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Controlled oxidative protein refolding using an ion-exchange column

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Abstract

Column-based refolding of complex and highly disulfide-bonded proteins simplifies protein renaturation at both preparative and process scale by integrating and automating a number of operations commonly used in dilution refolding. Bovine serum albumin (BSA) was used as a model protein for refolding and oxido-shuffling on an ion-exchange column to give a refolding yield of 55% after 40 h incubation. Successful on-column refolding was conducted at protein concentrations of up to 10 mg/ml and refolded protein, purified from misfolded forms, was eluted directly from the column at a concentration of 3 mg/ml. This technique integrates the dithiothreitol removal, refolding, concentration and purification steps, achieving a high level of process simplification and automation, and a significant saving in reagent costs when scaled. Importantly, the current result suggests that it is possible to controllably refold disulfide-bonded proteins using common and inexpensive matrices, and that it is not always necessary to control protein–surface interactions using affinity tags and expensive chromatographic matrices. Moreover, it is possible to strictly control the oxidative refolding environment once denatured protein is bound to the ion-exchange column, thus allowing precisely controlled oxido-shuffling.

Keywords: Oxidative; Protein; Refolding; Ion-exchange; Chromatography; Column

1. Introduction

Escherichia coli is an important host for the efficient expression of valuable recombinant proteins. However, high-level expression of recombinant proteins often leads to the formation of dense, insoluble protein aggregates, called inclusion bodies [1]. Although the production of protein in inclusion bodies can be advantageous, as it allows high protein concentrations, limits proteolytic degradation and reduces toxicity to the host cells, solubilization and subsequent refolding is necessary to obtain biologically active protein. The main challenge in the production of biologically active proteins via inclusion bodies is to refold the protein at reasonably high efficiency [2].

Various process operations have been used for refolding, including diafiltration, size-exclusion chromatography (SEC) and dilution. Dilution is the simplest and most widely used method, and involves refolding initiation by reducing the denaturant concentration. However, at high protein concentrations, dilution refolding usually leads to protein aggregation. By carefully controlling the protein concentration at a low level, the formation of protein aggregates can be minimized [3]. In large-scale protein refolding this leads to a high cost for reagents and buffers and an additional concentration step is often required for further processing after dilution. For diafiltration, accumulation of denatured protein on the membrane is a major limitation, whereas low column efficiency limits protein load in large-scale SEC operations. Interestingly, these operations are better able to impose an optimized redox environment on the refolded protein than dilution refolding. In dilution refolding, residual reducing agents carried from the solubilization/denaturation buffer can drastically reduce refolding yield for some proteins [4]. In such cases, dilution refolding must be preceded by a buffer exchange operation (e.g. diafiltration or SEC).

An ideal method for the refolding of commercially valuable proteins would be scale invariant, easily automated, generic for a broad range of similar proteins and economical [5]. Column-based refolding is potentially superior to all

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of the refolding methods mentioned, as it is the most likely to fulfill all of these criteria (subject to column scaling difficulties). Using this method, protein aggregation during refolding can be minimized by spatially isolating the molecules on a solid matrix. By binding the proteins reversibly to the matrix, intermolecular interactions between partially folded proteins, and hence aggregate formation, can be prevented or at least minimized. The ability to refold on a matrix is, however, protein dependent and can be hindered by protein–matrix interactions. Because of this, affinity interactions are generally preferred, since binding through specific domains leaves the bulk of the protein free from the surface and hence able to refold [5]. However, affinity matrices are often expensive at process scale and require the use of affinity-tagged proteins, from which the tag must be removed. Therefore, the use of common and less expensive matrices such as ion-exchange matrices is more feasible, in particular for large-scale applications. As these matrices are generally used for purification and concentration after protein refolding, process intensification can be achieved by using the matrix to integrate refolding, purification and concentration. Moreover, their use facilitates buffer exchange prior to dilution thus enabling, in a very simple way, the imposition of an optimized redox environment.

Refolding on an ion-exchange matrix was first shown by Creighton in 1986 [6] for horse cytochrome *c* and bovine pancreatic trypsin inhibitor. Suttnar et al. [7] subsequently used a strong anion exchanger to refold recombinant protein derived from *E. coli* inclusion bodies. Refolding of lysozyme and superoxide dismutase on an ion-exchange column has also been reported [8,9]. Several methods have been developed to increase refolding yields for refolding on ion-exchange matrices, ranging from two and three-buffer systems to dual-gradient chromatography [10]. While all the proteins described above have a relatively simple structure, the refolding of more complex proteins is often complicated by the formation of multiple disulfide bonds, which is often the rate-limiting step in refolding [11]. As the number of possible disulfide bond combinations increases dramatically with the number of cysteine residues in the protein, more time is needed to form the correct conformational state. Careful optimization of the redox conditions in the refolding buffer is necessary to facilitate correct disulfide bond formation. This is usually achieved by the addition of a redox couple to buffer, which facilitates correct disulfide bond formation in a process known as ‘oxido shuffling’ [12].

This study reports the development of a column-based ion-exchange refolding procedure for complex and highly disulfide-bonded proteins using BSA, which contains 17 disulfide bonds, as a model protein. The effects of incubation time and maximum protein load on refolding yield were investigated. The result demonstrates that the on-column refolding process is able to intensify and automate the bioprocessing of highly disulfide-bonded proteins without the need for specific orientational control of the protein–matrix interaction.

2. Experimental

Fatty-acid-free BSA was purchased from Roche (Sydney, Australia). Tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), and reduced (GSH) and oxidized glutathione (GSSG) were from Sigma. Urea was from Applichem (Darmstadt, Germany) and acetonitrile (HPLC grade) was purchased from Lab-Scan (Bangkok, Thailand). Dithiothreitol (DTT) was from Progen Biosciences (Brisbane, Australia). Q-Sepharose Fast-Flow HiTrap ion-exchange columns (1 ml) were obtained from Amersham Biosciences (Sydney, Australia). All ion-exchange chromatography experiments were performed on an ÄKTAexplorer workstation (Amersham Biosciences).

2.1. RP-HPLC analysis

RP-HPLC analysis was performed on a Shimadzu HPLC-system. A C_5 reversed-phase column (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) was from Phenomenex (Cheshire, UK). Samples containing between 0 and 10 μg protein were injected onto the reversed-phase column. After a 9.5 min equilibration period at 40% (v/v) acetonitrile, protein samples were eluted using a linear acetonitrile gradient containing 0.05% (v/v) trifluoroacetic acid (TFA), starting at 40% (v/v) acetonitrile and increasing at 0.4% (v/v) acetonitrile/min for 25 min. Protein elution was performed at room temperature and monitored by measuring absorbance at 214 nm. The mass of total protein eluted from the RP-HPLC column was quantitatively determined by peak integration, based on a standard curve obtained from calibration using native and denatured BSA standards. Peak tailing was excluded from peak integration when determining concentrations of native and denatured–reduced BSA in every protein sample, including the standards.

2.2. BSA denaturation–reduction

Denatured and reduced BSA was prepared by incubating a 20–100 mg/ml solution for at least 3 h at room temperature in denaturing buffer (50 mM Tris–HCl, 3 mM EDTA, 8 M urea, 0.1 M DTT, pH 8.5), prior to analysis by RP-HPLC to confirm the presence of denatured–reduced protein.

2.3. Refolding by dilution

To remove DTT from samples, 2.5 ml of concentrated denatured–reduced protein (7–100 mg/ml) was applied to a PD10 desalting column (Amersham Biosciences) for exchange into DTT-free denaturing buffer. After buffer exchange, the protein was RP-HPLC analyzed to determine protein concentration after the PD-10 step, and then immediately diluted 100× into refolding buffer (50 mM Tris–HCl, 1 mM EDTA, 1.1 mM GSSG, 2.2 mM GSH, pH 8.5) to final

protein concentrations ranging from 50 μg/ml to 0.5 mg/ml. Dilution refolding was performed in a glass beaker by adding 50 μl of denatured protein to 4.95 ml refolding buffer, under constant stirring conditions. Samples were then incubated for 96 h at room temperature. Two-hundred microlitre samples were taken for RP-HPLC analysis to monitor refolding progress over time.

Refolded protein yield and total protein recovery represent the amounts of refolded protein and total protein. The latter consists of both intermediate and refolded protein species, obtained after dilution, given as a percentage of the amount of denatured BSA recovered from the PD-10 column. Quantification was by RP-HPLC.

2.4. Refolding by ion-exchange chromatography

Refolding was conducted on a 1 ml HiTrap Q-Sepharose column. A constant flow rate of 0.5 ml/min was used for all chromatographic steps except on-column protein incubation, which was done under no-flow conditions. The column was equilibrated with buffer A (50 mM Tris–HCl, 3 mM EDTA, 8 M urea, pH 8.5) for 10 column volumes (CVs), before loading with a 1ml sample containing 0.5–20 mg/ml denatured–reduced BSA in denaturing buffer. After washing away DTT and unbound protein with 5 CVs of buffer A, refolding was initiated by switching from buffer A to buffer B (50 mM Tris–HCl, 1 mM EDTA, 79 mM urea, 1.1 mM GSSG, 2.2 mM GSH, pH 8.5; composition identical to that obtained after a 100-fold dilution in the dilution refolding protocol), for 5 CVs. Flow rate was then set to zero, and the protein was left on the column to incubate for up to 40 h, followed by a washing step with 6 CVs of Tris–EDTA buffer (50 mM Tris–HCl, 1 mM EDTA, pH 8.5). Elution was then initiated by a salt gradient. A 20 CVs linear gradient from 100% Tris–EDTA buffer containing no NaCl to that containing 1 M NaCl was applied to elute protein from the column. Flow through and eluate were collected in 1 ml fractions and assayed for protein. Protein-containing fractions were pooled and analyzed by RP-HPLC to determine the concentrations of refolded and partially folded protein. Total protein recovery and refolding yield were calculated as described for the dilution refolding study, and are given as a percentage of the amount of denatured protein loaded.

After each run, precipitated and tightly bound protein was removed from the Q-Sepharose column by washing with at least 5 CVs of both 1 M NaOH and 70% ethanol.

2.5. Circular dichroism (CD) spectra

Far-UV CD spectra of denatured, native and refolded BSA (dilution and column refolded) were measured on a Jasco-810 spectrapolarimeter (Jasco, Japan) using a quartz cuvette with 1 mm pathlength (Hellma, Essex, UK). Spectra were corrected by subtracting the buffer baseline, and were averaged over 10 scans for far-UV CD measurements. Native and refolded protein samples were solubilised in 10 mM sodium phosphate, pH 7.4. For CD analysis of on-column refolded BSA, the 40 h on-column incubated fraction was used. Denatured protein was prepared by denaturing–reducing the protein in 8 M urea, 0.1 M DTT, followed by DTT removal using a PD-10 column, as DTT absorbs in the region of the spectrum used. Protein concentration of 0.2 mg/ml, as quantified by RP-HPLC, was used. CD measurements were at room temperature, 23 °C.

3. Results and discussion

3.1. Dilution refolding

BSA refolding by dilution was performed as a control experiment. In the absence of a redox couple, no formation of completely refolded BSA was observed. Various ratios of reduced to oxidized glutathione were investigated and the best result was obtained when a 2:1 reduced to oxidized glutathione ratio was used (data not shown). It was also found that the presence of NaCl in the refolding buffer, at concentrations of up to 0.1 M, had no effect on refolding yield.

Residual DTT is known to have a negative impact on refolding yields, even at concentrations lower than 1 mM [4]. Complete DTT removal prior to dilution is necessary for optimal refolding yields, despite the need to incorporate an additional unit operation in the process. RP-HPLC analysis of denatured BSA after buffer exchange into denaturing buffer lacking DTT revealed that partial oxidation of the denatured protein occurred after DTT removal, resulting in a slight shift in retention time. This shift can be seen in Fig. 1 as a shoulder on the denatured protein peak after the PD10 step. Dilution

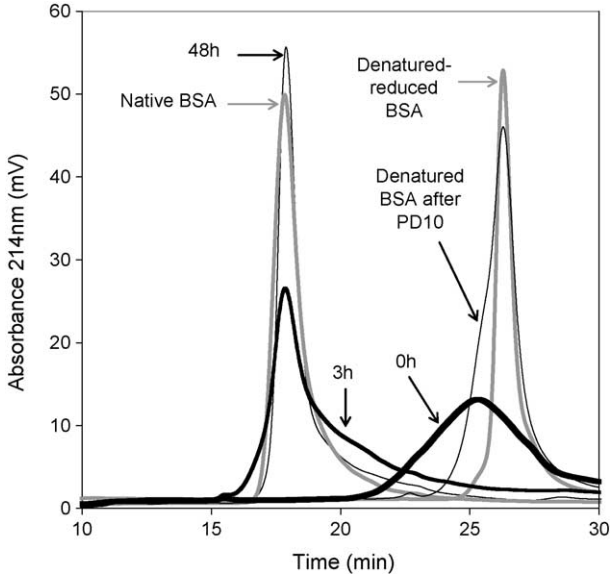

Fig. 1. RP-HPLC chromatograms showing the development of correctly folded BSA during refolding. Denatured BSA was diluted 100-fold to 50 μg/ml into the refolding buffer and 50 μl samples were injected onto the column.

was performed immediately after this buffer exchange step. A 100-fold dilution factor was chosen to ensure sufficient dilution of denaturants thus enhancing refolding yield. This choice led to constraints on the maximum protein concentration after dilution, as solubility and viscosity problems limited the concentration of the denatured–reduced BSA solution and its application in subsequent buffer exchange. Because of these practical restrictions, the maximum protein concentration after 100-fold dilution was limited to 0.5 mg/ml.

The refolding yield and protein recovery after dilution to a concentration of 50 μg/ml, were monitored over time as shown in Fig. 2. Analysis of the samples by RP-HPLC revealed that no spontaneous refolding of BSA into the native conformation occurred immediately after dilution ($t = 0$ h in Fig. 1). Instead, the broadness of the peak shown at 0 h in Fig. 1 suggests the denatured protein was re-oxidized into various misfolded or partially folded protein species. After 3 h of incubation, a large part of the protein present had refolded completely into the native conformation, giving a 45% refolding yield. Misfolded or partially folded protein was still visible in the RP-HPLC chromatogram as peak tailing at both sides of the 'refolded' peak. However, tailing was also observed for the native standard, but to a much lesser extent. Analysis of this native standard on size exclusion chromatography showed that this tailing is due to higher molecular weight impurities, present in the start material (data not shown). The total amount of protein recovered after 3 h of incubation, consisting of refolded, partially folded and misfolded protein species, was approximately 90% (Fig. 2). Total protein recovery did not change with time after dilution and was approximately independent of protein concentration in the tested range of 0.05–0.5 mg/ml after 100-fold dilution.

Analysis of the refolding mixture over an extended period of incubation showed that refolding yield increased significantly with time over the first 48 h after dilution, after which it reached a stable plateau. This increase results from the conversion of misfolded or partially folded protein into correctly refolded protein. This process is known to be inherently slow, due to the rate-limiting 'oxido shuffling' process [13]. The final refolded protein yield achieved was approximately 82%.

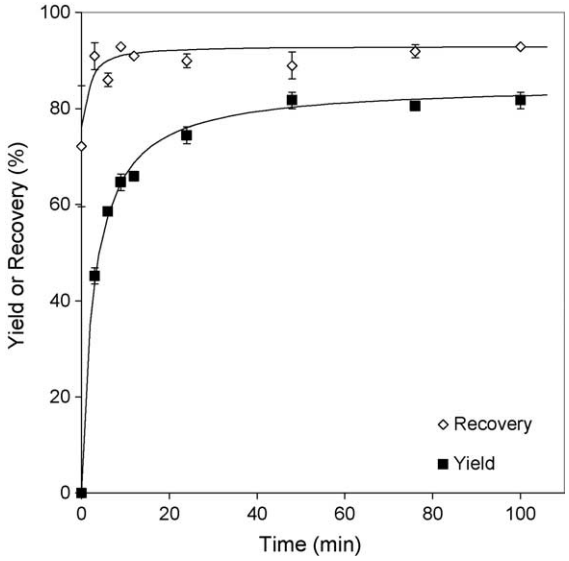

Fig. 2. Refolding yield and total protein recovery for dilution refolding as a function of time. The protein concentration after 100-fold dilution was 50 μg/ml.

3.2. Refolding by ion-exchange chromatography

Optimized buffer conditions which gave the highest yield in dilution refolding were used for the column-based refolding study (see Section 2). Elution profiles obtained after an extended period of incubation of 2 mg protein bound on the column are shown in Fig. 3. No protein was lost in the flow through during column loading. The elution profile showed a single broad protein peak when elution was initiated immediately after refolding buffer was introduced to the column (0 h). RP-HPLC analysis of the eluted protein (Fig. 4) revealed that this peak corresponds to misfolded or partially folded protein; neither correctly refolded protein, nor completely denatured–reduced protein, was present. Strikingly, the total amount of protein recovered from the column was less than 20%, when elution was done immediately, indicating that most protein had precipitated on the column during the refolding and/or elution step(s).

The elution profile changed significantly over time with the emergence of a second peak after 3 h of incubation on the column. RP-HPLC analysis of the protein from this peak revealed a clear overlap with native BSA (Fig. 4). This result shows that refolded protein bound less tightly to the ma-

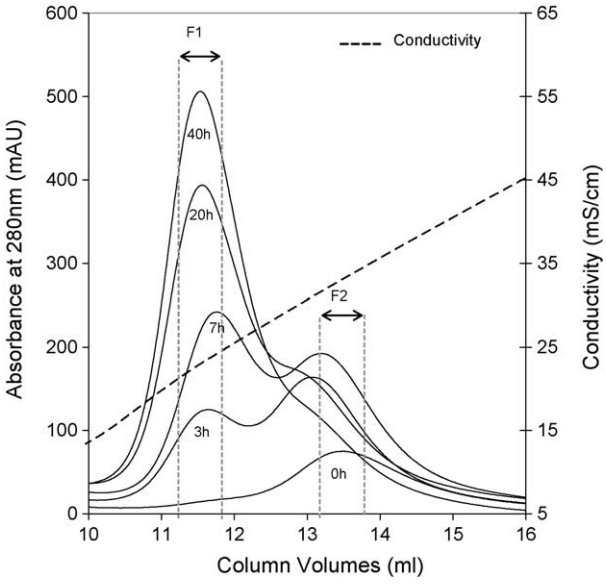

Fig. 3. Chromatograms representing the elution profiles of BSA from the ion-exchange column after extended column incubation. A 6 CVs wash step with Tris–EDTA buffer was carried out before elution buffer was introduced. F1 and F2 represent the fractions which were RP-HPLC analyzed, as shown in Fig. 4.

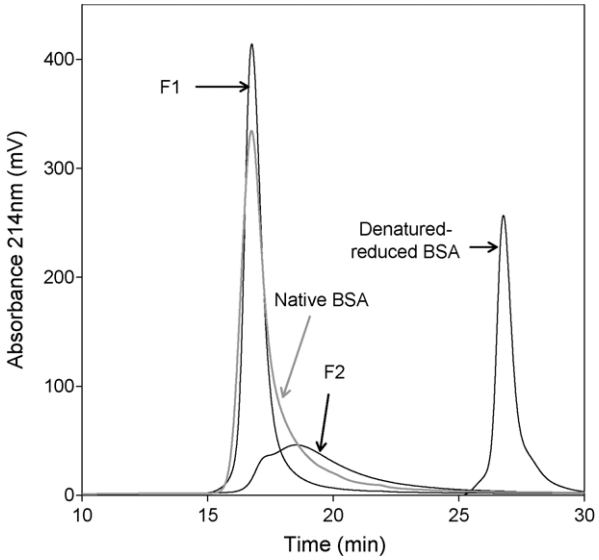

Fig. 4. RP-HPLC chromatograms of fractions eluted from the ion-exchange column as indicated in Fig. 3. F1 and F2 are the eluted fractions after 40 and 0 h column incubation, respectively.

trix compared with the other protein conformations, allowing separation of completely refolded BSA from misfolded and partially folded BSA. The refolding yield appeared to increase with incubation time, reaching 55% after a 40 h incubation period (Fig. 5). This increased refolding yield with time indicates that the protein–matrix interaction was highly reversible, allowing the structural re-arrangements necessary for the protein to refold into its most stable form.

The total amount of protein recovered from the column followed a similar trend to refolding yield. After 40 h of incubation, 67% of total protein was recovered. Inability to achieve 100% protein recovery suggests that some protein aggregated and precipitated after being desorbed from the matrix. In fact, a higher protein recovery (approximately 90%) was achieved when 8 M urea was added to the elution buffer (data not shown). The extent of aggregation could be reduced by extending the incubation period of protein on the column. This approach allows a higher proportion of intermediate protein species to refold into the stable native conformation, hence reducing the amount and concentration of protein that potentially participates in aggregate formation. The increase in refolding yield and total protein recovery with time support this hypothesis and suggests that the formation of irreversible aggregates during incubation is not causing the low recovery observed for short term incubations, although the formation of aggregates during incubation cannot be completely excluded.

For column refolding to be suitable for large-scale operations, the binding capacity of the protein on the column is an important factor to be considered. In this study, binding of various amounts of denatured–reduced protein onto the ion-exchange column was investigated, and the yield and recovery were quantified (Fig. 6). It was found that 10 mg of denatured–reduced protein can be bound per ml of Q-Sepharose resin, without any protein lost in the flow through. Not surprisingly, refolding yield decreased with increasing protein load on the column. Total protein recovery also decreased upon increased protein load, ranging from 67 to 48% for 2 and 10 mg bound, respectively. This is because higher protein load affects the spatial isolation of the protein molecules on the matrix and consequently promotes aggregation of incompletely refolded protein. Partially folded intermediates will also occur at a higher local concentration during elution. Nonetheless, higher protein loads lead to in-

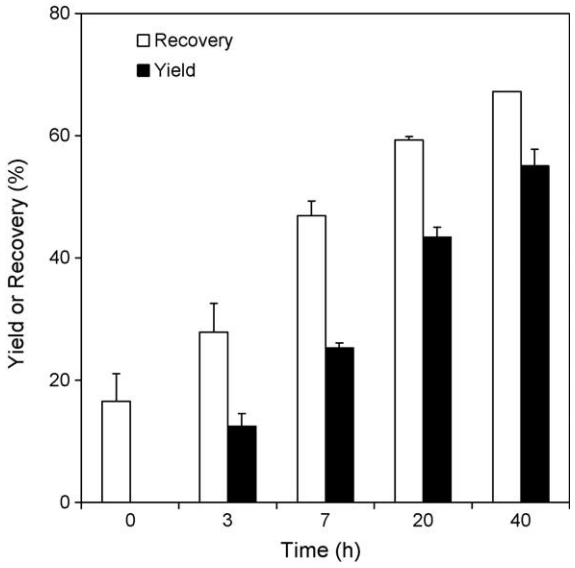

Fig. 5. Increase in refolding yield and total protein recovery with time for on-column refolding of BSA. A 1 ml fraction containing 2 mg/ml denatured–reduced BSA was loaded onto the Q-Sepharose column and eluted after incubation for the time indicated.

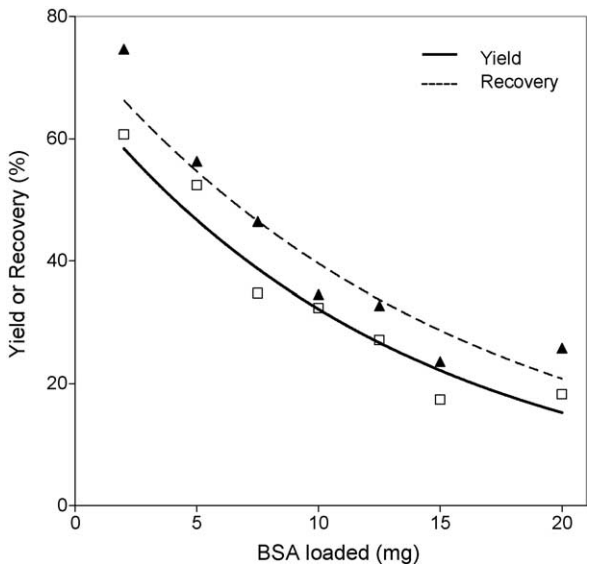

Fig. 6. Refolding yield and total protein recovery as a function of the total amount of denatured–reduced BSA loaded on the column. A 1 ml sample containing between 2 and 20 mg/ml denatured–reduced BSA was loaded onto the column and eluted after an incubation period of 40 h.

Table 1
Mass balance for BSA on-column refolding

Nominal BSA load (mg)	Actual BSA load (denatured) (mg in 1 ml)	Actual BSA load (total) (mg in 1 ml)	Native BSA in eluate (mg in 7 ml)	Total BSA in eluate (mg in 7 ml)
2	1.60	1.88	0.91	1.26
5	4.01	4.69	2.10	2.64
7.5	5.90	7.06	2.05	3.28
10	8.00	9.86	2.58	3.40
12.5	9.84	11.76	2.67	3.84
15	11.81	14.12	2.04	3.33
20	16.04	18.76	2.93	4.84

Actual BSA load (denatured) was determined by RP-HPLC, neglecting peak tailing. Actual BSA load (total) was obtained by full integration of the entire RP-HPLC peak, including tailing. BSA loaded did not contain any native protein.

creased refolded protein concentrations in the eluate, with concentrations of more than 3 mg/ml when 10 mg was bound to the column.

A mass balance for BSA on-column refolding is given in Table 1. All protein amounts were quantified by RP-HPLC.

3.3. Circular dichroism spectrometry

Circular dichroism (CD) was used to compare the physical characteristics of dilution and column refolded BSA against native BSA. The secondary structure of refolded BSA was determined by measuring its CD spectrum in the far UV region (190–250 nm). The spectra of both dilution and column refolded protein were very comparable, whereas the unfolded protein showed a completely different spectrum (see Fig. 7).

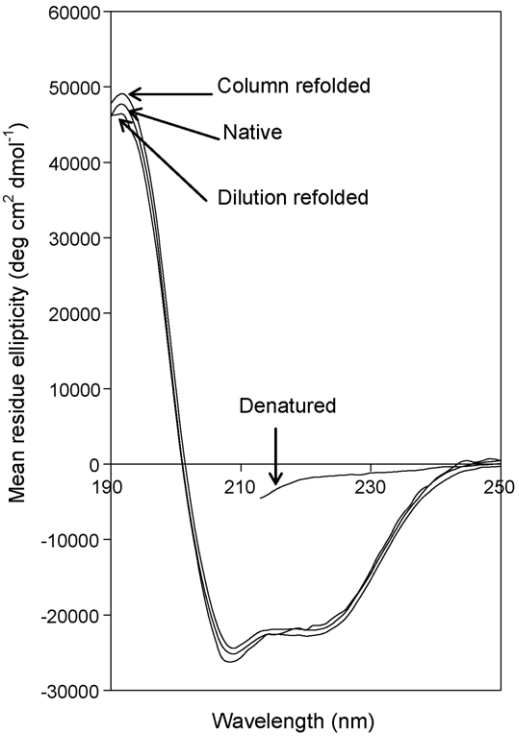

Fig. 7. Far-UV CD spectra of 0.2 mg/ml native, (dilution and on-column) refolded and denatured BSA.

3.4. Comparison of dilution refolding and ion-exchange refolding

The refolding yields achieved in this study were higher in dilution refolding than after on-column refolding. In a direct comparison, refolding at a protein concentration of 0.5 mg/ml on-column was compared to refolding at 0.5 mg/ml after dilution. This resulted in refolding yields of 55 and 80% after 40 h incubation for column refolding and dilution refolding, respectively. Additionally, it is clear by comparing the time-profiles shown in Figs. 2 and 5, that dilution refolding is a quicker process. After an incubation period of 3 h, a refolding yield of 45% was achieved with dilution refolding, whereas a yield of only 12.5% was achieved for on-column refolding. NaCl was added to the refolding buffer in an attempt to increase the on-column refolding rate as addition of NaCl was expected to weaken the reversible protein–matrix interaction and therefore enhance refolding rate. However, the NaCl concentrations tested did not appear to accelerate refolding. High salt concentrations >100 mM even appeared to reduce refolding yield, probably by promoting hydrophobic interactions between partially folded proteins, leading to increased protein aggregation.

The benefits of on-column refolding lie in a different area. One obvious advantage of column refolding over dilution refolding is that refolding can occur at much higher protein concentrations. It was found that BSA could be refolded on-column from starting concentrations of up to 10 mg/ml, without any protein loss observed in the flow through. These are concentrations which cannot be reached in refolding by 100-fold dilution, because of the constraints discussed earlier. At large-scale processing this eliminates the need to use large buffer volumes, hence reducing running costs. Furthermore, the ability to recover refolded protein with concentrations of more than 3 mg/ml during elution, overcomes the need for a concentration step, which is generally required after dilution refolding. Another advantage of column refolding over dilution refolding is that the final concentration of buffer components can be controlled carefully during on-column refolding, whereas carry-over of denaturants and reducing agents will always be an interfering factor in the dilution refolding process. An extra purification step is almost always necessary to completely remove them prior to dilution refolding.

In this study, it was also found that correctly refolded BSA could be easily separated from misfolded or incompletely folded protein, showing the ability of on-column refolding to allow simultaneous purification and refolding in an easily automated format.

4. Conclusion

This work demonstrates that a commonly used and relatively inexpensive ion-exchange matrix can be used for the refolding of BSA with high yields of up to 55%. Refolding was not inhibited by protein–matrix interactions, thus preventing the need for tagged protein in an affinity based method. Refolded protein was directly obtained from the column in a highly concentrated form and free from incorrectly or incompletely refolded protein. This demonstrates that the proposed method could contribute significantly to bioprocess intensification as it integrates the reducing-agent removal, refolding, concentration and purification unit operations, used in dilution refolding, into an easily automated process. Therefore, the method is an interesting alternative for large-scale or preparative oxidative refolding of complex and highly disulfide-bonded proteins.

References

[1] F.A.O. Marston, Biochem. J. 240 (1986) 1.
[2] E. De Bernadez Clark, Curr. Opin. Biotechnol. 12 (2001) 202.
[3] E. De Bernadez Clark, Curr. Opin. Biotechnol. 9 (1998) 157.
[4] H. Lanckriet, A.P.J. Middelberg, J. Chromatogr. A 1022 (2004) 103.
[5] A.P.J. Middelberg, Trends Biotechnol. 20 (2002) 437.
[6] T.E. Creighton, World Patent WO 86/05809 (1986).
[7] J. Suttnar, J.E. Dyr, E. Hamsikova, J. Novak, V. Vonka, J. Chromatogr. B 656 (1994) 123.
[8] M. Li, G. Zhang, Z. Su, J. Chromatogr. A 959 (2002) 113.
[9] M. Li, Z. Su, Biotechnol. Lett. 24 (2002) 919.
[10] M. Li, Z. Su, J.-C. Janson, Protein Expr. Purif. 33 (2004) 1.
[11] E. De Bernadez Clark, D. Hevehan, S. Szela, J. Maachupalli-Reddy, Biotechnol. Prog. 14 (1998) 47.
[12] B. Fischer, I. Sumner, P. Goodenough, Biotechnol. Bioeng. 55 (1993) 3.
[13] S.J. Milner, J.A. Carver, F.J. Ballard, G.L. Francis, Biotechnol. Bioeng. 62 (1998) 693.