepared by incubating 8–20 mg ml^{-1} lysozyme for 2 h at 37 °C in 0.1 M Tris–HCl, 1 mM EDTA, 6 M urea and 32 mM DTT, buffered at pH 8.1. Reduction of disulphide bonds and loss of activity was confirmed by both HPLC analysis and enzyme activity assay.

2.3.2. DTT-free denatured lysozyme

DTT was removed from a standard denatured lysozyme solution by performing a buffer exchange on a prepacked 5 ml HiTrap desalting column (Amersham Biosciences). The column was equilibrated with 6 M urea, 0.1 M Tris–HCl and 1 mM EDTA at pH 8.1. This buffer was also used for elution of standard denatured and reduced lysozyme. A flow rate of 0.5 ml min^{-1} was used throughout the whole procedure.

2.4. Refolding

The same refolding buffer (2 M urea, 0.1 M Tris, 1 mM EDTA, 3 mM cysteine and 0.3 mM cystine, pH 8.1) was used both for batch dilution refolding and size-exclusion refolding. This composition is similar to that used by Batas and Chaudhuri [11] except that a cysteine/cystine redox couple was used instead of the more expensive glutathione couple.

2.4.1. Batch dilution refolding

Denatured lysozyme of different concentrations (9.0–19.3 mg ml^{-1}) was refolded by diluting it 25–200 times with refolding buffer. Whilst shaking the refolding buffer in a test tube, 30 μl of denatured lysozyme was injected. Refolding yield was assessed by enzyme activity assay within 24 h.

2.4.2. Batch SEC refolding

A 60 cm × 2.6 cm column prepacked with Sephacryl S200 HR (Amersham Biosciences) was equilibrated with three column volumes of refolding buffer. Two milliliters of fully denatured protein solution was loaded onto the column and renaturation buffer was then pumped through at a constant flow rate of 2 ml min^{-1}. Eluate was continuously monitored at 280 nm and fractions of 15 ml were collected for enzyme activity determination within 24 h. Fractions containing refolded lysozyme were pooled and total protein was determined using Coomassie Plus Protein Assay Reagent (Pierce). Samples were stored at 4 °C before analysis.

2.4.3. P-CAC SEC refolding

The P-CAC system (Prior Separation Technologie, Goetzis, Austria) comprised an annular column of height 60 cm, outer diameter of 15 cm and inner diameter of 13 cm, giving a bed volume of 2.6 l. The column was packed with Sephacryl S200 HR resin (Amersham Biosciences). A 2.6 l volume of resin was diluted to 5.2 l with 20% (v/v) ethanol. While stirring, a peristaltic pump transported this dilute slurry into the rotating column (7 rpm) at a flow rate of 40 ml min^{-1}. After 1 h, the flow rate was reduced to 13 ml min^{-1}. When all the resin was pumped into the column, the bed was further compressed by an increase in flow rate to 23.4 ml min^{-1}. This flow rate was maintained for 1 h.

Before loading the protein feed, the column was equilibrated for 3 h with refolding buffer at a flow rate of 0.99 l h^{-1} (16.5 ml min^{-1}). After equilibration denatured protein was fed at 0.09 l h^{-1} (1.5 ml min^{-1}) and the flow rate of refolding buffer was reduced to 0.90 l h^{-1} (15.0 ml min^{-1}). Fractions were collected from the 90 outlets distributed over 360° (4° per outlet) 3.5 h after protein feeding commenced. Enzyme activity of these samples was measured within 24 h of collection. Samples were stored at 4 °C.

3. Results and discussion

3.1. Refolding by batch dilution

Injecting denatured lysozyme in a larger volume of refolding buffer whilst vigorously mixing will cause the denaturant molecules to be dispersed throughout the total volume causing the unfolded protein molecules to collapse to a more compact state. This state will either develop to a correctly folded specie, a misfolded specie or an aggregated specie. Standard denatured lysozyme solution (30 μl) was injected into six different volumes of refolding buffer resulting in a dilution factor varying between 25 and 200. This was done for denatured lysozyme concentrations between 9 and 20 mg ml^{-1}. The refolding yield was quantified by an enzyme activity assay. The yield versus dilution profiles shown in Fig. 1 indicate that refolding yield in these experiments was not dependent on the protein concentration at a given dilution factor. Approximately, equal yields were observed for equal dilution factors, e.g. a 50× dilution from 19.3 to 0.4 mg ml^{-1} or from 9.5 mg ml^{-1} diluted to 0.2 mg ml^{-1} gave approximately 4% yield. This suggests that, in our experiments, dilution factor and not protein concentration was the variable controlling yield, presumably because refolding yield is affected by differences in the chemical composition of the refolding environment after dilution. Injection of a fixed volume of denatured lysozyme, containing 6 M urea and 32 mM DTT, into different volumes of refolding

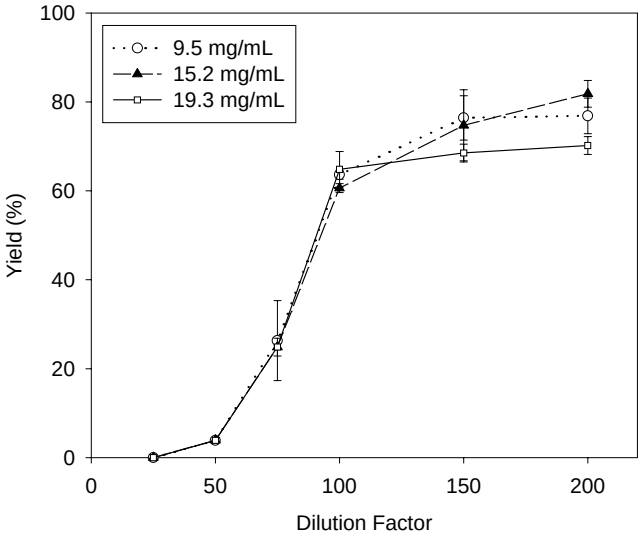

Fig. 1. Refolding yield as function of dilution factor for different initial concentrations of denatured lysozyme (9.5–19.3 mg ml^{-1}) in batch dilution refolding experiments.

buffer will lead to different final compositions of the refolding solution, and hence to differences in redox potential. This current finding complements previous studies where yield variability occurs due to changes in protein concentration [21–26]. The concentrations of cysteine (CysH) and cystine (CySSyC) in the refolding buffer used in this study were set at 3 and 0.3 mM, respectively. This gives an optimal CysH/CySSyC ratio of 10 [27]. The urea concentration was set at 2 M to prevent the formation of large aggregates [11]. However, when a solution containing denatured and reduced lysozyme is injected into the refolding buffer, the concentration of urea and redox agents will be changed. These differences in chemical composition have notable effects on the refolding yield. Extensive work done on the chemistry of the disulphide bonds in proteins has shown that refolding yield is extremely sensitive to redox potential [28].

To confirm the effect of DTT carry-over in a cysteine–cystine refolding system, we characterised lysozyme refolding in the standard refolding buffer but with variation in the DTT concentration (0.3–5 mM). Lysozyme and urea concentrations in the refolding buffer were kept constant at 0.1 mg ml^{-1} and 2 M, respectively. The results of these experiments are shown in Fig. 2. As expected, a strong dependence of refolding yield on DTT concentration was observed. When the DTT concentration in the refolding buffer increased above 1 mM, a sharp decrease in refolding yield occurred. In experiments with cysteine and cystine as the redox couple, DTT carried over from the denatured lysozyme sample will alter the redox-ratio, i.e. [cysteine]/[cystine], by reducing the cystine concentration hence inducing a sub-optimal ratio for oxidative refolding. The sub-optimal redox effect is obviously more pronounced at low dilution factors as this induces the largest impact on buffer composition. The dependence of protein refolding yield on the composition of the refolding buffer was also confirmed by refolding on size-exclusion matrices, as will be discussed subsequently.

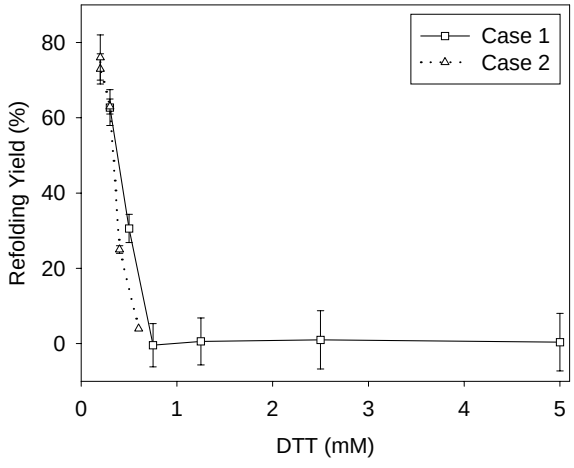

Fig. 2. Yield vs. concentration of DTT added to the standard refolding buffer for batch dilution refolding. In case 1, the protein concentration (0.10 mg ml^{-1}) and the urea concentration (2 M) in the refolding solution after dilution were kept constant (0.10 mg ml^{-1}). In case 2, both protein concentration and urea concentration were changing with the dilution factor.

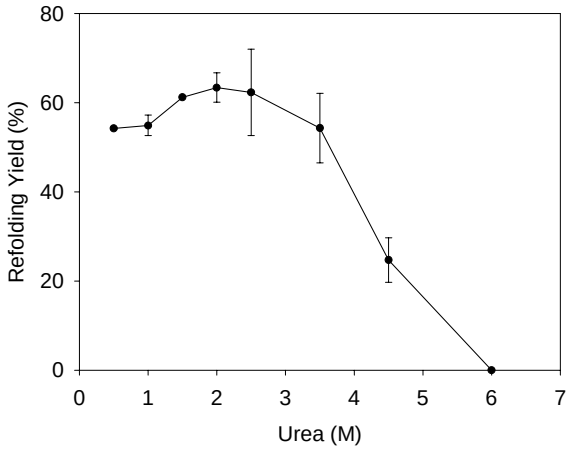

Fig. 3. Lysozyme refolding yield vs. concentration of urea in the refolding solution after dilution. The protein concentration (0.10 mg ml^{-1}) and the DTT concentration (0.32 mM) in the refolding solution after dilution were kept constant.

As a control, urea concentration was varied (0.5–6 M) while DTT and protein concentrations were kept constant at 0.3 mM and 0.1 mg ml^{-1}, respectively. Fig. 3 confirms that 2 M urea is the optimal urea concentration for lysozyme refolding for a cysteine/cystine redox couple, as shown previously for lysozyme refolding with reduced and oxidised glutathione [29]. However, within the range of 2–2.5 M urea, corresponding to the differences in urea concentration observed in the experiments reported in Fig. 1, no significant effect on the refolding yield could be observed. This confirms that, for these experiments, the lower yield is caused predominantly by an increase in DTT concentration.

3.2. Size-exclusion refolding

A 2 ml sample of denatured lysozyme in 6 M urea and 32 mM DTT was injected on a SEC column equilibrated with refolding buffer. Small molecules, like DTT and urea, enter the pores of the resin and are separated from unfolded protein molecules having a higher hydrodynamic radius. As the chaotrope concentration surrounding the unfolded species is reduced, the protein starts to refold. This yields more compact partially folded protein molecules, with a smaller hydrodynamic radius. It has been proposed that these smaller molecules can enter the pores of the resin where refolding can be completed with a reduced likelihood of aggregation [11]. It has been shown that eluting with a urea gradient enhances the refolding yield for lysozyme [30]. By applying a gradient through the column, a very smooth and slow change from strong denaturing conditions to an environment that promotes refolding and/or aggregation is induced. It is proposed that one can create a similar effect by reducing the rate at which the urea from the denatured sample and

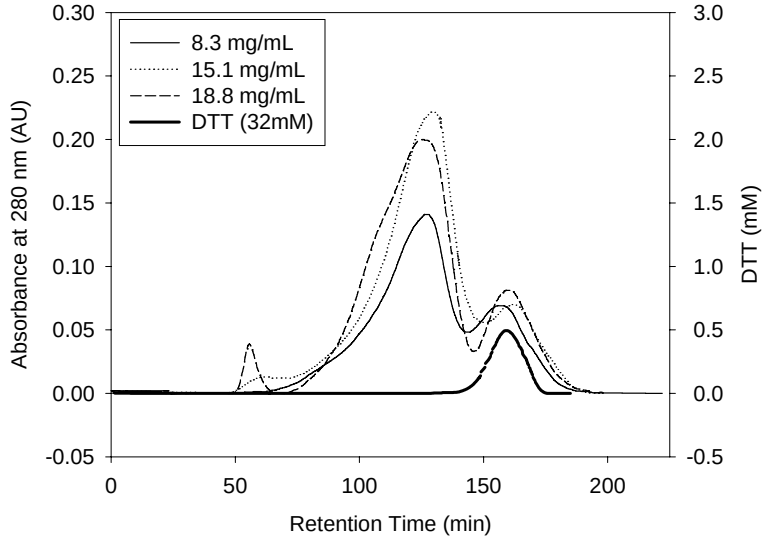

Fig. 4. Chromatograms for size-exclusion refolding on a 60 cm × 2.6 cm Sephacryl S200 packed bed for different protein feed concentrations and chromatogram of a DTT pulse (2 ml, 32 mM). Lysozyme denatured and reduced in 6 M urea and 32 mM DTT was used as protein feed.

the denatured protein molecules are separated, i.e. one can apply a resin with larger pores. For this work, Sephacryl S200 resin was therefore chosen as its fractionation range for globular proteins is between M_r 5000 and 250,000, corresponding to pore radii between 1.2 and 6.3 nm. Unfolded lysozyme having a hydrodynamic radius of about 4.5 nm can enter the larger pores but will still be separated from folded lysozyme, which penetrates deeper and more easily into the resin due to its smaller hydrodynamic radius (1.8 nm).

Different concentrations of denatured lysozyme (8.3, 15.1 and 18.8 mg ml^{-1}) were injected on a column packed with Sephacryl S200 HR size-exclusion matrix. Refolding buffer (2 M urea, 0.1 M Tris, 1 mM EDTA, 3 mM cysteine, and 0.3 mM cystine, pH 8.1) was used as the mobile phase. The chromatograms obtained for different concentrations of denatured lysozyme are shown in Fig. 4. The peak eluting near 160 min was assigned to be DTT and urea, as this peak has the same retention time as a pulse of DTT eluted under similar conditions. Partial overlap with the lysozyme peak also means that this peak probably contains lysozyme, possibly in a partially reduced, incompletely renatured state. Refolded lysozyme eluted between 85 and 155 min with a peak maximum at 125 min. Fractions collected between 85 and 155 min were pooled and the concentration refolded lysozyme was determined by enzyme activity assay. Protein recovery was determined by the Bradford assay (Table 1).

For 18.8 mg ml^{-1} denatured lysozyme, a small peak eluted near 55 min. No lysozyme activity could be measured. It was assumed that this peak corresponded to larger protein aggregates at this high feed concentration. For the lowest feed concentration of 8.3 mg ml^{-1} of denatured lysozyme, no peak at 55 min was detected, indicating a lack of aggregation. For higher concentrations of denatured lysozyme, a decrease in refolding yield was observed because of this aggregation (Table 1).

3.3. Comparison of batch dilution and SEC refolding

One of the major differences between batch dilution refolding and SEC refolding is the final concentrations of urea and DTT in the refolding buffer. As shown above, batch dilution causes crucial differences in the chemical composition of the refolding buffer. In contrast, elution on a SEC matrix causes a separation of the denaturing and refolding buffers, ensuring that the protein experiences the designed redox environment for renaturation. Hence, the major factor affecting yield for lysozyme refolding in size-exclusion matrices was expected to be protein concentration, which is confirmed by the data shown in Table 1.

Table 1
Summary for batch size-exclusion refolding

Concentration denatured lysozyme (mg ml^{-1})	Total protein recovered (mg)	Recovery (%)	Pooled elution volume (ml)	Refolded protein concentration (mg ml^{-1})	Total protein concentration	Refolding yield (%)
8.3	16.4	98	135	0.08	0.11	71
15.1	28.7	95	150	0.11	0.18	59
18.8	32.1	85	130	0.15	0.23	53

Lysozyme was refolded on a 60 cm × 2.6 cm Sephacryl S200 packed bed equilibrated with refolding buffer. Denatured and reduced lysozyme (8.3–18.8 mg ml^{-1} lysozyme, 6 M urea, 32 mM DTT) was used as protein feed.

A second important issue regarding SEC refolding is the width of the elution peak. The total dilution factor was calculated over the entire peak giving an averaged concentration for comparison with batch dilution refolding data. Nonetheless, the protein concentration in an infinitesimal elution volume around the peak maximum will be significantly higher than the averaged concentration, and vice versa for an infinitesimal elution volume in one of the peak tails. Hence, aggregation will be more likely in the central zone and less likely in the peak tails. For illustration, the central peak area was arbitrarily defined as the zone with a UV absorbance above 45% of the maximum peak absorbance. The lysozyme concentration in this central area was calculated to be six times higher than that in the tails, demonstrating a clear difference between batch refolding at uniform protein concentration and SEC refolding at spatially varying concentrations. These substantial differences between the two methods makes it difficult to directly compare SEC and batch dilution refolding yields.

By performing a buffer exchange into TE buffer (0.1 M Tris, 1 mM EDTA) containing 6 M urea, DTT can be removed from the standard denatured lysozyme solution. Results for batch dilution experiments with such a denatured lysozyme solution and for SEC refolding are summarised in Table 2. Both experimental set-ups gave very similar results, i.e. decreased yield for increased protein concentration. However, batch dilution refolding gave slightly higher yields for all conditions. This contrasts with previous findings that SEC consistently gave a higher refolding yield than batch dilution columns, although these previous studies were conducted at similar column loading conditions. We believe these previous conclusions relate to the sub-optimality of batch dilution refolding caused by the carryover of DTT from the denatured protein solution. In effect, previous comparisons between the two systems were not done on an equal redox basis.

This current finding suggests that, in many instances, SEC refolding may provide no significant yield advantage over dilution refolding, at least for lysozyme. However, yield is only one criterion for selecting a refolding method. SEC refolding is a valuable alternative to batch dilution refolding as it facilitates immediate separation of reducing compounds such as DTT, protein aggregates and properly refolded species. Hence, it allows easy implementation of an optimised redox environment and therefore avoids the necessity for two purification steps, one prior to refolding to remove excess reducing compounds and one after refolding to remove aggregated species from the refolded product.

Table 2
Comparison between batch dilution refolding and batch size-exclusion refolding

Protein concentration (mg ml^{-1})		Batch dilution		SEC	
Initial	Final	Yield (%)	S.D. (%)	Yield (%)	S.D. (%)
8.3	0.11	78	9	71	2
15.1	0.18	65	5	59	2
18.8	0.23	63	6	53	2

Denatured and reduced lysozyme (8.3–18.8 mg ml^{-1} lysozyme, 6 M urea) was used as protein feed for batch dilution experiments, similar protein solutions containing 32 mM DTT were used for SEC experiments.

To confirm the influence of the chemical composition on refolding yield, denatured lysozyme was eluted with different refolding buffers. The composition of the buffers was chosen in such a way that they were equal to the final composition established by 100-, 50- and 25-fold batch dilution experiments. The chromatograms are given in Fig. 5. When the refolding buffer contained 1.2 mM DTT, a tall and narrow single peak eluting after 100 min was observed. It is suggested that this peak corresponds to incompletely renatured lysozyme, as it elutes earlier than refolded lysozyme and because no enzymatic activity could be registered. Decreasing the DTT concentration in the refolding buffer resulted in a broadening of this peak and a shift toward the retention time of refolded lysozyme. In addition, the collected fractions were analysed by RP-HPLC. When elution occurred with a refolding buffer containing more than 0.63 mM DTT, no refolded lysozyme could be recovered. Refolded lysozyme was recovered for refolding buffers containing less DTT. Refolded lysozyme elutes as two peaks around 4.75 ± 0.15 min. It is suggested that these correspond to species having different disulfide conformations, but both show enzyme activity when diluted into the buffer used to assess enzyme activity. The concentration of refolded lysozyme was estimated by integrating both peaks. The results corresponded to the concentrations obtained from the enzyme activity assay (data not shown). HPLC analysis is particularly useful for fractions where the DTT and protein peaks overlap. As expected traces of denatured lysozyme were observed in fractions collected in this overlapping zone (Fig. 6) confirming once more the sensitivity of lysozyme refolding to DTT concentration in the refolding buffer.

3.4. Continuous refolding: P-CAC

As stated above, SEC refolding will be the preferred mode of renaturation in some cases, especially where the denatured feed contains contaminants and when the protein is aggregative. To overcome the capacity limitations inherent in batch chromatography, a continuous SEC refolding was developed. A schematic comparison of both batch and continuous SEC refolding is given in Fig. 7.

The data acquired for batch SEC refolding allows modelling of such a process by P-CAC. The retention factor, k, was calculated for each component (Table 3), i.e. aggregated lysozyme, refolded lysozyme and the small molecules like DTT and urea, using Eq. (1):

$$k = \frac{t_r}{t_m} - 1 \quad (1)$$

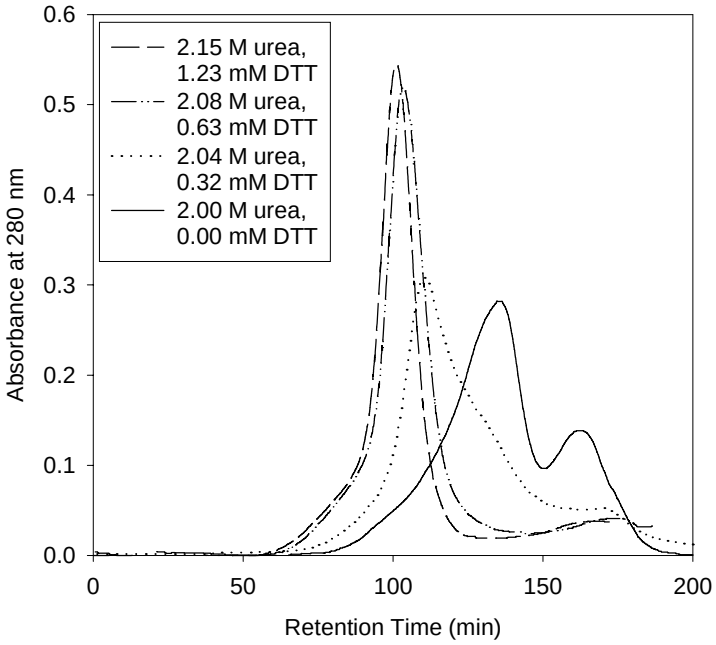

Fig. 5. Effect of DTT and urea in size-exclusion refolding. Chromatograms for SEC refolding experiments with refolding buffers with different urea (2.15–2 M) and DTT (1.23–0 mM) concentration.

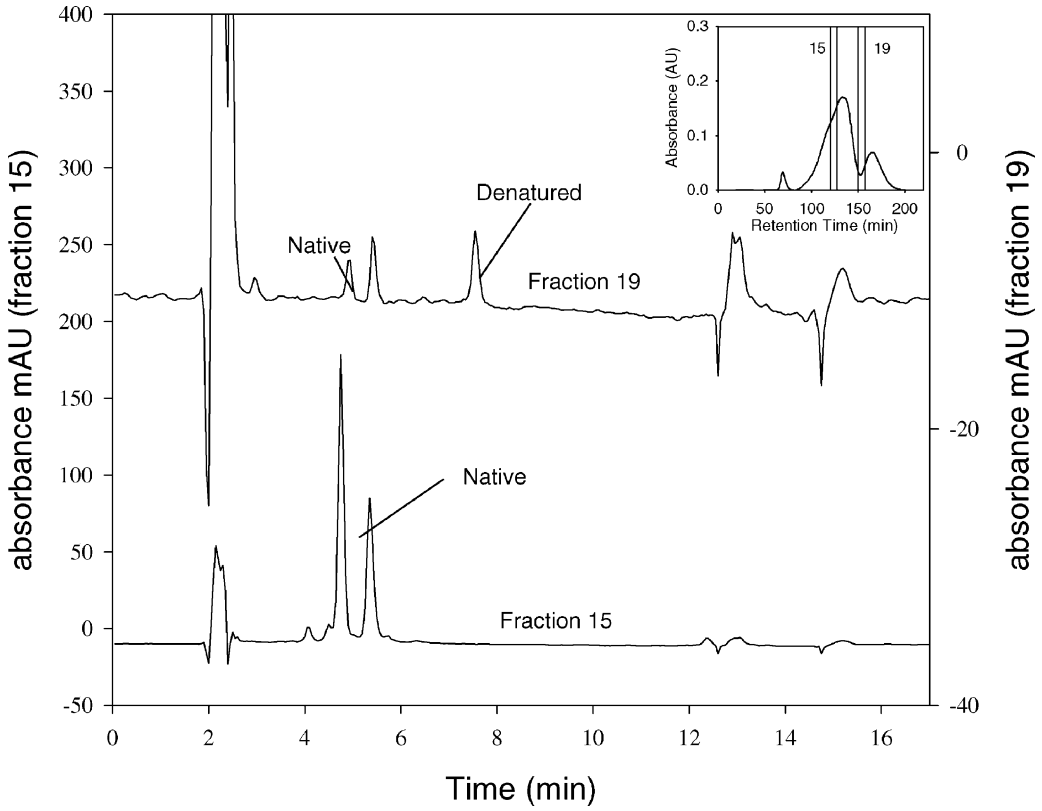

Fig. 6. RP-HPLC chromatograms of the different fractions collected when 2 ml of 18.8 mg ml^{-1} denatured and reduced lysozyme was refolded on the batch SEC column with standard refolding buffer.

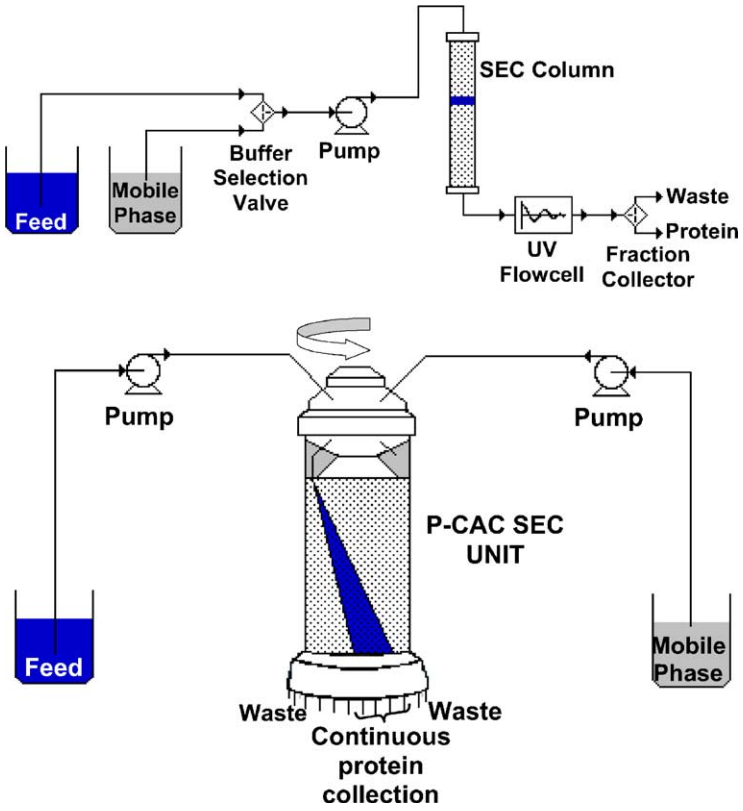

Fig. 7. Diagram comparing batch column chromatography with preparative continuous annular chromatography (P-CAC).

where t_r (min) represents the retention time of the species for which the retention factor is determined and t_m (min) is the dead time, i.e. the retention time of large tracer molecule such as blue dextran. The retention factor can be used to calculate the retention times for the different components for any given bed length at any given flow rate. For continuous annular chromatography, retention time is translated into retention angle, θ, defined as the angle between the point of feeding and point of elution, i.e. $\theta = t_r\omega$. The peak spreading whilst moving through the packed bed can be estimated using Eq. (2):

$$W_z = 4\frac{\sqrt{HZ}}{u_m}(1+k) \quad (2)$$

where u_m (cm min^{-1}) is the linear velocity, Z (cm) the depth in the bed, W_z (min) the spreading of the peak at this depth and H (cm) is the height equivalent theoretical plate, experimentally determined for a 60 cm batch SEC column. This equation can be converted for use in P-CAC by multiplying W_z with the angular rotation speed ω ($^\circ$ min^{-1}) and by superimposing the angular spreading of the feed at the top of the column (β), is given by Eq. (3) [31]:

$$\beta = \frac{Q_f}{Q_t}360 \quad (3)$$

Q_f (ml min^{-1}) is the feed flow rate and Q_t is the total flow rate. Hence, the total peak spreading for P-CAC is equal to $\sqrt{(W_z\omega)^2 + \beta^2}$. Using these equations, it is possible to estimate the angle at which refolded lysozyme will elute for any given flow rate, bed length and angular rotation speed.

A 60 cm Sephacryl S200 HR bed was used for stationary SEC refolding. The P-CAC was therefore packed with the same resin constructing a 60 cm tall, 1 cm thick annular bed. For batch refolding, the flow rate of the refolding buffer was set at 2 ml min^{-1}; the feed pulse was loaded at the same flow rate. For a column of 2.6 cm diameter, this corresponds to a flux of 0.38 ml min^{-1} cm^{-2}. Hence, the flow rate on the P-CAC was set to 16.5 ml min^{-1} to obtain the same flux through the resin. Using simple model equations (Eqs. (1)–(3)), an appropriate flow rate for the feed and angular rotation speed, which would result in acceptable resolution, was estimated. It was predicted that feeding denatured protein at 1.50 ml min^{-1} and rotating the column at 120° h^{-1} would give good resolution of aggregated and refolded protein, as shown in Fig. 8. However, operating under these conditions results in a protein load of 10% of the total bed volume, which is 10 times the load applied on the batch SEC column.

Table 3
Retention factors for elution on a Sephacryl S200 packed bed

Compound	k
Refolded lysozyme	1.27
Aggregated lysozyme	0.00
DTT/urea	1.92

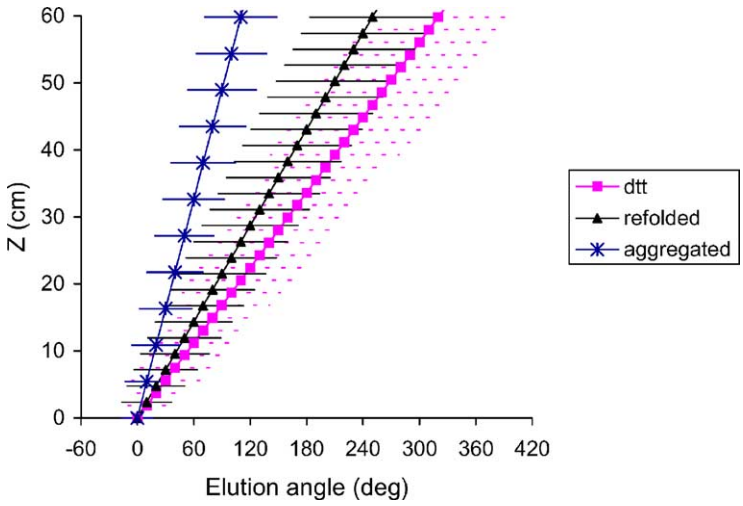

Fig. 8. Predicted elution profile for P-CAC SEC refolding. Total flow rate 16.5 ml min^{-1}, feed flow rate 1.5 ml min^{-1} and ω 120° h^{-1}.

Based on the high yields obtained for refolding of ~9 mg ml^{-1} denatured and reduced lysozyme on the batch column, a similar denatured lysozyme solution (10.5 mg ml^{-1}) was continuously applied on the P-CAC system. The flow distribution through the P-CAC was monitored by plotting the flow rate observed at each outlet normalised with respect to the average flow rate (Fig. 9). The flow distribution shows some scatter, but as most compounds do not elute in one fraction this variance will average out when fractions are pooled. After 3.5 h, when the system had reached steady state, an elution profile for refolded lysozyme was obtained by performing enzyme activity analysis on the 90 collected fractions, as shown in Fig. 10. As predicted, the refolded lysozyme elutes at around 240° from the feed point and is spread over approximately 130°.

To illustrate the transferability of a process from small-scale batch columns to a large-scale P-CAC system the retention angles were converted back to retention times to allow direct comparison with the retention times obtained on the batch SEC system (Fig. 11). The P-CAC profile is exactly like a batch column, except that processing is continuous. Besides comparing elution profiles, we also investigated the performance of both systems. The refolded lysozyme concentration, which was determined experimentally for each fraction, was multiplied by the volume registered during this 20 min period to give the amount of refolded lysozyme in each fraction. Adding up these masses and dividing by the total mass of denatured lysozyme applied to the column during this 20 min period gives the refolding yield. The refolding yield was 72%, which is comparable to the yield obtained for a 2 ml pulse of 9 mg ml^{-1} denatured

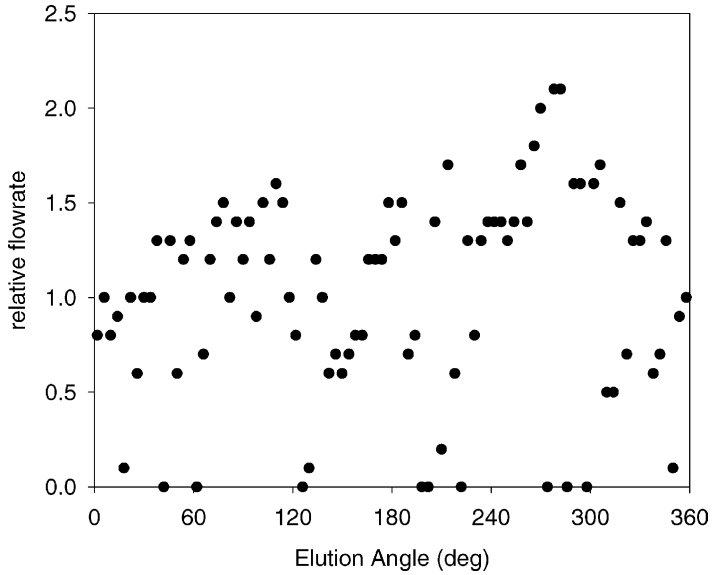

Fig. 9. Flow distribution during elution on P-CAC. The relative flow rate is obtained by normalising the flow rate observed at every outlet with respect to the average flow rate.

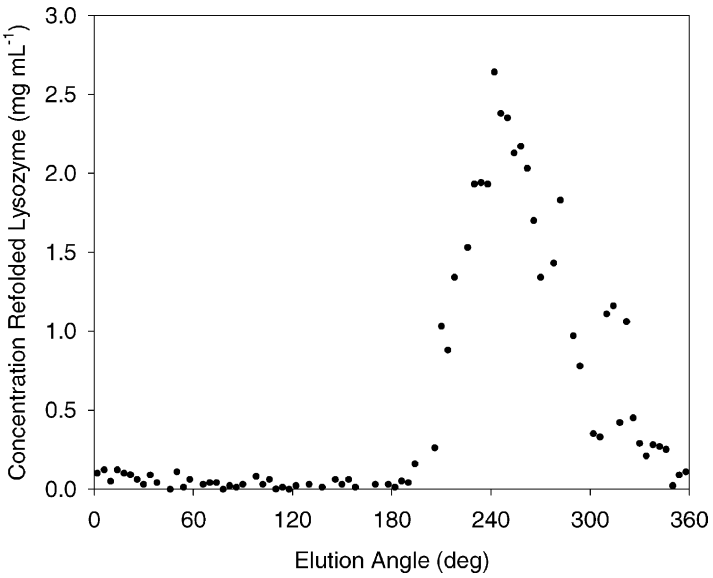

Fig. 10. Refolded lysozyme concentration for the different elution angles determined by enzyme activity assay of fractions collected for 20 min, 3.5 h after feeding of 10.5 mg ml^{-1} denatured and reduced lysozyme commenced.

lysozyme on a batch SEC refolding (71%) (Table 4). However, one very important difference is the concentration of refolded lysozyme after pooling the fractions comprising the refolded lysozyme peak. For batch SEC, this was calculated to be 0.11 mg ml^{-1}. For P-CAC, the pooled concentration was much higher, 1.2 mg ml^{-1}. Apparently the dilution fold caused by elution on P-CAC in these tests was an order of magnitude smaller than the dilution caused by batch SEC refolding. The refolding yield does not, however, appear to be affected by this higher eluate protein concentration.

This finding can be understood when considering the P-CAC angular rotation speed. In translating the batch column protocol to P-CAC, it was decided to keep the values for directly transferable parameters such as flux, linear velocity and bed length equal to values for the batch column. To obtain resolution, the model for P-CAC elution suggested a rotation speed of 120° h^{-1}. However, rotation speed also has a significant effect on protein load. If the P-CAC behaves like a system consisting of an infinite number of batch columns in series, then the rotation speed will determine how long an infinitesimal column with infinitesimal surface area dA will remain in the 'feeding zone'. The width of this zone is only determined by the ratio of feed flow over main eluent flow [5]. It was calculated that for the batch column the load corresponded to 1% of the total bed volume, while for the P-CAC setup this corresponded to 10% of the bed

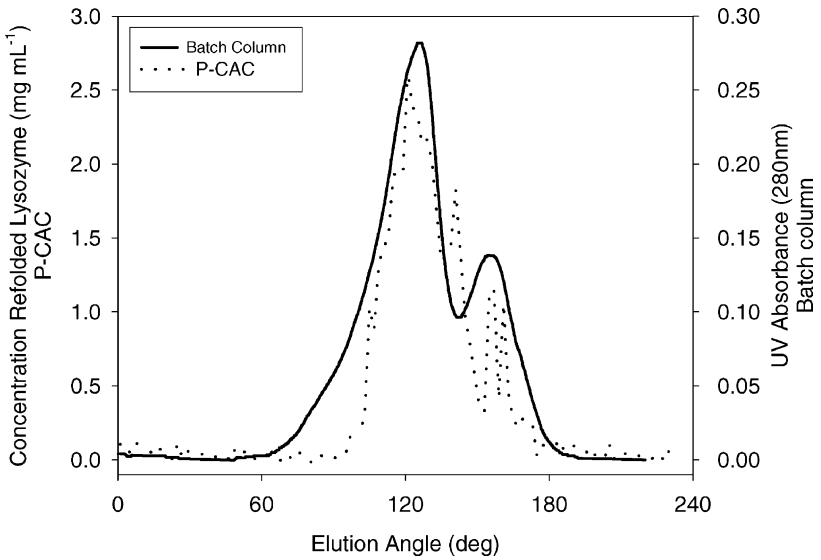

Fig. 11. Comparison between batch SEC refolding and P-CAC SEC refolding. For P-CAC, elution angle were converted to retention on dividing by the angular rotation speed.

Table 4
Comparison between batch and continuous SEC refolding

	Concentration ($mg\,ml^{-1}$)		Load (% vol. of column)	Yield (%)
	Initial	Final		
Batch	8.3	0.11	1	71
Continuous	10.5	1.20	10	72

Standard denatured and reduced lysozyme (8.3–10.5 $mg\,ml^{-1}$ lysozyme, 6 M urea, 32 mM DTT) was used as protein feed.

volume, giving approximately a 10-fold increase in elution concentration. Under these conditions, it appears that the SEC matrix does indeed enhance refolding yield; dilution refolding yield at a final concentration of 1.2 $mg\,ml^{-1}$ would be reduced substantially below the current 72%. This finding suggests that the feed concentration to the SEC column, rather than the final eluate concentration, has the dominant effect on refolding yield. Subsequent work will investigate this conclusion in greater detail.

4. Conclusions

The refolding yield data obtained from batch dilution refolding experiments demonstrates the sensitivity of lysozyme refolding to the concentration of redox agents in the refolding buffer. It was shown that carry-over of DTT from the denatured solution, in particular, inhibited lysozyme refolding.

Refolding on a SEC matrix was not affected by DTT present in the injected sample of denatured lysozyme, as it is separated from the protein fraction whilst moving through the bed. A general trend of increased yield for decreased protein concentration in the feed was observed for both batch dilution and SEC refolding. SEC refolding was shown to be a good alternative for batch dilution refolding, as yields were slightly lower at the feed concentration. Moreover, SEC facilitates immediate separation of reducing compounds such as DTT, protein aggregates and properly refolded species. Hence, it avoids the necessity for two purification steps, one prior to refolding to remove excess reducing compounds and one after refolding removing aggregated species from the desired refolded product.

As batch-mode productivity would be the limiting step in a column-based refolding downstream process, the stationary column refolding method was implemented on a continuously operating P-CAC system. It was shown that P-CAC behaves like an infinite series of stationary columns enabling easy insertion into a downstream processing chain after optimisation of the refolding process on a batch SEC system. The benefits of SEC refolding are high yield at high protein concentration (>1 $mg\,ml^{-1}$), simultaneous refolding and separation of aggregated species, and elimination of problems associated with the carry-over of DTT and other interfering small molecules from the denatured protein solution. P-CAC enables these advantages to be realised in a continuous format, giving an enormous process advantage.

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