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Refolding of Low Molecular Weight Urokinase Plasminogen Activator by Dilution and Size Exclusion Chromatography—A Comparative Study

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Refolding of Low Molecular Weight Urokinase Plasminogen Activator by Dilution and Size Exclusion Chromatography—A Comparative Study

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ABSTRACT

Overexpression of the serine protease domain of urokinase plasminogen activator (u-PA) in *Escherichia coli* (BL-21) results in the production of inclusion bodies. Batch dilution refolding of the u-PA fragment was investigated. The effect of denaturant concentration, redox potential, pH, and temperature on the recovery of u-PA activity was determined. It was found that u-PA is very susceptible to aggregation and therefore required a high concentration of urea (3 M) in the refolding buffer. It has recently been established that size exclusion chromatography can perform the buffer exchange to initiate protein refolding while minimizing aggregation. Using the best refolding buffer (3 M urea, 50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.5 mM reduced glutathione, 0.5 mM oxidized glutathione) determined from batch dilution refolding, the effects of sample concentration, sample volume, and flow rate were investigated. It was found that size exclusion refolding is particularly sensitive to volume of denatured sample applied to the column. Finally, a comparison between batch dilution and size exclusion refolding established that size exclusion refolding resulted in a higher recovery of u-PA activity, below batch dilution factors of 40.

Key Words. Urokinase plasminogen activator; Inclusion body; Refolding; Size exclusion chromatography

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INTRODUCTION

Recombinant proteins are routinely expressed in host cells, such as *Escherichia coli*. However, over expression often results in the production of inclusion bodies. These are insoluble masses of biologically inactive protein found in the cell cytosol or periplasm (1). Inclusion bodies have been found in all host-vector systems, and their physical properties have been studied in detail (2, 3). They are produced due to several factors: the host cell lacks the necessary posttranslational enzymes, the conformation of the recombinant protein may not be adapted to conditions prevailing in the host cell cytosol, or the protein is overexpressed at a rate where the physiological solubility limit of the protein is exceeded (4). There is no simple relationship between the formation of inclusion bodies and any one factor, genetic, physiological, or chemical.

Activity is recovered through purification of the inclusion bodies, dissolution in a strong denaturant, and refolding by the controlled removal of the denaturant (5). Currently, batch dilution is the preferred method for refolding most recombinant proteins due to its simplicity. It can, however, be a very inefficient process, producing large volumes of dilute protein, which increases the cost of the subsequent downstream processing. Protein recoveries above 5% are often considered adequate on an industrial scale. The majority of protein is lost through nonspecific hydrophobic interactions between folding intermediates, resulting in insoluble aggregates of inactive protein (6). The final protein concentration has been identified as the major limiting factor in batch refolding (7). Various experimental strategies have been employed to reduce aggregation at high protein concentrations: the use of polyethylene glycol to stabilize refolding intermediates (8), binding the unfolded protein to a strong anion-exchange resin (9), and passage through a column of immobilized molecular chaperones (10). Such techniques have been developed using a single model protein, and the high refolding efficiency is often protein specific. Batch dilution remains the only generic method to refold recombinant proteins.

It has recently been reported that size exclusion chromatography (SEC) can perform the buffer exchange necessary to initiate protein refolding while separating folding intermediates. Refolding of lysozyme from a starting concentration of up to 80 mg/mL resulted in a 46% recovery of fully active lysozyme (11). However, the effects of all parameters for this process have not been fully identified. In this study we compare the refolding of the serine protease domain of human urokinase plasminogen activator (u-PA) by both batch dilution and size exclusion chromatography.

Urokinase plasminogen activator is recognized as an important pharmaceutical target. There is experimental evidence that suggests u-PA may play an important role in tumor biology (12). u-PA (E.C. 3.4.21.73) is a 45 kDa multidomain glycoprotein which contains 411 residues and 12 disulfide bonds

(13). Figure 1 shows the expression of HMW u-PA and activation to the two chain form. u-PA is synthesized as a single-chain glycoprotein (HMW u-PA) which possesses a very low amidolytic activity. HMW u-PA is converted to the highly active two-chain u-PA by cleavage of two peptide bonds located at Lys135–Lys136 and Lys 158–IIe 159. Cleavage is catalyzed by plasmin in a positive feedback mechanism. The resulting polypeptide consists of amino acids 136–158 joined by a disulfide bridge at Cys-148 and Cys 279 to amino acids 159–411. This 33 kDa fragment is termed low molecular weight u-PA (LMW u-PA), which contains six disulfide bonds (14). When overexpressed in *E. coli*, unactivated, single chain LMW u-PA (amino acids 136–411) readily produces inclusion bodies.

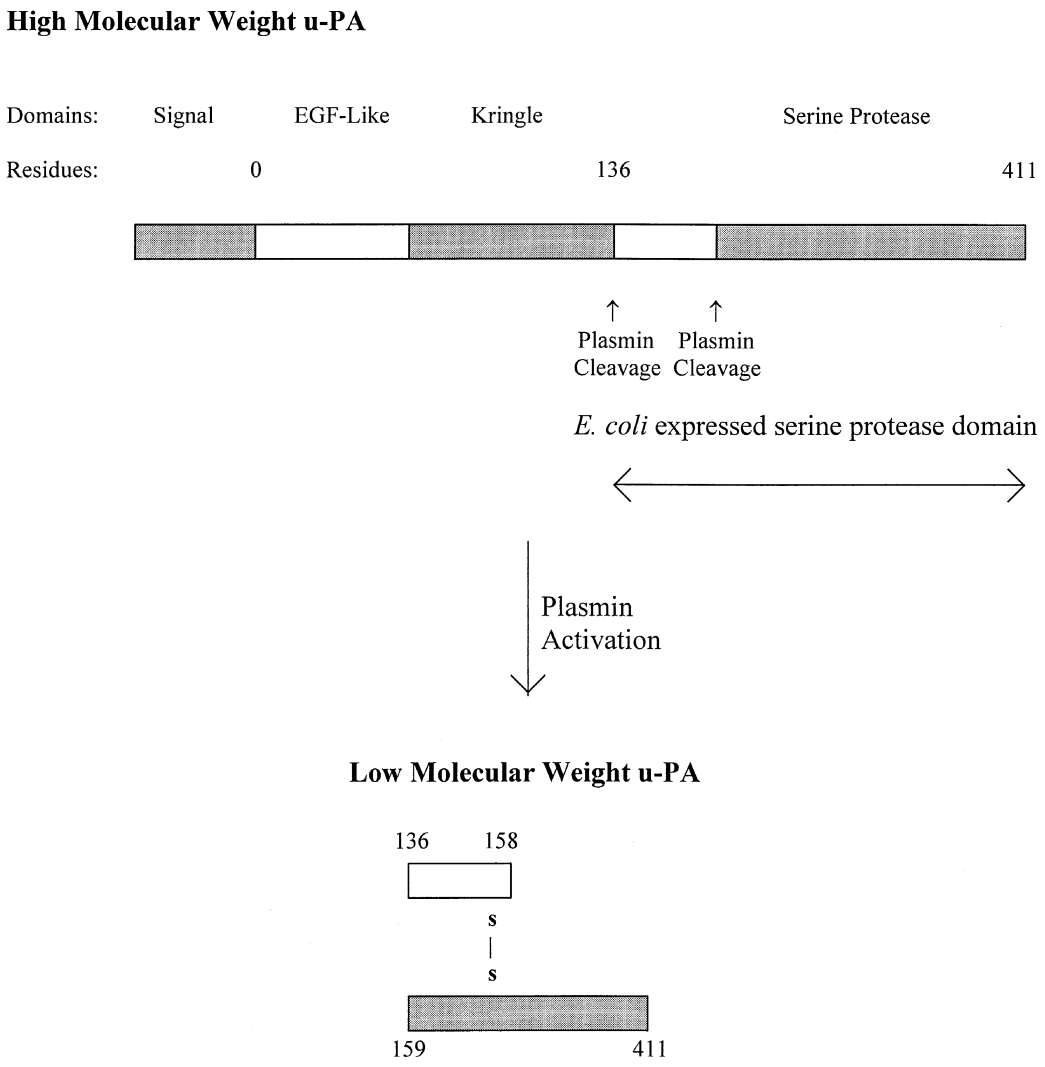

FIG. 1 Schematic diagram of u-PA expression and activation to the two chain low molecular weight form.

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MATERIALS AND METHODS

Materials

Bis-Tris-propane, β-mercaptoethanol, (βME), dithiothreitol (DTT), EDTA, reduced glutathione (GSH), oxidized glutathione (GSSG), guanidine hydrochloride (GuHCl), plasmin, sucrose, Trizma-base, and urea were all purchased from Sigma (Poole, Dorset, UK). u-PA substrate, Chromozym U, was purchased from Boehringer Mannheim (Lewes, East Sussex, UK). Electrophoresis gels, buffers, and standards were purchased from Novex (San Diego, California, USA). Water to 18 MΩ quality was obtained using a Prima and Maxima system, ELGA (High Wycombe, Buckinghamshire, UK). Size exclusion media and columns were purchased from Amersham Phamacia Biotech (Amersham, Bucks, UK). Chromatographic separations were performed using a Biologic Workstation (Biorad, Hemel Hempstead, Hertfordshire, UK). *E. coli* (BL-21) cell paste containing the overexpressed serine protease domain of u-PA (amino acids 136–411) was kindly provided by Pfizer Central Research (Sandwich, Kent, UK).

Methods

All centrifugations were performed in 250 mL Sorvall centrifuge tubes using a superlite GSA rotor in a Sorvall 5C centrifuge. Each inclusion body pellet was resuspended in the wash buffer using a benchtop homogenizer (IKA-Ultraturrax, Laborteknik, Fisons, Loughborough, UK) at 18,000 rpm, and was stirred at room temperature (22°C) for 2 hours (Triton X-100) or 1 hour (urea).

u-PA Inclusion Body Isolation

50 g of *E. coli* cell paste was resuspended in 11 of lysis buffer (Table 1), and cells lysed by six passes through a high pressure homogenizer (APV Manton

TABLE 1
List of Buffers

Lysis buffer	50 mM Tris-HCl pH 7.5, 5 mM EDTA
Detergent buffer	1% Triton X-100, 25% sucrose, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA
1st urea buffer	0.5 M urea, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA
2nd urea buffer	4 M urea, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA
Denaturing buffer	6 M Gu-HCl, 5 mM DTT, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA
Refolding buffer	3 M urea, 50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.5 mM GSH, 0.5 mM GSSG

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Gaulin) at 41 MPa. The lysate was centrifuged at 10,400*g* for 10 minutes, and the inclusion body pellet was resuspended in a detergent wash buffer (Table 1). Inclusion bodies were repelleted by a centrifugation at 10,400*g* for 60 minutes and resuspended in the first urea wash buffer (Table 1). The suspension was centrifuged at 10,400*g* for 10 minutes, and the pellet was resuspended in the second urea wash buffer (Table 1). The suspension was centrifuged at 10,400*g* for 10 minutes, and the purified inclusion body pellet was dissolved in the denaturing buffer (Table 1), 1 g wet weight of pellet in 15 mL denaturing buffer. The denatured solution was mixed (10 hours) on a rotary mixer at room temperature. Any insoluble particles were removed by centrifugation at 10,400*g* for 60 minutes, followed by filtration (0.22 μM).

Batch Dilution Refolding of Solubilized u-PA

980 μL of refolding buffer (Table 1) was aliquoted into microcentrifuge tubes and equilibrated to the desired temperature (4–37°C). Unfolded u-PA (20 μL at 8 mg/mL) was pipetted into the refolding buffer and incubated for 24 hours, at which point refolding was complete. The results shown are an average of four refolding experiments.

Refolding of Solubilized u-PA Using Size Exclusion Chromatography

Size exclusion refolding was performed using a XK26/100 column packed with Sephacryl S-300 gel media 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 refolding buffer (Table 1). 1–16 mL of 2–16 mg/mL of denatured/reduced u-PA was injected onto the column through a static loop and eluted at a flow rate of 0.5–4.5 mL/min. Fractions were analyzed 24 hours following sample injection for u-PA activity.

Analytical Methods

Partition of the inclusion bodies between the supernatant and pellet was determined by SDS-PAGE (4–20% Tris-glycine). SDS-PAGE gel analysis was performed using Quanti-scan software (Biosoft, UK). Protein concentration was determined by absorbance at 280 nm ($\varepsilon^{1\%} = 15.5$) (15) or by Coomassie assay (16). Protein aggregation was measured by sample absorbance at 450 nm. Guanidine elution was monitored by conductivity. Refolded single chain 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 hour. Activated two chain u-PA was then assayed by adding 5 μL of 9 mM Chromozym U and measuring the rate of change of absorbance at 405 nm.

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RESULTS

Purification of u-PA Inclusion Bodies from *E. coli* Cell Lysate

Inclusion bodies are very dense particles which are resistant to high shear forces. Their physical characteristics facilitate their isolation. Figure 2 shows an SDS gel summarizing the u-PA inclusion body purification procedure used here. Centrifugation in the lysis buffer removes the bulk of soluble *E. coli* protein while centrifugation in 25% sucrose removes light insoluble cell debris. The urea wash steps remove *E. coli* membrane proteins which adhere to the inclusion body surface during cell disruption. Analysis of the SDS-PAGE gel revealed a u-PA purity in excess of 90% in the final pellet (results not shown).

Characterization of the Batch Dilution Refolding of Solubilized u-PA

To characterize the refolding of the u-PA fragment, a broad range of solubilizing and refolding conditions was investigated. Urea and guanidine are commonly used to solubilize and unfold proteins. Solubilization in 8 M urea resulted in a maximum protein concentration of 3–4 mg/mL, with the remain-

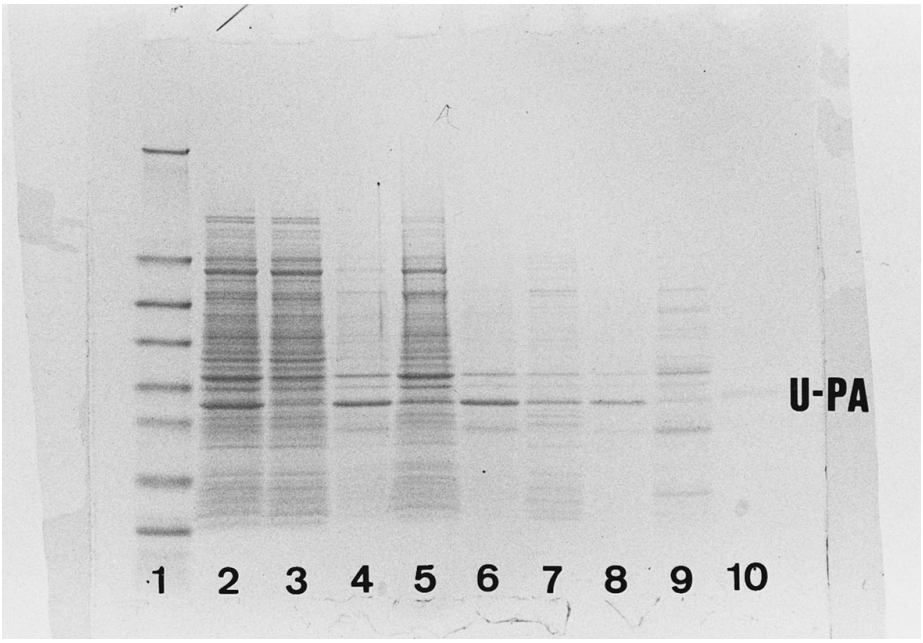

FIG. 2 SDS gel showing the purification of u-PA inclusion bodies. Lane 1, protein standards; 2, cell lysate; 3, supernatant in lysis buffer; 4, pellet in lysis buffer; 5, supernatant in detergent buffer; 6, pellet in detergent buffer; 7, supernatant in 0.5 M urea; 8, pellet in 0.5 M urea; 9, supernatant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

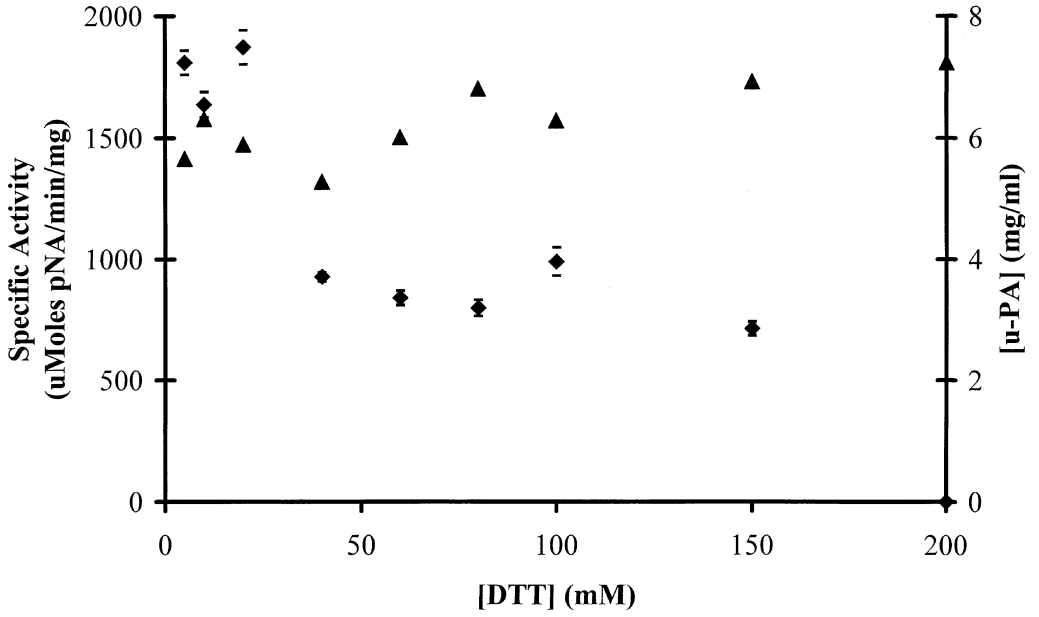

FIG. 3 Effect of DTT concentration on the u-PA solubility (▲) and recovery of u-PA activity (◆).

der of u-PA left as a visible insoluble mass in the denaturing buffer. Solubilization in 6 M guanidine increased the protein concentration to 4.7–6.5 mg/mL, while disruption of the inclusion body pellet in the denaturing buffer (Table 1), using high speed homogenization, increased the u-PA concentration to 14–16 mg/mL.

Reducing agents, such as DTT and β-ME, are required to fully solubilize inclusion bodies by reducing all disulfide bonds. Figure 3 shows the effect of DTT concentration in the denaturing buffer (Table 1) on the solubilization of the inclusion bodies and the subsequent recovery of u-PA activity. Increasing the DTT concentration had little effect on the solubility of u-PA. Solubility was slightly enhanced above 60 mM DTT, although all samples reached a concentration of 5.6 ± 0.9 mg/mL. However, an increased DTT concentration had a marked effect on the subsequent refolding of u-PA. Increasing the DTT concentration resulted in a reduced recovery of u-PA activity, with concentrations above 20 mM resulting in a 50% loss in recovered u-PA activity.

It has been demonstrated that moderate concentrations of denaturant in the refolding buffer can improve refolding yield. We therefore investigated the effect of urea and guanidine in the refolding buffer (Table 1). Figure 4 shows the effect of urea concentration on the recovery of u-PA activity. There is an increase in the recovery of u-PA activity from 0 to 3 M urea, with a peak of u-PA activity at 3 M urea. Urea concentrations greater than 3 M show a sharp decrease in recovery of u-PA activity. Absorbance measurements at 450 nm

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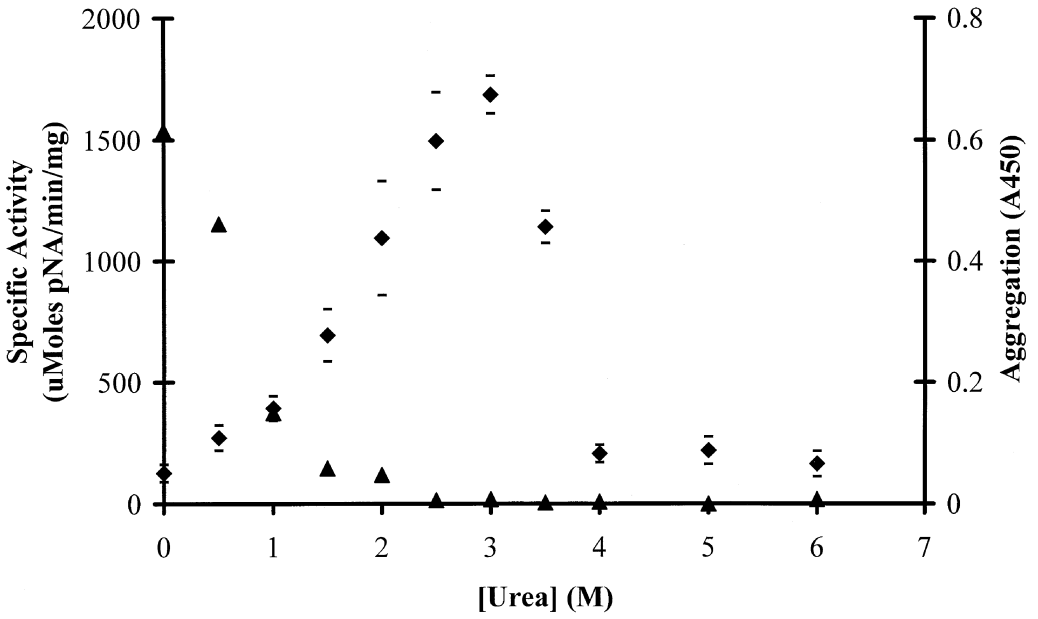

FIG. 4 Effect of urea concentration on the recovery of u-PA activity (◆) and aggregation (▲).

show aggregation predominates at low urea concentrations (0–1 M) and is completely suppressed above 2.5 M urea. Figure 5 shows the effect of guanidine concentration on the recovery of u-PA activity. There is an increase in recovery of u-PA activity from 0 to 1 M guanidine, at which there is a plateau of recovered u-PA activity between 1 and 1.25 M guanidine. Guanidine concentrations above 1.25 M result in a sharp decrease in the recovery of u-PA activity. Aggregation is completely suppressed at a much lower concentration of guanidine (0.75 M) compared to urea (2.5 M). The maximum recovery of u-PA activity in guanidine is 76% of that obtained with urea.

The appropriate redox environment is essential to successfully refold proteins containing disulfide bonds. Figure 6 shows the effect of the glutathione ratio (reduced:oxidized) on the recovery of u-PA activity. Maximum u-PA activity is achieved in a neutral redox environment with a plateau of maximum u-PA activity at 3 equal molar ratios of reduced:oxidized glutathione. Increasing the reducing or oxidizing potential of the refolding buffer resulted in a lower recovery of u-PA activity.

The effects of both pH and temperature on u-PA refolding were investigated (data not shown). The effect of pH on the recovery of u-PA activity was determined by substituting Bis-Tris-propane for the Tris-HCl buffer. Bis-Tris-propane has a large pH buffering range, and the pH of the refolding buffer is easily adjusted by the addition of concentrated HCl or NaOH. There is a peak of u-PA activity at a pH of 8.5, with a broad range of similar activities recovered between a pH of 7.75 and 8.75. The effect of temperature on the recov-

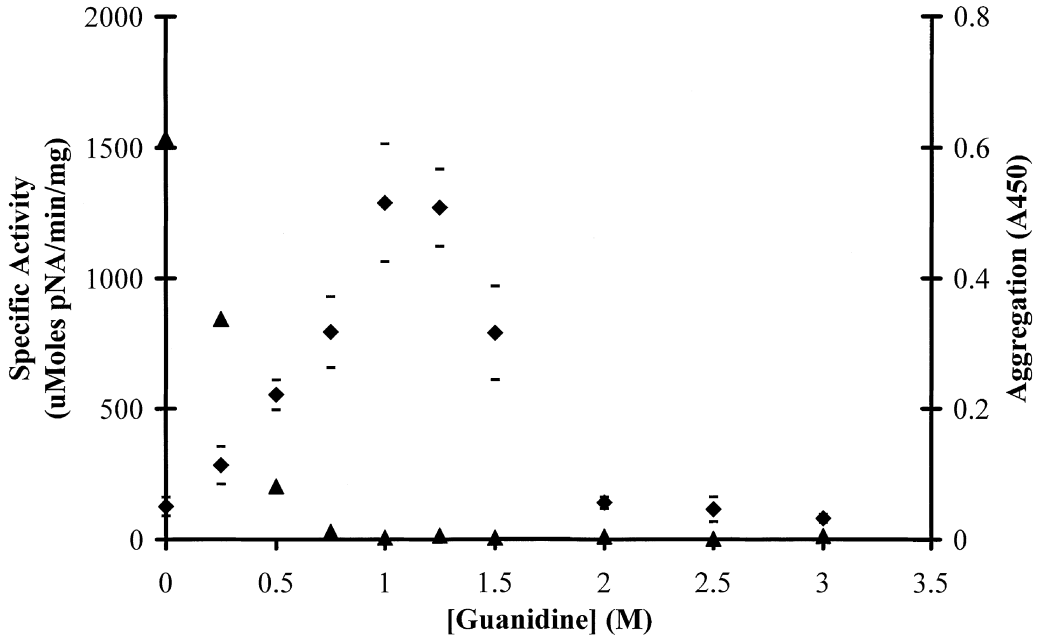

FIG. 5 Effect of guanidine concentration on the recovery of u-PA activity (◆) and aggregation (▲).

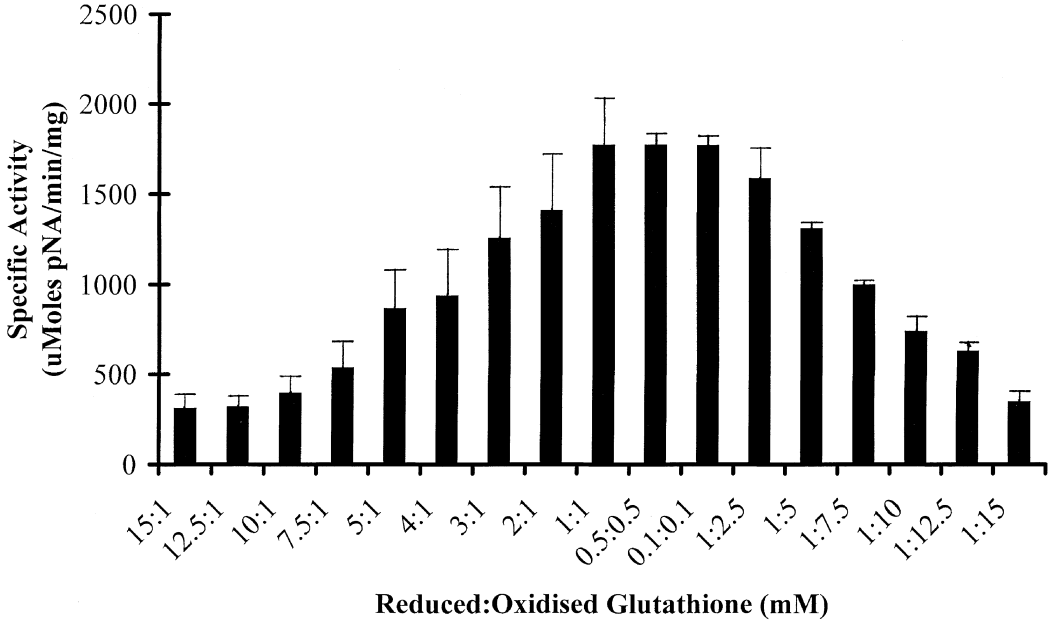

FIG. 6 Effect of glutathione ratio on the recovery of u-PA activity.

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ery of u-PA activity was determined at four temperatures (3.7, 8.6, 22, and 37°C). Recovery of u-PA activity is inversely proportional to the refolding buffer temperature, with a maximum activity achieved at 3.7°C.

Refolding of Solubilized u-PA Using Size Exclusion Chromatography

Characterization of the batch dilution refolding conditions for the u-PA fragment led to a defined mobile phase for the size exclusion refolding. Previous studies have determined that use of Sephacryl S-300 size exclusion media results in the greatest recovery of u-PA activity and the best resolution of aggregates, active u-PA, and denaturant (17). Figure 7 shows the effect of initial protein concentration on the recovery of u-PA activity and the total amount of aggregation. The recovered activities have been normalized to the amount of u-PA loaded onto the column. High initial u-PA concentrations result in an increase in aggregation coupled with a decrease in the recovery of u-PA activity. As the initial u-PA concentration decreases, less aggregation is observed, resulting in an increase in the recovery of u-PA activity.

One of the limitations of size exclusion chromatography is the volume of sample that can be resolved. Sample volume is limited to 1–2% of the column volume for preparative SEC and 0.3% for analytical SEC (18). The effect of increasing the sample volume was investigated to test the limitations of SEC to refold larger quantities of recombinant protein. Figure 8 shows that the to-

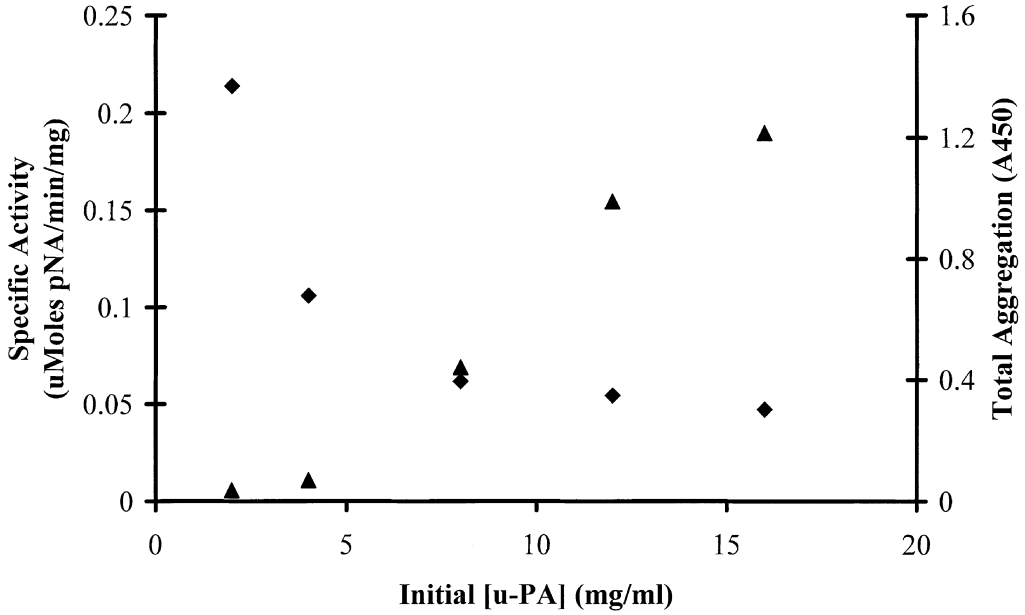

FIG. 7 Effect of initial u-PA concentration on the recovery of u-PA activity (◆) and aggregation (▲) by size exclusion refolding.

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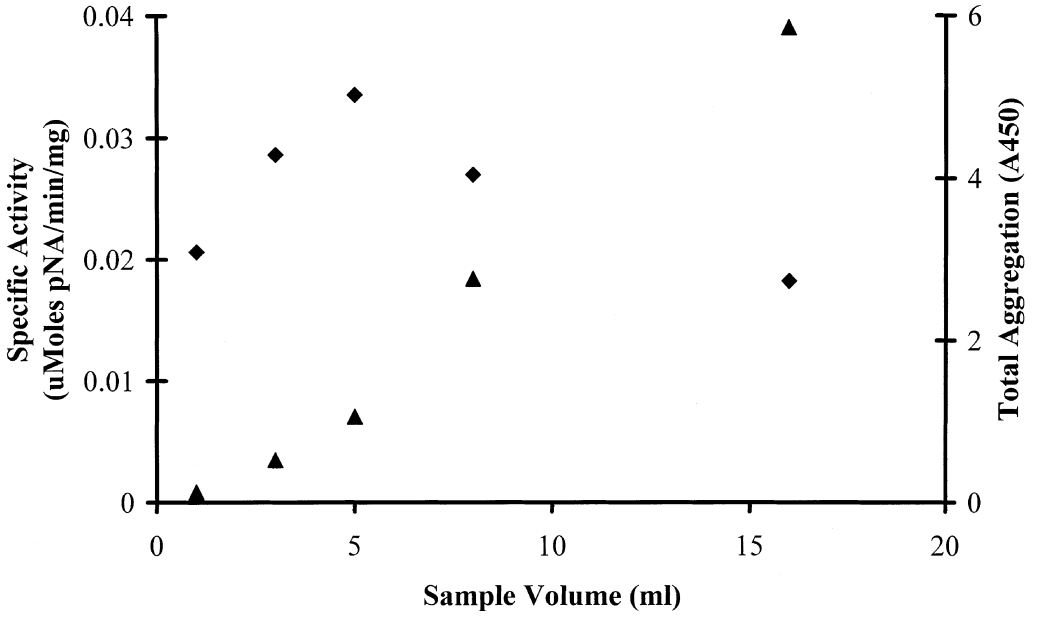

FIG. 8 Effect of sample volume on the recovery of u-PA activity (◆) and aggregation (▲) by size exclusion refolding.

tal amount of aggregation linearly increases with the sample volume. However, the total amount of activity recovered also increases with sample volume (data not shown). If the results are normalized to the mass of u-PA loaded onto the column, a direct comparison of the effect of sample volume is obtained. The normalized data show recovery of u-PA activity increases to a maximum at a sample volume of 5 mL, which is 1.07% of the column volume, with larger sample volumes resulting in a lower recovery of u-PA activity.

Volumetric flow rates were also tested over a range for their potential to refold u-PA by SEC. Figure 9 shows that increasing the volumetric flow rate results in a greater retention of the aggregate peaks on the SEC column. Figure 10 shows the total amount of aggregation decreases as flow rate increases, which is coupled to an increase in the recovery of u-PA activity.

Determination of the Relative Efficiency of Size Exclusion Refolding

To quantify the relative efficiencies of batch dilution and size exclusion refolding, identical u-PA samples were refolded by each technique. Figure 11 shows the relative efficiency of batch dilution compared to size exclusion refolding. The ordinate represents the recovery of u-PA activity by batch dilution refolding compared to that from size exclusion chromatography. The abscissa represents various batch dilution factors. At 100% on the ordinate, batch dilution and size exclusion refolding have the same efficiency, which

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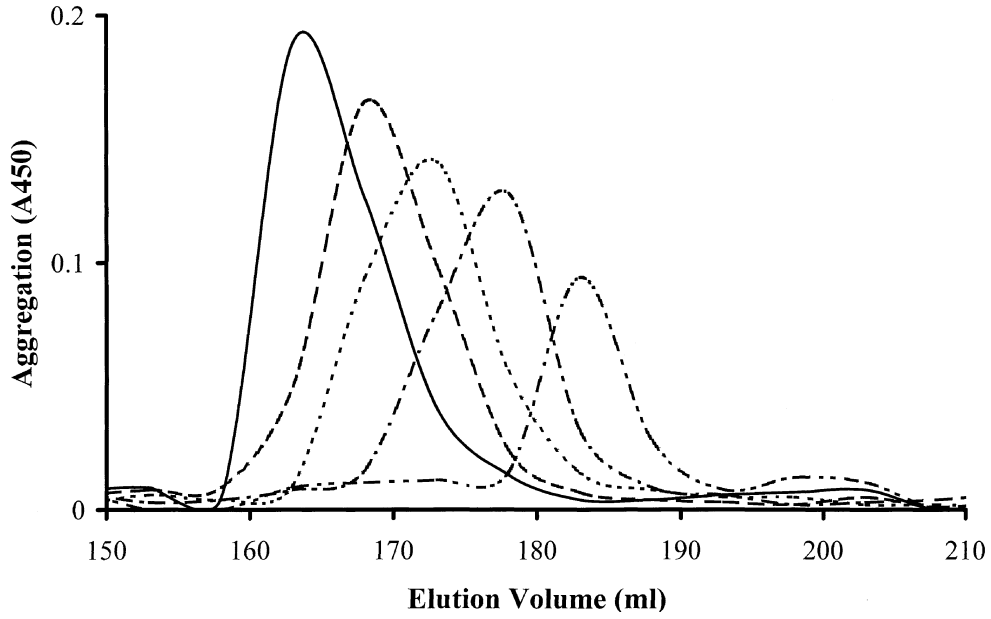

FIG. 9 Effect of volumetric flow rate on the elution of aggregate peaks: 0.5 mL/min (——), 1.5 mL/min (– –) 2.5 mL/min (- - -), 3.5 mL/min (– - –), 4.5 mL/min (– - - –).

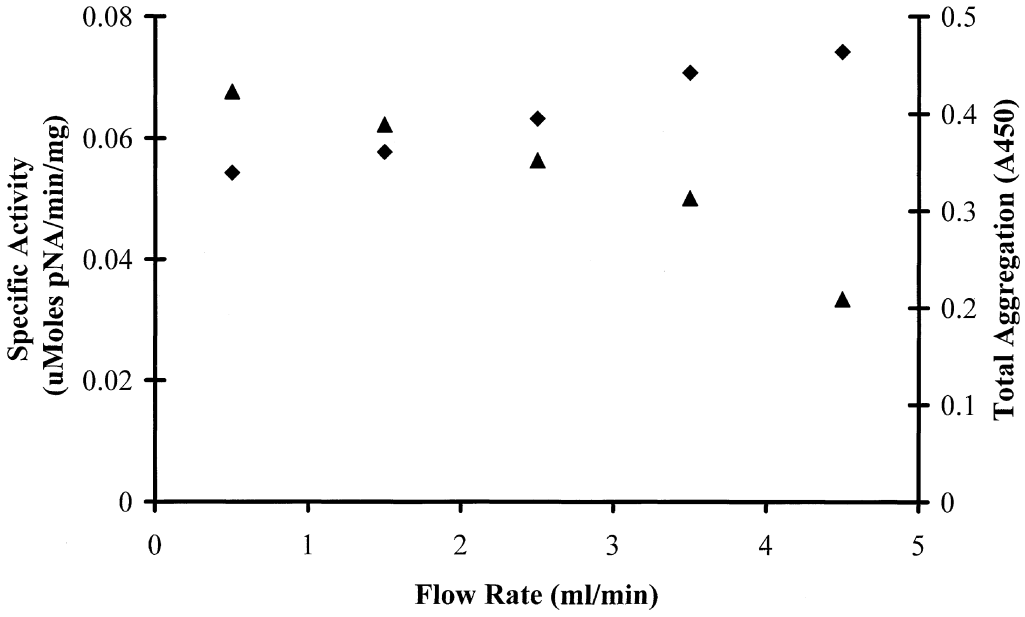

FIG. 10 Effect of mobile phase flow rate on the recovery of u-PA activity (◆) and aggregation (▲) by size exclusion refolding.

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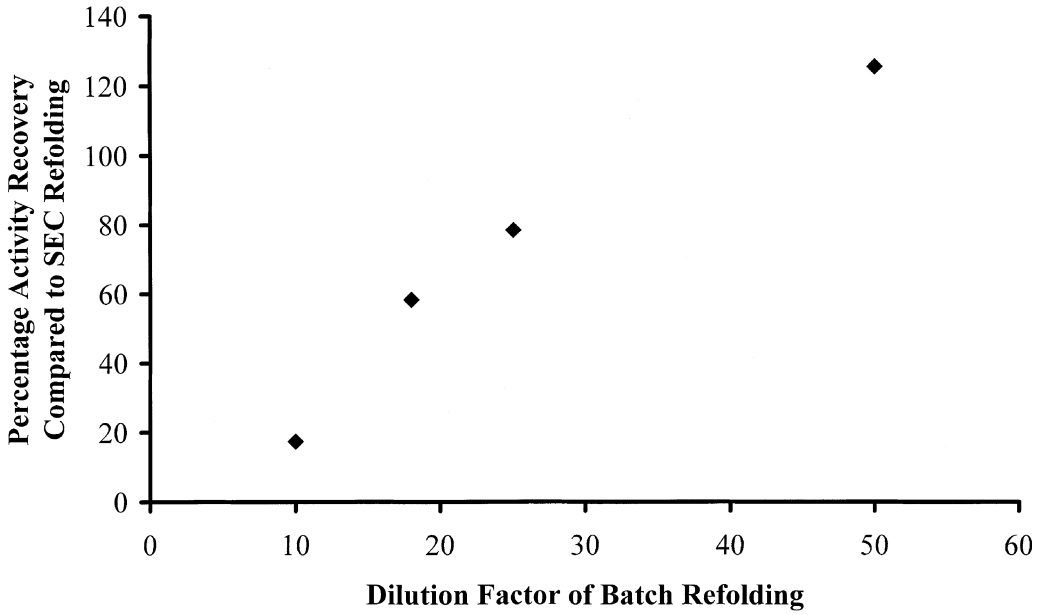

FIG. 11 Comparison of the relative efficiencies of size exclusion and batch dilution refolding.

corresponds to a dilution factor of 40. Thus, size exclusion refolding is more efficient than batch refolding at dilution factors less than 40. Dilution on the SEC column is calculated through the elution of guanidine. Typical dilution factors for a 90-cm column packed with 500 mL of settled gel are between 18 and 20.

DISCUSSION

It has been shown that HMW u-PA is very difficult to refold by dilution (19). The denaturing and reducing conditions required for protein solubilization are usually overlooked in refolding studies. High concentrations of guanidine or urea are arbitrarily used to unfold proteins, though it has recently been established that the recovery of lysozyme activity is heavily dependant on the denaturant used (20). Urea is a weak chaotropic agent, and a concentration of 8 M was unable to completely denature all of the u-PA inclusion bodies present. Guanidine was therefore used to fully denature the u-PA inclusion bodies. It is thought that homogenizing the u-PA inclusion body pellet in the denaturing buffer increases solubility by increasing the surface area open to guanidine denaturation and prevents particles from "gelling" to an insoluble mass, which was observed during urea denaturation.

HMW u-PA inclusion bodies are partially linked by intermolecular disulfide bonds (19). An excess of reducing agent is commonly used to fully reduce all disulfide bonds. Lysozyme is solubilized in a buffer containing 10 M urea

and 150 mM DTT without any adverse effects on the recovery of active protein (21). DTT concentrations above 5 mM did not significantly enhance the solubility of the u-PA inclusion bodies. The small increase in solubility is attributed to the interaction of DTT with the Coomassie dye. However, high concentrations of DTT in the denaturation buffer did have a marked effect on the recovery of u-PA activity. Higher concentrations of β-ME (50 mM) have been previously used to unfold HMW u-PA (22). However, the excess reducing agent was removed by dialysis prior to refolding, which is costly and time consuming on an industrial scale. The optimal concentration of reducing agent in the denaturing buffer should be determined in conjunction with the glutathione ratio in the refolding buffer, because an excess of DTT or β-ME will convert oxidized glutathione to the reduced form according to Eq. (1).

$$\text{DTT (red)} + \text{GSSG} \rightarrow \text{DTT (ox)} + 2\ \text{GSH} \quad (1)$$

shifting the overall redox potential in the refolding buffer. Maximum recovery of u-PA activity is obtained between 5 to 20 mM DTT, which suggests the refolding of u-PA is very sensitive to a reducing redox potential.

The final denaturant concentration is arguably the most important variable in the refolding buffer. Relatively high final concentrations of urea or guanidine are routinely included to suppress aggregation (23, 24). In the absence of denaturant, aggregation is clearly visible, indicating the majority of u-PA is in a misfolded form. Aggregation is totally suppressed at 3 M urea and 1 M guanidine without affecting the recovery of u-PA activity. At higher denaturant concentrations the unfolded protein cannot fold to the native state, which allows stable folding intermediates to predominate, reducing the recovery of u-PA activity.

The effect of cysteine on the refolding of lysozyme was first studied in 1967 (25), where it was established that proteins folded via oxido-shuffling of their disulfide bonds. Refolding is a net oxidative process, but the highest refolding yields are often obtained under reducing conditions, with ratios of 10:1 and 5:1 of reduced:oxidized glutathione being common (21, 26). This is linked to in vivo folding in the cytosol, which occurs under reducing conditions. Glutathione has been found in cell cytosols at millimolar concentrations in ratios of 20:1 and 100:1 reduced:oxidized (27). It has been hypothesized that a reducing system will allow the breakage of incorrectly formed disulfides but will not have sufficient reducing potential to reduce correctly formed disulfides. The results show that increasing the reducing or oxidizing potential of the refolding buffer has an adverse effect on the recovery of u-PA activity. This is in agreement with previous results which established an excess of DTT reduced the recovery of u-PA activity. Even though maximum recovery of u-PA activity is obtained under neutral redox conditions, the refolding buffer will have a net reducing potential due to the residual concentration of DTT

(0.1 mM), which converts oxidized glutathione to the reduced form according to Eq. (1).

pH affects the solubility of native proteins and is known to affect the rate and yield of protein refolding. Below a pH of 7 it is likely that the thiol groups on the cysteine residues are not sufficiently ionized to allow the formation of disulfide bonds (28). At higher pH values the refolding buffer will have a pH close to the isoelectric point of the protein, decreasing its solubility. Most proteins have a broad range of pH values, where pH has little effect on the refolding yield. Maximum refolding yield is usually achieved between pH 7 to 9. It is therefore not surprising that the highest recovery of u-PA is obtained between a pH of 7.75 and 8.75, with a maximum recovery of u-PA activity at a pH of 8.5. Under alkaline conditions oxido-shuffling of disulfide bonds via the disulfide exchange system will be at an optimum (29). This result corresponds well to previous data published for HMW u-PA (15, 19).

The refolding buffer temperature is crucial for the suppression of aggregation (23). The fact that u-PA requires a high concentration of denaturant in the refolding buffer established that u-PA is very susceptible to aggregation. Recovery of u-PA activity is inversely proportional to the temperature of the refolding buffer. Aggregation predominates at elevated temperatures, resulting in a decrease in the recovery of u-PA activity.

Size exclusion chromatography is a well-established method for isolating biological compounds due to their physical characteristics (18). The gel media separates solutes by partitioning solutes according to their Stokes radius. Size exclusion chromatography is generally used as a polishing technique within a purification protocol because of its low capacity. However, it is often used to perform buffer exchange as an alternative to tangential flow filtration (30). Its ability to perform the buffer exchange has been utilized to refold a variety of recombinant proteins: lysozyme (11), RNase (31), and leukocyte protease inhibitor (32).

Unfolded proteins exhibit a random coil with little secondary or tertiary structure, and have a Stokes radius in excess of the native (33). Therefore, a size exclusion column calibrated with native proteins will elute an unfolded protein in a smaller volume compared to its native form. Thus the unfolded protein will appear to have a larger effective molecular weight when compared to the native protein standards. Unfolded u-PA has a Stokes radius of 5.3 nm, calculated from Stokes radius–molecular weight plots of unfolded proteins (34), which corresponds to an effective molecular weight of 231 kDa using Stokes radius–molecular weight plots of native proteins (35). Denatured u-PA will therefore have limited access to the pores within the Sephacryl S-300 gel matrix, which has a fractionation range of 20 to 1500 kDa. Guanidine, being a low molecular weight salt, will have full access to the network of pores within the gel matrix. Following sample application, separation of the two

species will occur, and the guanidine concentration will decrease, initiating the refolding of u-PA. Separation of u-PA and guanidine is a gradual process, and the unfolded u-PA will experience a linearly decreasing concentration of guanidine. At a guanidine concentration specific for u-PA, the protein structure collapses to a molten globule state. Aggregation is most likely to occur at this point due to exposure of hydrophobic regions on the polypeptide chain. However, the collapse of the protein structure involves a large reduction in the Stokes radius, which increases the partition coefficient of the u-PA between the mobile and stationary phases. It has been proposed that the partitioning of folding intermediates is responsible for lowering the probability of aggregation, maximizing the recovery of active protein (11). Any aggregates formed during sample application or during refolding will be separated from the refolded protein due to their larger Stokes radius. A typical elution profile will consist of two protein peaks, aggregated and active, followed by the denaturant peak. Ideally, the resolution of the three peaks should be maximized to achieve a baseline separation.

Even though this technique has been successfully demonstrated with lysozyme and carbonic anhydrase, the effect of all process parameters has yet to be fully elucidated, though sample application is known to be crucial (11). The denatured u-PA sample is applied through a static loop, designed for standard protein separations. Mixing of the denatured u-PA and refolding buffer will occur at the point of sample application within the dead volume of the column adapter. u-PA has been shown to be very susceptible to aggregation, and mixing prior to the column will result in aggregation. To determine the effect of sample application, three process variables were investigated.

The initial and final protein concentrations are major limiting factors to the recovery of active protein during batch dilution refolding (7). Elution of a specific concentration of refolded protein cannot be achieved using size exclusion chromatography. Therefore, the effect of initial protein concentration on the recovery of u-PA activity was investigated. The results established that the recovery of u-PA activity is indirectly proportional to the initial protein concentration, which is in agreement with the batch dilution refolding (21). At high u-PA concentrations there is an increased probability of collision between folding intermediates at the point of sample application and during denaturant removal, resulting in an increase in aggregation and a decrease in the recovery of u-PA activity.

To further investigate the effect of sample application, increased sample volumes were applied to the SEC column. It was hypothesized that as sample volume is increased, there would be an increased shielding effect for the bulk of denatured u-PA entering the column adapter. This theory is partly proven by the results which established the recovery of u-PA activity is directly proportional to sample size, to a volume of 5 mL. Sample volumes above 5 mL

resulted in a lower recovery of u-PA activity. The increased amount of aggregation in sample volumes above 5 mL is probably a result of prolonged exposure to intermediate concentrations of denaturant due to the increased sample volume.

To increase the dispersion at the point of sample application, a range of volumetric flows was tested. Higher flow rates increased the recovery of u-PA activity and decreased the total amount of aggregation. However, the mechanism for this increase in recovery of u-PA activity is not known. The higher flow rate introduces a number of effects: an increase in the dispersion at sample application, a decrease in the number of theoretical plates in the column, and an increased rate of denaturant removal during passage through the column. Further experiments are required to separate and quantify these effects.

Comparison of refolding by batch dilution and size exclusion determined that size exclusion refolding results in a greater recovery of u-PA activity when compared to batch refolding below dilution factors of 40. HMW u-PA has been successfully refolded using dilution factors between 20 and 30 (19). It was therefore concluded that size exclusion refolding of u-PA offers an increase in process efficiency compared to batch dilution refolding.

CONCLUSIONS

u-PA does not readily refold using batch dilution refolding. Solute and physical conditions can be characterized to enhance recovery. Misfolded u-PA predominates, and recoveries of 10 to 15% active u-PA are typical.

u-PA can be refolded using size exclusion chromatography without prior experimental experience. However, for this technique to become a generic refolding tool, all process parameters have to be characterized. Sample concentration, sample volume, and volumetric flow rate effect the recovery of u-PA activity. Future work will be directed toward developing new injection protocols to limit aggregation and enhance the recovery of activity.

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