ELSEVIER

Journal of Chromatography B, 737 (2000) 225–235

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Refolding and purification of a urokinase plasminogen activator fragment by chromatography

Edward M. Fahey[a,*], Julian B. Chaudhuri[a], Peter Binding[b]

[a]*Department of Chemical Engineering, University of Bath, Bath BA2 7AY, UK*
[b]*Pfizer Central Research, Sandwich, Kent CT13 9NJ, UK*

Abstract

A fragment of recombinant urokinase plasminogen activator (u-PA), was expressed in *E. coli* in the form of inclusion bodies. Purification and renaturation was achieved in a three-stage process. Capture of the inclusion bodies was achieved by coupling wash steps in Triton X-100 and urea with centrifugation. Solubilised inclusion bodies were then renatured by buffer exchange performed by size-exclusion chromatography (SEPROS). Use of size-exclusion media with higher fractionation ranges resulted in an increase in the recovery of u-PA activity, to a maximum fractionation range of M_r 10 000–1 500 000 after which recovery is reduced, due to a low resolution between the refolded u-PA and denaturant. Fractions of refolded u-PA were concentrated using cation ion-exchange chromatography, which selectively binds correctly folded u-PA. The result is concentrated, active, homogeneous u-PA.

Keywords: *Escherichia coli*; Refolding; Purification; Enzymes; Urokinase plasminogen activator

1. Introduction

Purification of recombinant proteins is a crucial step in the production of many biopharmaceuticals. Soluble recombinant proteins are isolated using a series of purification steps progressing from low resolution high capacity techniques, to more specific lower capacity polishing steps, as the protein load is reduced. Chromatography has emerged as one of the most useful tools and provides the basis to most modern purification processes. Most chromatographic separations are well established, and detailed protocols are readily available.

Over-expression of recombinant proteins, however, often results in the production of inclusion bodies, intracellular masses of biologically inert protein. The physical characteristics of inclusion bodies offer several advantages for their purification [1]. However, the process advantages of expressing the gene product as inclusion bodies can only be exploited, if the subsequent refolding yields are high. Protein refolding methods have been recently reviewed [2,3]. To date, batch dilution refolding remains the preferred technique to refold recombinant proteins, mainly due to its simplicity. However, refolding yields are typically low, with recoveries of 5% and above being considered adequate on an industrial scale. Low refolding yields are attributed to loss of protein by aggregation, due to non-specific hydrophobic interactions. It is well established that aggregation is proportional to the initial protein concentration [4], so refolding is performed in dilute solutions. However, this significantly increases subsequent downstream processing volumes and costs.

*Corresponding author. Tel.: +44-1225-826-349; fax: +44-1225-826-894.
E-mail address: cesjbc@bath.ac.uk (E.M. Fahey)

0378-4347/00/$ – see front matter
PII: S0378-4347(99)00360-6

It has recently been reported that size-exclusion media has the potential to perform buffer exchange for protein refolding, whilst separating folding intermediates, and thus reducing aggregation [5]. This technique has been demonstrated with pure, denatured enzymes such as lysozyme and carbonic anhydrase. The model enzyme used in this study is the serine protease domain of urokinase plasminogen activator (u-PA).

Urokinase plasminogen activator is recognised as an important pharmaceutical target. There is experimental evidence suggesting u-PA may play an important role in tumour biology [6]. u-PA (E.C. 3.4.21.73) is a multi-domain glycoprotein (M_r 45 000), which contains 411 residues and 12 disulphide bonds [7]. Fig. 1 shows the expression of u-PA and activation to the two-chain form. u-PA is synthesised as a single-chain glycoprotein, high-molecular-mass (HMM u-PA), which possesses a very low amidolytic activity. HMM u-PA is converted to the highly active two-chain u-PA by cleavage of two peptide bonds located at Lys 135–Lys 136 and Lys 158–Ile 159. Cleavage is catalysed by plasmin, in a positive feedback mechanism. The resulting polypeptide consists of amino acids 136–158 joined by a disulphide bridge, at Cys 148 and Cys 279, to amino acids 159–411. This fragment (M_r 33 000) is termed low-molecular-mass u-PA (LMM u-PA), and contains six disulphide bonds [8]. When over-expressed in *Escherichia coli*, LMM u-PA readily produces inclusion bodies.

This paper describes the isolation, folding and concentration of the serine protease domain of u-PA from inclusion bodies. The latter two steps are performed with the chromatographic techniques of size-exclusion chromatography and ion-exchange chromatography, respectively.

2. Experimental

2.1. Materials

Trizma-Base, 2-(*N*-morpholino)ethanesulphonic acid (MES), EDTA, sodium chloride, sucrose, Triton X-100, guanidine hydrochloride (GuHCl), urea, dithiothreitol (DDT), β-mercaptoethanol (BME), reduced glutathione (GSH), oxidised glutathione (GSSG) and plasmin were purchased from Sigma (Poole, UK). u-PA substrate, Chromozym U, was purchased from Boehringer Mannheim (Lewes, UK). Electrophoresis gels, buffers and standards were purchased from Novex (San Diego, CA, USA). Water to 18 MΩ quality was obtained using a Prima and Maxima system, ELGA (High Wycombe, UK). Size-exclusion refolding and ion-exchange separations were performed on a Biologic Workstation, Bio-Rad (Hemel Hempstead, UK). Columns and media were purchased from Amersham Pharmacia Biotech (St. Albans, UK). *E. coli* (BL-21) cell paste containing the over-expressed serine protease domain of u-PA (amino acids 136–411) was kindly provided by Pfizer Central Research (Sandwich, UK).

2.2. Isolation of the u-PA inclusion bodies

A 50-g amount of *E. coli* cell paste was resuspended in 1 l of lysis buffer (Table 1) and lysed by six passes through a high-pressure homogeniser (APV Manton Gaulin), at 41 MPa. All subsequent centrifugations were performed in 250-ml Sorvall centrifuge tubes using a superlite GSA rotor, in a Sorvall 5C centrifuge. Rotor accelerations ranged between 5000 *g* and 14 000 *g*, with durations between 10 and 60 min. All inclusion body wash steps were performed at room temperature (22°C), for either 2 h (Triton X-100) or 1 h (urea). Each inclusion body pellet was resuspended in the wash buffer using a bench top homogeniser (IKA-Ultraturrax, Laborteknik, Fisons, Loughborough, UK), at 18 000 rpm. The final inclusion body pellet was solubilised by homogenisation (IKA-ultraturax) in a denaturing buffer (Table 1) for 4 min, followed by gentle agitation on a rotary mixer (22°C, 10 h). Any insoluble particles were removed by centrifugation at 10 400 *g* for 60 min, followed by filtration (0.22 μ*M*).

2.3. Refolding of solubilised u-PA

Size-exclusion refolding was performed using a XK26/100 column packed with Sephacryl gel media (S-100, S-200, S-300 and S-400) to a bed height of 87–91 cm. Prior to sample application, the chromatographic apparatus was cooled to 4°C and the column was equilibrated with one column volume of

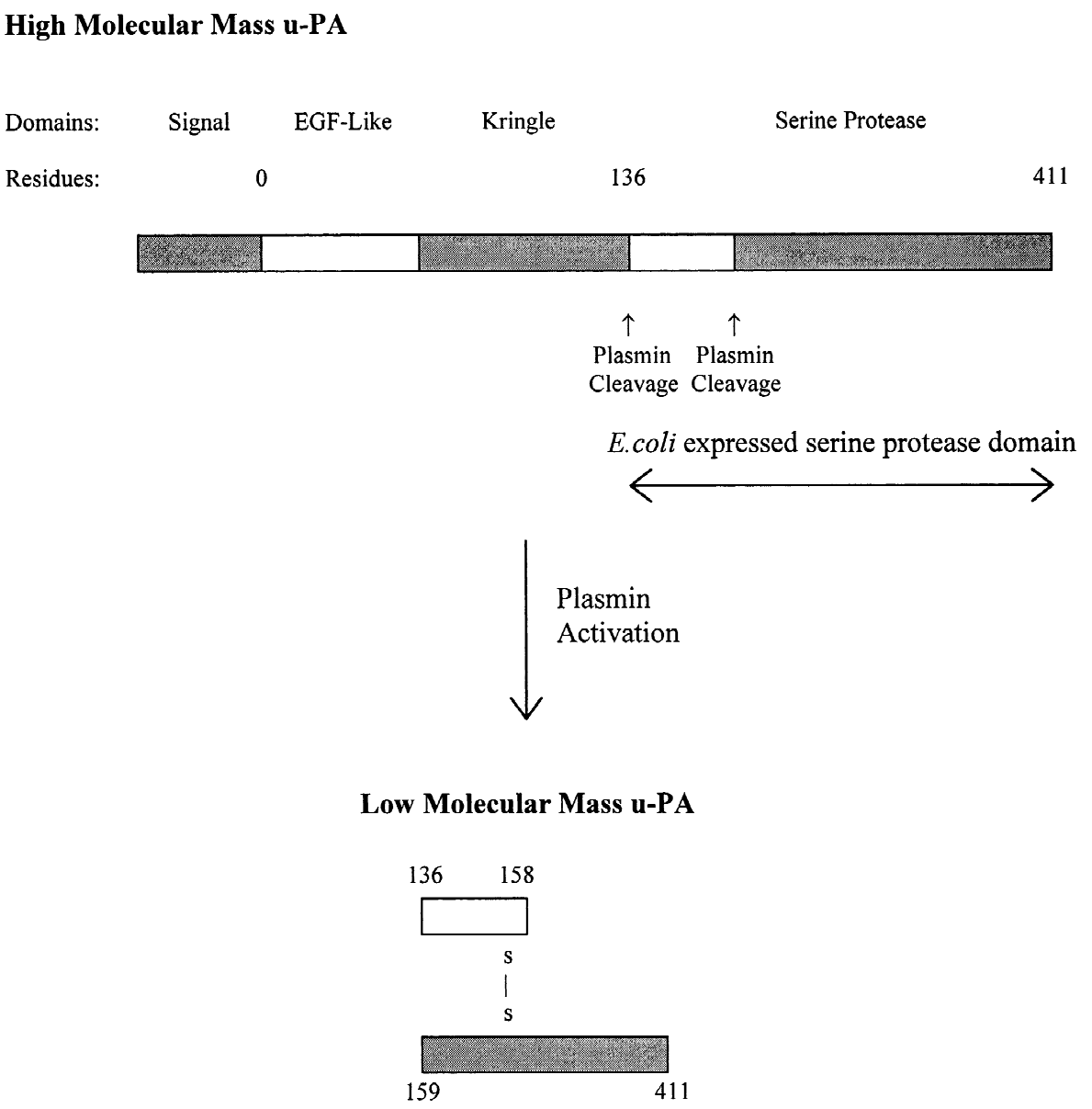

Fig. 1. Schematic diagram of u-PA expression and activation to the two-chain low-molecular-mass form.

refolding buffer (Table 1). A 2-ml volume of 8 mg/ml denatured u-PA was injected through a static loop and eluted at a flow-rate of 0.5 ml/min.

2.4. Concentration of refolded u-PA

Ion-exchange chromatography was performed using a 5 ml Hi-Trap SP column. Gel filtration fractions containing u-PA activity (170–345 ml) were pooled and filtered using a Whatman GF/B filter (1 μm) under vacuum. The filtered sample was acidified to pH 6.5, using 1 *M* MES. The refolded u-PA was loaded at 5 ml/min at room temperature. The column was washed with two column volumes of equilibration buffer (Table 1), then bound u-PA was eluted with a linear gradient of elution buffer

Table 1
List of buffers

Lysis buffer	50 m*M* Tris–HCl, pH 7.5, 5 m*M* EDTA
Detergent buffer	1% Triton X-100, 25% sucrose, 50 m*M* Tris–HCl, pH 7.5, 5 m*M* EDTA
0.5 *M* urea buffer	0.5 *M* urea, 50 m*M* Tris–HCl, pH 7.5, and 5 m*M* EDTA
4 *M* urea buffer	4 *M* urea, 50 m*M* Tris–HCl, pH 7.5, and 5 m*M* EDTA
Denaturing buffer	6M GuHCL, 5 m*M* DTT, 50 m*M* Tris–HCl pH 7.5, 5 m*M* EDTA
Refolding buffer	3 *M* urea, 50 m*M* Tris–HCl, 5 m*M* EDTA, 0.5 m*M* GSH, 0.5 m*M* GSSG, pH 8.5 at 4°C
Equilibration buffer	50 m*M* Tris–HCl, pH 7.5, 5 m*M* EDTA
Elution buffer	1 *M* NaCl, 50 m*M* Tris–HCl, pH 7.5, 5 m*M* EDTA

(Table 1), over 100 ml. Fractions containing the protein peak were assayed for u-PA activity, and desalted by dialysis.

2.5. Analytical methods

Partition of the inclusion bodies between the supernatant and pellet was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (4–20% Tris–Glycine). SDS gel analysis was performed using Quanti-scan software (Biosoft, Cambridge, UK). Protein concentration was determined by absorbance at 280 nm ($\epsilon^{0.1\%}=1.55$) [9], or by Coomassie assay [10]. Protein aggregation was measured by sample absorbance at 450 nm. Guanidine elution was monitored by a conductivity meter at the outlet of the column. Refolded u-PA (20 μl) was activated by incubation with 1 mg/ml plasmin (20 μl) in 960 μl of Tris–HCl, pH 8.5 at 22°C for 1 h. Activated u-PA was then assayed by adding 5 μl of 9 m*M* Chromozym U and measuring the rate of change of absorbance at 405 nm. The isoelectric point of refolded u-PA was estimated using GCG Wisconsin Package (Version 7, Genetics Computer Group, Madison, WI, USA).

3. Results

3.1. Isolation of u-PA inclusion bodies

The high density of the inclusion bodies facilitates their isolation by centrifugation. Coupling wash steps with centrifugation provides a quick, high capacity method to purify recombinant proteins. *E. coli* cells were resuspended in a lysis buffer (Table 1) and disrupted by high pressure homogenisation. The inclusion bodies were recovered using a range of centrifugation times and accelerations. Centrifugation at 10 400 *g* for 10 min provided the best separation of inclusion bodies and soluble *E. coli* protein (data not shown). The inclusion body pellet was resuspended in detergent buffer (Table 1) and incubated for 2 h. Fig. 2 shows an SDS gel of the composition of supernatant and pellet following washing in the detergent buffer, from a range of centrifugation times all at 10 400 *g*. Only the 60 min centrifugation recovered the majority of the inclusion bodies present. Centrifugation of less than 60 min resulted in increased amounts of u-PA remaining in the supernatant.

During solubilisation of the u-PA inclusion bodies, it was noted that a concentration of 8 *M* urea was insufficient to raise the protein concentration above 4 mg/ml. Low concentrations of urea have been previously used to selectively solubilise the contaminated proteins, without affecting the bulk of the inclusion body protein [11]. Fig. 3 shows the composition of the supernatant following a 1 h incubation of inclusion body pellets with increasing concentrations of urea (0.1–9 *M*). Urea concentrations up to 6 *M* were unable to solubilise a significant amount of u-PA inclusion body protein. However, an incubation at 0.5 *M* urea removes two proteins at M_r 39 000 and 41 500, whilst an incubation at 4 *M* urea removes a band of protein at M_r 27 000. Thus two additional wash steps in 0.5 *M* and 4 *M* urea were introduced to further purify the u-PA inclusion bodies. Fig. 4 shows an SDS gel of the complete inclusion body isolation procedure, combining the detergent and urea wash steps with the centrifugations. The percentage purity of u-PA compared to other *E. coli* proteins was determined by scanning densitometry of the sample lanes in Fig. 4. Table 2

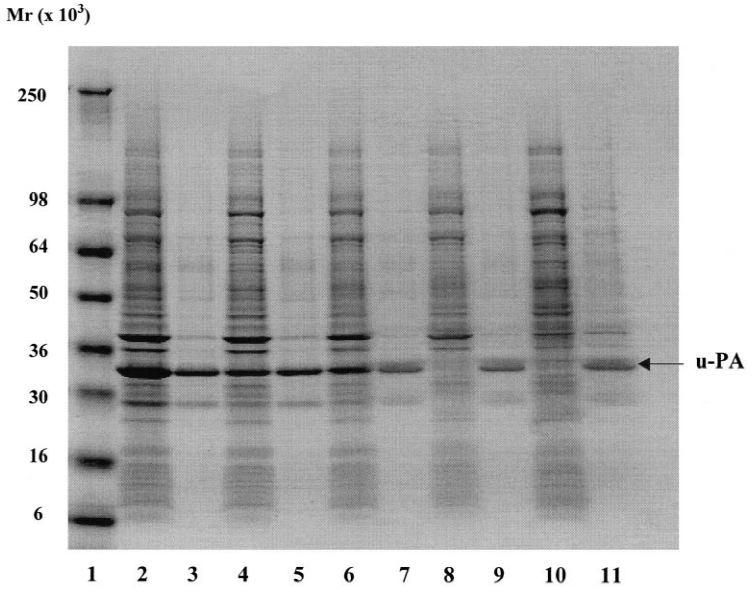

Fig. 2. SDS gel showing the composition of supernatant and pellet following washing with Triton X-100. Lanes: 1=protein standards, 2=supernatant 10 min, 3=pellet 10 min, 4=supernatant 30 min, 5=pellet 30 min, 6=supernatant 45 min, 7=pellet 45 min, 8=supernatant 60 min, 9=pellet 60 min, 10=supernatant following centrifugation of cell lysate, 11=pellet following centrifugation of cell lysate.

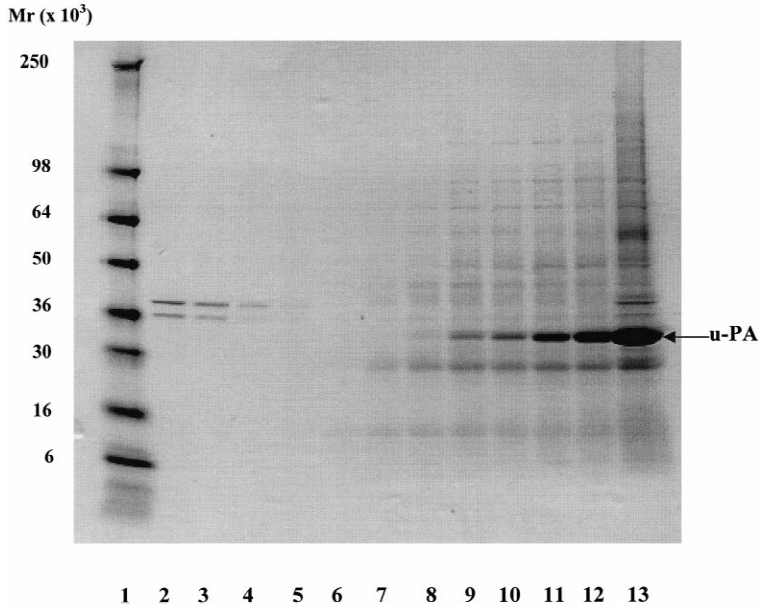

Fig. 3. SDS gel showing the effect of an increasing concentration of urea on the solubility of u-PA inclusion bodies. Lanes: 1=protein standards, 2=0.1 *M* urea, 3=0.5 *M* urea, 4=1 *M* urea, 5=2 *M* urea, 6=3 *M* urea, 7=4 *M* urea, 8=5 *M* urea, 9=6 *M* urea, 10=7 *M* urea, 11=8 *M* urea, 12=9 *M* urea, 13=starting u-PA pellet.

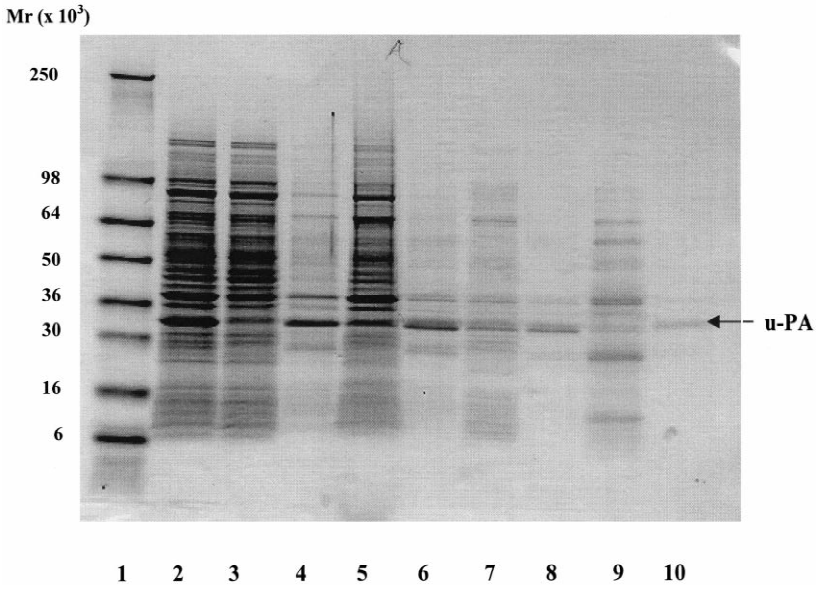

Fig. 4. SDS gel showing the purification of u-PA inclusion bodies. Lanes: 1=protein standards; 2=cell lysate; 3=supernatant in lysis buffer, 4=pellet in lysis buffer, 5=supernatant in detergent buffer, 6=pellet in detergent buffer, 7=supernatant in 0.5 *M* urea, 8=pellet in 0.5 *M* urea, 9=supernatant in 4 *M* urea, 10=final purified u-PA inclusion body pellet.

shows a summary of the change in u-PA purity following each stage of the purification.

3.2. Refolding of the solubilised u-PA by size-exclusion chromatography

Size-exclusion chromatography can perform the buffer exchange necessary to initiate protein folding, and aggregate removal in a single process [5]. Buffer exchange is performed by the separation of sample denaturant and unfolded protein, which equilibrates with the mobile phase. The resolution of the refolded protein and denaturant is dependent on the fractionation range of the gel.

The refolding efficiency of four gel types (Sephacryl S-100, S-200, S-300 and S-400) was tested. Fractionation range increases from S-100 (M_r 1000–100 000) to S-400 (M_r 20 000–8 000 000). Fig. 5 shows the variation of protein elution with gel fractionation range. Elution volume of the refolded protein peak increases with the fractionation range of the gel, which was expected. There is however, a large change in peak symmetry, which does not occur during native protein separations. The elution profile of the S-100 gel type shows a sharp leading edge with a small tail area. The peak of protein concentration is eluted at a volume equivalent to the void volume of the column, determined by the elution of blue dextran (M_r 2 000 000). The protein peak is mainly comprised of aggregated u-PA and contains little u-PA activity. As the fractionation range increases, the peak of protein concentration decreases and the tail region increases, resulting in a greater elution volume. Fig. 6 shows the total aggregation observed with each gel type. The decrease in aggregation is linked to the decrease in the peak of u-PA concentration. Fig. 7 shows the elution of activity for each gel tested. The activity peak is increasingly retarded, as with the protein concentration peak. However, there is less change in peak symmetry. Fig. 8 shows a histogram of the total u-PA

Table 2
Summary of u-PA purification from inclusion bodies

Purification stage	Purity (% u-PA)
Cell lysate	11.0
First centrifugation	27.7
Detergent wash	39.0
0.5 *M* urea wash	67.0
4 *M* urea wash	100.0

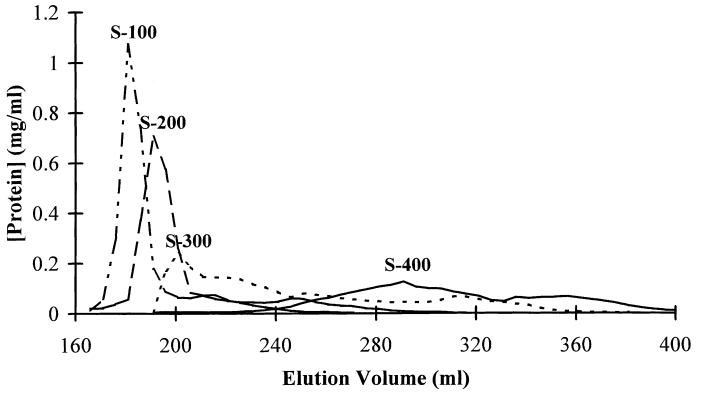

Fig. 5. Protein elution during size-exclusion refolding.

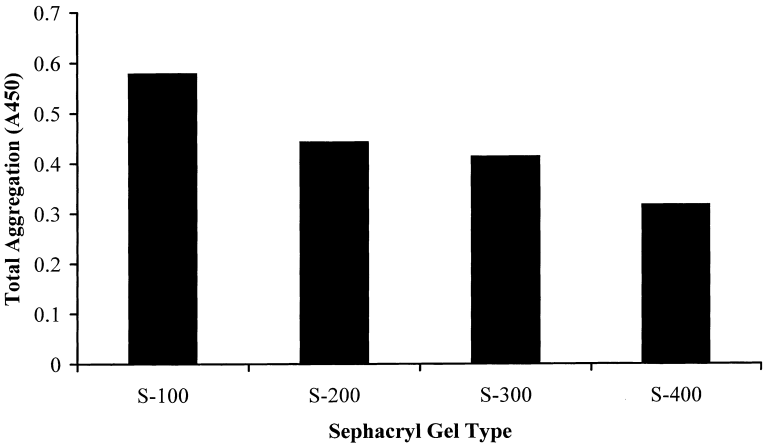

Fig. 6. Total aggregation of u-PA during size-exclusion refolding.

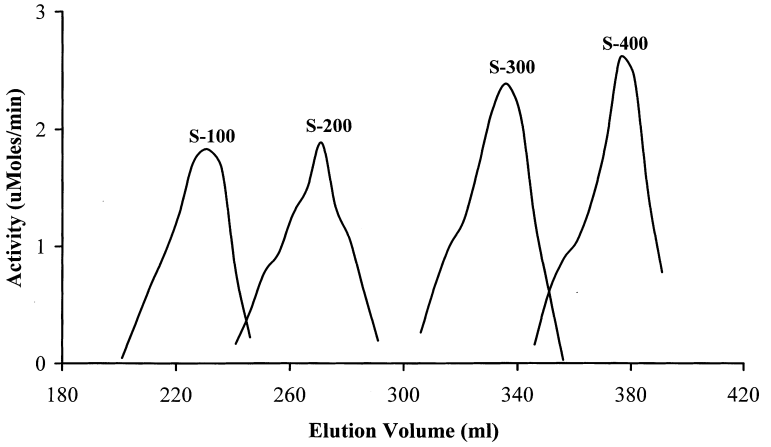

Fig. 7. Elution of u-PA activity during size-exclusion refolding.

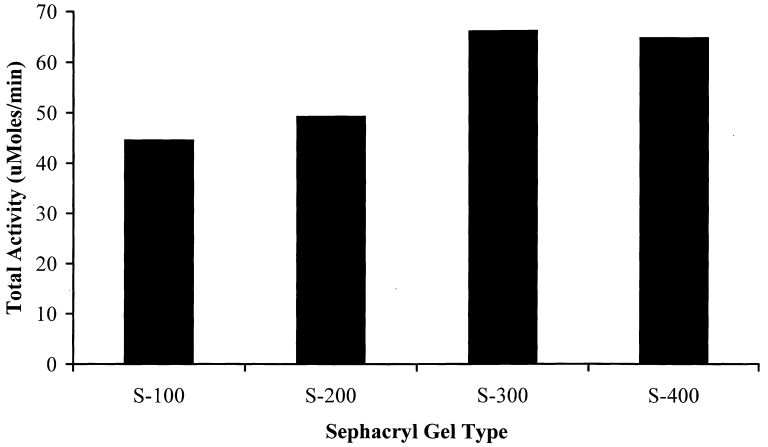

Fig. 8. Total recovery of u-PA activity from each gel type.

activity recovered, calculated from the peak areas, using linear regression of the leading and tailing edge of the peak. Refolding using the S-300 gel type results in the maximum recovery of u-PA activity, and the greatest resolution of the three species being separated: aggregates, active protein and denaturant.

3.3. Concentration of the refolded u-PA

Conventional purification of refolded u-PA is performed using affinity chromatography, which requires the activation of the u-PA by plasmin [12]. We investigated the use of ion-exchange chromatography as an alternative for the purification of u-PA. An isoelectric point of 7.97 was estimated for the refolded fragment u-PA. It was therefore decided that a strong cation exchanger, such as SP Sepharose could concentrate the refolded u-PA. The rigidity of the agarose matrix allows high flow-rates (5ml/min), reducing the time taken to load the refolded sample (175 ml).

Fig. 9 shows a chromatogram of the purification using a NaCl gradient. A single protein peak is observed at 36% of 1 *M* NaCl. No additional protein was eluted upon washing with 1 *M* NaOH. Fractions were also collected during sample loading to test binding of the refolded u-PA, and to detect breakthrough of u-PA. Analysis of these fractions showed

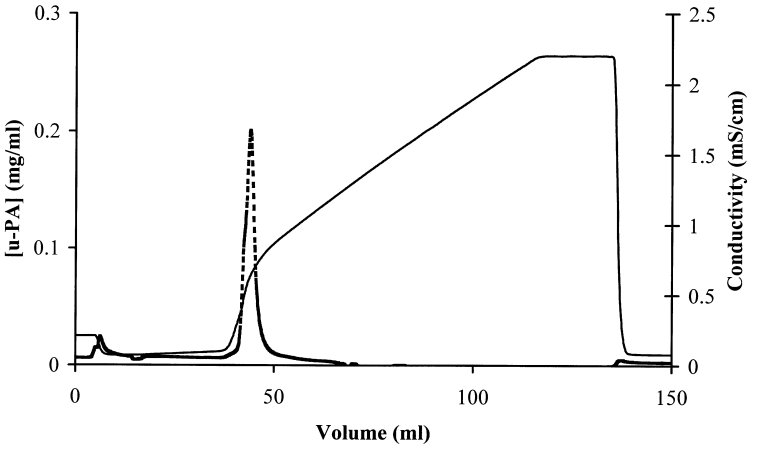

Fig. 9. Concentration of u-PA using ion-exchange chromatography.

constant elution of a protein (M_r 33 000) which exhibited no u-PA activity. The eluted protein peak contained 2.45 mg of active u-PA, which corresponds to a 15.3% refolding yield from the initial inclusion body material. This ion-exchange step resulted in an 11-fold increase in the specific activity for the refolded u-PA.

4. Discussion

Refolding efficiency of the solubilised inclusion body protein can be affected by the presence of *E. coli* contaminants [13]. Such contaminants include nucleic acids, proteins and phospholipids. Removal of these impurities has resulted in improved refolding yields [14], or had no effect [15]. Generally, purification of the inclusion bodies prior to refolding is desirable, to lower the load of protein on subsequent down stream processes [3]. The first stage of an inclusion body purification should capture of the bulk of over-expressed protein. Microfiltration [16], chromatography [17], and centrifugation [18] have all been used to isolate inclusion body protein.

In this study, centrifugation provided a high capacity technique that was quick and cheap. It utilised the density difference between the insoluble u-PA inclusion bodies, the lighter cell debris and the soluble *E. coli* protein. Once, the bulk of the soluble *E. coli* protein had been removed by the initial centrifugation, following steps either prevented insoluble material from co-sedimenting with the inclusion bodies or selectively removed contaminating proteins. It has been shown that insoluble cell debris can be removed by increasing the viscosity of the solution being centrifuged with 25% sucrose [19]. Whilst, detergents such as SDS [20], Triton X-100 [21], Berol 185 [22] and sodium deoxycholate [23] have been successfully used to strip away adhering membrane proteins from the inclusion body surface. Triton X-100 being a weaker anionic detergent removed loosely bound proteins, without strongly binding to the inclusion bodies, which would effect subsequent purification steps. Washing the inclusion bodies in Triton X-100 is combined with centrifugation in sucrose, because contaminants removed by the Triton X-100 may still be insoluble and will sediment if a high enough centrifugal force is applied. Though, according to Stokes' law particle sedimentation is dependent on the size and density of the particle, viscosity of the solution, and the speed and duration of the centrifugation [24]. Therefore, either the centrifugation time or acceleration had to be increased, to compensate for the increased viscosity of the sucrose buffer. A centrifugation of 60 min at 10 400 *g* was the minimum duration required to pellet all of the inclusion body present.

Subsequent wash steps concentrated on stripping away the remaining membrane proteins from the inclusion body surface. The 0.5 *M* urea wash step successfully removed two distinct bands of protein, which are likely to be *E. coli* outer membrane proteins OmpA and OmpC, whose presence in inclusion bodies has been previously observed [25]. Phospholipids were removed in each step, by EDTA present in the wash buffer [26].

Combining all the washes and centrifugations results in an isolation protocol, which takes considerably less time than the processing of the same quantity of inclusion bodies by microfiltration [27]. Scanning densitometry analysis of the SDS gel (Fig. 4) determined a final u-PA purity in excess of 90%.

Presently, batch dilution is the most popular method to refold recombinant proteins, due to its simplicity and immediate results. Although dialysis [28], ion-exchange chromatography [29], molecular chaperones [30], hollow fibre membranes [31] and reverse micelles [32] have been used to refold proteins with limited success. Recently it has been shown that size-exclusion gel media can perform the necessary buffer exchange, to initiate protein refolding, whilst separating folding intermediates [5]. Refolding of lysozyme from a starting concentrations up to 80 mg/ml resulted in a 46% recovery of fully active protein. It was proposed that the reduced diffusion within the size-exclusion media increases refolding yield by suppressing the non-specific hydrophobic interactions of folding intermediates, thus minimising aggregation. This led to an increase in refolding studies using size-exclusion chromatography [33–35].

The mechanism for size-exclusion refolding has not been fully elucidated, but an initial explanation has been proposed [5]. The unfolded protein exhibits a random coil configuration, with a large Stokes radius. The increase in Stokes radius results in the

unfolded protein having a higher effective molecular mass in comparison to its native form, when determined by size-exclusion chromatography. During passage through the column the unfolded protein encounters a linearly decreasing concentration of denaturant, until it equilibrates with the mobile phase applied to the column. The reduction in denaturant concentration promotes protein folding, resulting in the collapse of the polypeptide chain to a compact native like structure with a reduced Stokes radius. As folding occurs the partition coefficient of the protein between the mobile phase and the gel increases. Within the gel matrix transport is diffusion limited [36], which will minimise aggregation by reducing non-specific hydrophobic interactions. Once protein folding is complete, the Stokes radius is constant and the protein is eluted in its native form. Any aggregates formed during refolding are eluted first, due to their larger Stokes radius.

The effective molecular mass of unfolded u-PA, on the size-exclusion column, may be estimated by considering the changes to the protein's Stokes radius between the folded and unfolded states. The Stokes radius for u-PA unfolded in GuHCl was determined from a correlation of Stokes radii with molecular mass for several GuHCl-denatured proteins [37]. Thus, the Stokes radius of unfolded u-PA was estimated to be 5.3 nm (as compared to its estimated native size of 2.6 nm). Using a correlation of Stokes radius for native proteins against molecular mass, the unfolded Stokes radius of u-PA corresponds to an effective relative molecular mass of 231 000 [37].

Lysozyme was successfully refolded from urea by size-exclusion chromatography using a matrix (S-100) with a globular protein fractionation range of M_r 10 000–100 000 [5]. Using the correlations cited above, lysozyme unfolded in urea has an effective relative molecular mass of 64 000, and thus is not fully excluded from the S-100 gel matrix. However, because unfolded u-PA has an effective relative molecular mass of 231 000 it is fully excluded from the S-100 gel. Transport of the unfolded u-PA will initially occur in the void volume of the column, resulting in a reduced volume available for the u-PA to refold. The unfolded u-PA will therefore have an increased local concentration, which will increase the probability of aggregation, reducing the recovery of u-PA activity. As the fractionation range of the gel matrix increases from S-100 to S-400 the partition coefficient of unfolded u-PA increases, increasing the volume available for refolding, and decreasing the probability of aggregation. The result is a decrease in the total amount of aggregation as fractionation range increases. Aggregation and refolding are competing reactions [4] therefore by minimising aggregation, more protein is available for refolding resulting in a higher recovery active protein. Total recovery of active u-PA increases with fractionation range until the S-400 gel type. The lower recovery using S-400 is attributed to the lower resolution between the active protein and denaturant.

Refolding produces a dilute solution of native and aggregated protein. The final purification should remove any misfolded protein and concentrate the correctly folded protein for subsequent studies. Separation of active and inactive species is difficult, due to the similar physical characteristics. Purification protocols for u-PA usually utilise benzamidine sepharose [12] to perform the final purification. This is an affinity matrix, which recognises active serine proteases. The refolded u-PA therefore has to be activated with plasmin, prior to purification, and purified samples will require inhibition if stored. Activation studies of the purified u-PA are therefore not possible, and alternatives to affinity chromatography were tested.

Ion-exchange chromatography has the potential to concentrate the refolded u-PA without the need to activate samples. Once the isoelectric point of u-PA was estimated, a suitable cation exchanger was selected. Hi-Trap SP columns have high binding capacities, and can be loaded at relatively high flowrates. Binding of the protein during sample application is dependent on the charge of the protein. At a pH of 6.5 the protein was sufficiently charged to bind to the matrix. No breakthrough of protein was observed on the chromatogram. There was, however, a constant elution of a protein (M_r 33 000) during loading, showing a percentage of the u-PA applied was unable to bind. When concentrated, the unbound protein showed no activity. It was therefore concluded that the cation exchanger has the ability to distinguish between correctly and incorrectly folded u-PA. This is supported by the order of magnitude increase in the specific activity of u-PA, showing that

this purification step had recovered the correctly folded u-PA from the refolding mixture.

5. Conclusions

Although inclusion bodies are seen as a hindrance to the production of recombinant protein, their production is often unavoidable. The physical characteristics of the inclusion body can be utilised to facilitate purification. The coupling of wash steps with centrifugation achieved >90% purity of the u-PA inclusion bodies. Renaturation of the denatured u-PA was enhanced by the use of size-exclusion chromatography, which is rapidly becoming an alternative to refolding by dilution. A number of proteins have been refolded successfully using this technique, although the effects of process parameters are not fully understood. This paper determined that the gel fractionation range affects protein recovery, and that the fractionation range should be sufficient to include the effective molecular mass of the unfolded protein. Finally it was shown that activation is not necessary for the final purification of refolded u-PA. Ion-exchange matrices can successfully discriminate between the mis-folded and correctly folded u-PA.

Acknowledgements

This work is supported by the EPRSC and Pfizer Ltd.

References

[1] F. Marston, Biochem. J. 240 (1986) 1.
[2] R. Rudolph, H. Lilie, FASEB J. 10 (1996) 49.
[3] D.R. Thatcher, A. Hitchcock, in: R.H. Pain (Ed.), Mechanisms of Protein Folding, IRL Press, New York, 1994.
[4] M.E. Goldberg, R. Rudolph, R. Jaenicke, Biochemistry 30 (1990) 2790.
[5] B. Batas, J.B. Chaudhuri, Biotechnol. Bioeng. 50 (1996) 16.
[6] R.L. Shapiro, J.G. Duquette, D.F. Roses, I. Nunes, M.N. Harris, H. Kamino, E.L. Wilson, D.B. Rifkin, Cancer Res. 56 (1996) 3597.
[7] U. Nowak, C. Dobson, Biochemistry 33 (1994) 2951.
[8] G.J. Steffens, W.A. Gunzler, F. Otting, E. Frankus, L. Flohe, Hoppe-Seylers Z. Physiol. Chem. 363 (1982) 1043.
[9] M. Winkler, M. Blaber, G.L. Bennett, W. Holmes, G. Vehar, Bio/Technology 3 (1985) 990.
[10] M.M. Bradford, Anal. Biochem. 72 (1975) 241.
[11] G. Kopitar, M. Dolinar, B. Strukelj, J. Pungercar, V. Turk, Eur. J. Biochem. 236 (1996) 558.
[12] M. Winkler, M. Blaber, Biochemistry 25 (1986).
[13] J. Maachupalli-Reddy, B.D. Kelly, E. De Bernardez Clark, Biotechnol. Prog. 13 (1997) 144.
[14] M.A. Shire, L. Bock, J. Ogez, S. Builder, D. Klied, D.M. Moore, Biochemistry 23 (1984) 6474.
[15] J. Buchner, R. Rudolph, Biotechnology 9 (1991) 157.
[16] S.M. Bailey, M.M. Meagher, Biotechnol. Bioeng. 56 (1997) 304.
[17] P. Ramage, Downstream, Amersham Pharmacia Biotech AB, 1998.
[18] T. Kleine, S. Bartsch, B.J.S. Schnier, M. Valentin, T. Gote, H. Tschesche, Biochemistry 32 (1993) 14125.
[19] B. Fischer, I. Sumner, P. Goodenough, Arzeim. Forschung. 12 (1992) 1512.
[20] R.P. Dempster, C.M. Robinson, G.B.L. Harrison, Parasitol. Res. 82 (1996) 291.
[21] B. Schmid, M. Kromer, G. Schulz, FEBS Lett. 381 (1996) 111.
[22] M. Belew, Y. Zhou, S. Wang, L. Nystrom, J. Janson, J. Chromatogr. A 679 (1994) 67.
[23] J. Suttnar, J. Dyr, E. Hamsikova, J. Novak, V. Vonka, J. Chromatogr. B 656 (1994) 123.
[24] H.H. Wong, B.K. O'Neill, A.P.J. Middelberg, Biotechnol. Bioeng. 55 (1997) 556.
[25] U.C. Rinus, T. Boone, J.E. Bailey, J. Biotechnol. 28 (1993) 313.
[26] R. Hlodan, S. Craig, R.H. Pain, Biotech. Genet. Eng. 9 (1991) 47.
[27] B. Batas, C. Schiraldi, J.B. Chaudhuri, J. Biotechnol. 68 (1999) 149.
[28] P. Valax, G. Georgiou, in: G. Georgiou, E. De Bernardez-Clark (Eds.), Protein Refolding, American Chemical Society, Washington DC, 1991.
[29] T.E. Creighton, in: D.L. Oxender (Ed.), UCLA Symposia on Molecular and Cellular Biology, Alan R. Liss, New York, 1986.
[30] Z. Xu, S. Yang, D. Zhu, J. Biochem. 121 (1997) 331.
[31] S.M. West, J.B. Chaudhuri, J.A. Howell, Biotechnol. Bioeng. 57 (1998) 590.
[32] A.J. Hagen, T.A. Hatton, D.I.C. Wang, Biotechnol. Bioeng. 35 (1989) 955.
[33] K. Hamaker, J. Liu, R. Seely, C. Ladisch, M. Ladisch, Biotechnol. Prog. 12 (1996) 184.
[34] R.H. Khan, K.B.C. Appa Rao, A.N.S. Eshwari, S.M. Totey, A.K. Panda, Biotechnol. Prog. 14 (1998) 722.
[35] M. Gauthier, Anal. Biochem. 248 (1997) 228.
[36] M. Potshka, J. Chromatogr. 648 (1993) 41.
[37] V. Uversky, Biochemistry 32 (1993) 13288.