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Continuous matrix-assisted refolding of proteins

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Abstract

A refolding reactor was developed for continuous matrix-assisted refolding of proteins. The reactor was composed of an annular chromatography system and an ultrafiltration system to recycle aggregated proteins produced during the refolding reaction. The feed solution containing the denatured protein was continuously fed to the rotating bed perfused with buffer promoting folding of the protein. As the protein passed through the column, it was separated from chaotropic and reducing agents and the refolding process took place. Native proteins and aggregates could be continuously separated due to different molecular size. The exit stream containing aggregates was collected, concentrated by ultrafiltration and recycled to the feed solution. The high concentrations of chaotropic and reducing agents in the feed solution enabled dissociation of the recycled aggregates and consequently were fed again to the refolding reactor. When the initial feed mixture of denatured protein is used up, only buffer-containing chaotropic agents and recycled aggregates are fully converted to native protein. This process resulted in a stoichiometric conversion from the denatured protein to its correctly folded native state. The system was tested with bovine α-lactalbumin as model protein. Superdex 75 PrepGrade was used as size-exclusion medium. The yield of 30% active monomer in the batch process was improved to 41% at a recycling rate of 65%. Assuming that the aggregates can be redissolved and recycled into the feed stream in a quantitative manner, a refolding yield close to 100% is possible. The method can be also applied to other chromatographic principles suited for the separation of aggregates.

Keywords: Annular chromatography; Matrix-assisted refolding; Proteins

1. Introduction

Escherichia coli is known as a common host cell organism for the production of recombinant proteins. When a heterologous protein is overexpressed, the production of the nascent polypeptide occurs with a faster kinetic then folding of the protein resulting in the formation of protein aggregates which are deposited in the cytoplasm of the bacterial cell as inclusion bodies, also called refractile bodies [1–3]. The general strategy for recovery of active protein from inclusion bodies involves cell lysis, extraction and washing of inclusion bodies, solubilization of inclusion bodies and refolding into the native conformation of the protein [4,5]. After dissolution of inclusion bodies in a buffer containing strong chaotropic agents, such as 8 *M* urea or 6 *M* guanidine hydrochloride, reducing agents such as dithiothreitol or β-mercaptoethanol are added to reduce all disulfide bonds. Then the denatured protein is transferred into a non-denaturating environment to shift the folding equilibrium towards its native conformation. This is normally achieved by dilution or dialysis [6]. Aggregate formation follows a reaction kinetic of

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second or higher order, while refolding of the protein follows first order reaction kinetic [7]. To prevent aggregation, refolding is usually performed at low protein concentrations within a range of 10 to 100 μg/ml [8,9]. Therefore refolding of recombinant proteins expressed in *E. coli* is still a bottleneck at the industrial scale, often requiring additional large refolding tanks. Such a refolding process has low productivity and the yield of native protein is usually low.

In other approaches, the in vivo folding pathway was emulated by adding chaperones or molecular mimicries of chaperones (minichaperones) [10,11] and enzymes catalyzing certain steps in the folding process such as disulfide bond formation or *cis–trans* peptide isomerization [12]. These compounds were also immobilized to a solid phase and the folding reaction was performed in a packed bed reactor or stirred tank. While denatured protein passes through the column it is separated form chaotropic agents and starts to refold while aggregation is reduced due to interchanges with the immobilized proteins [13,14]. Folding helper proteins act in a stochiometric manner, requiring almost the same amount of helper proteins as product. Also ATP is an essential component of such a refolding system. For both reasons, this strategy is very costly and currently not applied on an industrial scale.

Size-exclusion chromatography (SEC) comprises an efficient method for refolding with the advantageous features of high initial concentrations of denatured protein and high yield of active protein. The differences in yield of active protein between refolding by dilution and refolding by SEC cannot be simply explained by buffer exchange during the chromatographic separation. It has been suggested that differences in effective diffusivity of unfolded, partly folded, native and aggregated proteins lead to partial separation [15]. Therefore interactions of folding intermediates showing a strong tendency to aggregation, are reduced. This effect and additional gradual removal of denaturant during SEC impedes aggregation [16]. Refolding by SEC has been studied with pure proteins such as lysozyme and carbonic anhydrase with starting concentrations of up to 80 mg/ml [15] as well as urokinase plasminogen activator [17], heterodimeric platelet-derived growth factor [18] and recombinant hen egg white lysozyme expressed in *E. coli* as inclusion bodies [19]. Refolding by SEC represents an option to replace refolding by dilution at industrial scale because it is relatively easy to operate. Linear scale up has been shown [20] for other separation processes but the method is limited by the compressibility of chromatography media.

Irrespective of the method—refolding by dilution or SEC—the processes are not effective enough to convert the unfolded protein quantitatively into the native state. Due to kinetic reasons, a certain fraction is always lost as aggregates. The yield of any refolding process may be improved by running it continuously and recycling the separated aggregates into the feed stream. Neglecting all other losses such as protein degradation, adsorption, etc., a theoretical yield of 100% is achievable. Such a process would significantly reduce the tank size.

Here we describe a continuous refolding system using SEC with additional recycling of the aggregated protein fraction in a pressurized continuous annular chromatography (P-CAC) system. The original concept of P-CAC, as proposed by Martin [21] and realized by Fox and co-workers [22–24], was further developed at the Oak Ridge National Laboratory to operate the system under a certain pressure [25–27]. The company Prior Separation Technology (Götzis, Austria) improved the design and developed a unit that meets the requirements for biotechnology production purposes. The P-CAC system was designed as a closed system, where two concentric cylinders form an annulus, into which the chromatography medium is packed. The annulus rotates with an angular velocity usually ranging from 60 to 600°/h. The feed and eluent(s) are introduced continuously at a stationary entry at the top of the bed. The local separation of the feed solution into single components is caused by the rotation of the sorbent. The separated components appear as helical bands, each of which has a characteristic, stationary exit point. Three factors have an effect on the location of the exit point: (a) eluent velocity, (b) rotation rate, and (c) the distribution coefficient. Several reports were published describing the continuous separation of protein mixtures [28,29].

Bovine α-lactalbumin (α-LA) [30] was used as a model protein for continuous refolding by annular chromatography and recycling of aggregates. The

native protein contains 123 residues (M_r 14 176) and four disulfide bonds. The oxidative folding pathway is well characterized and the protein has an additional calcium-binding site which increases the stability of the native protein [31].

2. Theory

2.1. Refolding kinetics

According to Kiefhaber et al. [32], the kinetic competition between folding and aggregation can be described with a simplified model as shown in Fig. 1. The formation of intermediates (I) from unfolded protein (U) and native protein (N) can be described as first order reaction kinetic ($n=1$), whereas for the formation of aggregated protein (A) a higher order reaction can be considered ($n\geq 2$).

The change of concentration of unfolded protein with time is expressed as:

$$\frac{dU}{dt} = -(k_2 U + k_3 U^n) \quad (1)$$

and the relative amount of native protein as:

$$\frac{dN}{dt} = k_2 U \quad (2)$$

where k_2 is the net rate constant of folding and k_3 the net rate constant of aggregation. Evidence has indicated that the first intermediates resembling the molten globule form within milliseconds [33]. When the formation of intermediates from unfolded protein, U→I, is assumed to be an instantaneous reaction, the refolding kinetic can be simplified by neglecting k_1. U is the dimensionless concentration of unfolding protein, U/U_0, where U_0 is the initial concentration of the denatured protein. N is the dimensionless concentration of refolded protein and n is the reaction order. To solve this differential equation the initial conditions U_0, U at time $t=0$ must be known.

The time–profiles of refolding α-LA can be calculated with the kinetic model represented for $n=2$ by fitting k_2 and k_3 to experimental data. Eq. (1) was analytically solved for a second order reaction as follows:

$$N = \frac{k_2}{U_0 k_3} \cdot \ln\left[1 + \frac{U_0 k_3}{k_2} \cdot \{1 - \exp(-k_2 t)\}\right] \quad (3)$$

The final yield of native protein as time approaches infinity is then given by the following equation:

$$N = \frac{k_2}{U_0 k_3} \cdot \ln\left(1 + \frac{U_0 k_3}{k_2}\right) \quad (4)$$

2.2. Yield, productivity and recycling rate at continuous refolding operation

A schematical drawing of the system used for continuous refolding and recycling of aggregates is shown in Fig. 2. The main components of the experimental setup are the annular chromatograph and an ultrafiltration unit.

The amount of denatured protein after continuous addition of aggregates, c_1F_1, is given by the following mass balance:

$$c_1F_1 = c_0F_0 + c_4F_6 \quad (5)$$

The flow-rate of concentrated aggregates to the feed solution, F_6, is defined as:

$$F_6 = RF_1 \quad (6)$$

where R is the ratio of the recycling stream into the feed solution. The feed flow-rate, F_0, is:

$$F_0 = F_1 - F_6 \quad (7)$$

The refolding yield of native protein after passing through the column and collected in the monomer fraction, Y_1, can be calculated for steady state conditions as follows:

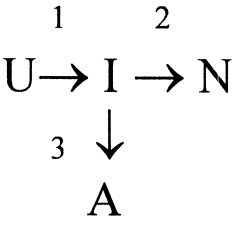

Fig. 1. Simplified protein folding model describing the competition between refolding and aggregation of the protein. U: Unfolded (denatured and reduced) state; I: intermediate state; A: aggregated state; N: native state.

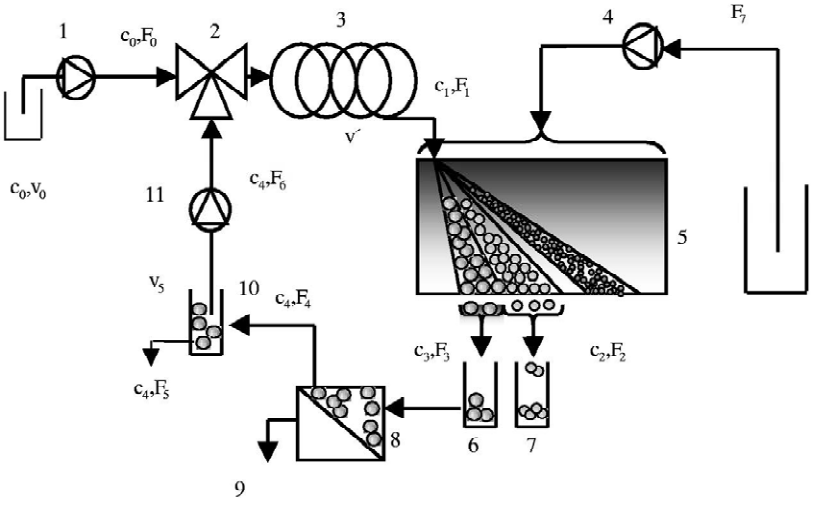

Fig. 2. Experimental setup of continuous refolding by annular chromatography with recycling of aggregates. 1 is the feed pump delivering the reduced and denatured protein; 2 is the mixer for blending of fresh feed with recycled feed after concentration by tangential flow filtration; 3 is the reaction loop to complete reduction of recycled aggregates; 4 is the eluent pump for the annular chromatography system; 5 is the annular chromatography system; 6 is a collecting device consisting of a simple glass bottle for aggregated protein fractions; 7 is the collecting device for monomeric protein fraction; 8 is a tangential flow filtration device; 9 is a permeate outlet; 10 is a vessel for collection of concentrated aggregates and 11 is the recycling pump.

$$Y_1 = \frac{c_2 F_2}{c_1 F_1} \tag{8}$$

Y_1 depends on the initial amount on denatured protein loaded onto the column. Additionally, the efficiency of the system with recycling of aggregates, Y_2, can be described as follows:

$$Y_2 = \frac{c_2 F_2}{c_0 F_0} \tag{9}$$

Y_2 depends on the refolding yield in the column and the recycling rate and increases with recycling of aggregates. The productivity, PR, of the system can be calculated as:

$$\mathrm{PR} = \frac{c_2 F_2}{V_{\mathrm{sp}}} = \frac{c_1 F_1 Y_1}{V_{\mathrm{sp}}} \tag{10}$$

where V_{sp} is the stationary phase volume.

2.3. Continuous separation

All the relevant chromatographic process parameters such as selection of a proper stationary phase, column length and diameter, initial concentration of denatured protein, loading factor and eluent velocity can be optimized in small-scale batch experiments. Optimization allows for the determination of elution positions and elution volumes for the various protein forms, i.e., native and aggregated forms. The transfer into a continuous separation mode is made by transformation of the elution time (t) and angular velocity (ω) into angular displacement (θ):

$$\theta = \omega t \tag{11}$$

Further optimization of the chromatographic response depending on the rotation rate can be done for a fixed bed operation by the following approximate solution for a feed pulse of infinitesimal width, neglecting axial dispersion and that the number of transfer units is larger than 5 [34]:

$$c\,(z,\hat{t}) = \frac{Q}{2\pi^{0.5}} \cdot \left\{ \frac{(k_0 a)^2}{u^3 z\,\hat{t} \cdot [(1-\varepsilon)K]^3} \right\}^{0.25} \cdot \exp\left\{ -\left[\left(\frac{k_0 a z}{u} \right)^{0.5} - \left(\frac{k_0 a \hat{t}}{K \cdot (1-\varepsilon)} \right)^{0.5} \right]^2 \right\} \tag{12}$$

In Eq. (12), c is the liquid-phase solute concentration, k_0a is the global interphase mass transfer coefficient, u is the superficial velocity, K is the distribution coefficient and z is the axial bed position. ε is the bed void fraction. Q is the quantity of solute injected with the feed mixture per unit cross-sectional area of the fixed bed and $\hat{t}$ corresponds to:

$$\hat{t} = t - \frac{\varepsilon z}{u} \tag{13}$$

k_0a can be calculated as follows:

$$k_0a = 16 \cdot \ln 2 \cdot \left(\frac{t_R}{\Delta}\right)^2 \cdot \frac{u}{z} \tag{14}$$

In Eq. (14), t_R is the peak elution time and Δ is the time interval at half of peak maximum concentration.

Eq. (12) can be used to predict the elution behavior of proteins in the continuous mode if angular dispersion is considered negligible and if the transformation $\theta = \omega t$ is made. Therefore as pointed out by Wankat [35] the unsteady-state, one-dimensional chromatographic process is equivalent to the steady-state, two-dimensional P-CAC process.

Additionally, $\hat{t}$ must be replaced with:

$$\hat{t} = \frac{\theta}{\omega} - \frac{\varepsilon z}{u} \tag{15}$$

and Q with:

$$Q = \frac{c_F u Q_F 360}{Q_T \omega} \tag{16}$$

where c_F is the solute concentration in the feed mixture, Q_F is the feed flow-rate and Q_T is the total flow-rate of fluid through the annular bed.

3. Materials and methods

3.1. Chemicals

Bovine α-lactalbumin (Ca^{2+}-depleted) was obtained from Sigma–Aldrich (Vienna, Austria) and used without further treatment. Guanidine hydrochloride (Gdn·HCl), dithiothreitol (DTT), cystine, cysteine, Tris–HCl and EDTA were obtained from Merck (Vienna, Austria). All other chemicals were of analytical grade. Superdex 75 PrepGrade was obtained from Amersham Biosciences (Uppsala, Sweden). Deionized water (specific conductivity of 1 μS/cm) was used for the preparation of all solutions.

3.2. Preparation of denatured and reduced protein

For batch refolding experiments α-LA was denatured and reduced in a buffer containing 0.1 *M* Tris–HCl, 6 *M* Gdn·HCl, 10 m*M* DTT and 1 m*M* EDTA, pH 8.0, and incubated for 1 h at room temperature. The concentration of denatured protein was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) as described below.

3.3. Refolding by dilution

Denatured and reduced aliquots at 2.5 to 37.5 mg/ml were rapidly diluted 1 by 25 into renaturation buffer consisting of 0.1 *M* Tris–HCl, 50 m*M* NaCl, 10 m*M* $CaCl_2$, 2 m*M* cystine and 2 m*M* cysteine, pH 8.5, to final protein concentrations of 0.05 to 1.5 mg/ml. The refolding volume was 2 ml. The protein was allowed to refold for 4 h at room temperature and analyzed for native protein by RP-HPLC and high-performance (HP) SEC.

3.4. Quenching of oxidative refolding

The kinetics of the oxidative refolding process was monitored at 0.05 and 1.0 mg/ml by removing 100 μl samples at specific time intervals and quenching the formation of disulfide bonds by addition of 5 μl 6.4% HCl, resulting in pH 2.

3.5. Analytical methods

Prior to analysis, all samples were centrifuged at 12 000 *g* for 3 min to remove insoluble material. For RP-HPLC a Vydac C_4 column (214TP54) was connected to an Agilent 1100 HPLC system (Agilent Technologies). Fully denatured α-LA was separated from oxidative folding intermediates and native protein by linear gradient elution from 30 to 45% acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) in 30 min at 1 ml/min and 30 °C. The

system was calibrated with solutions of native and denatured α-LA using a molecular extinction coefficient of 23 150 M^{-1} cm^{-1} [36]. No difference in absorbance at 214 nm was detected for equivalent amounts of injected samples of native and fully reduced protein. Total mass recovery from matrix-assisted refolding experiments was done by calculating the amount of protein recovered in all fractions. Therefore, samples of collected fractions were pooled, denatured and reduced by 1:6 dilution in 0.1 *M* Tris–HCl, 6 *M* Gdn·HCl, 50 m*M* DTT and 1 m*M* EDTA, pH 8.0, and analyzed by RP-HPLC.

Aggregation was analyzed with an analytical SEC column (Super SW2000, Toso Biosep), connected to a Merck–Hitachi HPLC system controlled by a personal computer. The column was equilibrated at room temperature with 10 m*M* phosphate buffer, pH 7.0, containing 150 m*M* NaCl at a flow-rate of 0.3 ml/min. The system was also calibrated with native α-LA and elution was monitored at 214 nm.

Far UV CD spectra of native, refolded and denatured α-LA were recorded on a Pistra-180 spectropolarimeter (Applied Photophysics, UK). The spectra were recorded at 25 °C in a cuvette with a path length of 1 mm. Protein concentrations were approx. 50 μg/ml. All spectra were recorded three times, averaged and smoothed and the mean of the three data points was taken. Due to the high amount of Gdn·HCl in the denatured protein sample, significant data could not be recorded at wavelengths below 210 nm.

3.6. Refolding by size-exclusion chromatography

Samples of α-LA were denatured and reduced in 6 *M* Gdn·HCl, 50 m*M* DTT, 0.1 *M* Tris–HCl, 1 m*M* EDTA, pH 8, at concentrations of 1 to 10 mg/ml. The denatured protein was applied onto a column (1.6 cm I.D., 37 cm bed height) packed with Superdex 75 PrepGrade (Amersham Biosciences) and connected to an Äkta Explorer 100 chromatography system (Amersham Biosciences). The column was equilibrated at 30 cm/h with the same refolding buffer as used in batch dilution experiments. The sample volume was 1.5% of the column volume and the column effluent was monitored at 280 nm. Samples of collected fractions were analyzed for native and aggregated protein after elution.

3.7. Continuous refolding of α-lactalbumin with annular chromatography

Further development of protein refolding by size-exclusion chromatography was the transfer of the refolding process from a batch to a continuous process. In the continuous process the collected aggregated protein fraction was recycled to increase the efficiency of the system. For continuous refolding experiments, the identical protein solutions and buffers as in discontinuous experiments were used. The P-CAC system used for continuous refolding studies was from Prior Separations Technology, Austria. It consists of two concentric cylinders forming an annulus into which the stationary phase is packed. The outer cylinder had a diameter of 15 cm and the inner one a diameter of 13 cm, resulting in an annulus width of 1 cm. The upper part of the outer cylinder is made of glass and the lower part of polypropylene. The inner cylinder is made of polypropylene and is shorter than the outer one, leaving a headspace at the top. Both cylinders are closed by a head made out of PEEK (polyether ether ketone) through which the eluent and feed streams are inserted. The feed stream was pumped at the top of the gel bed through a fixed feed nozzle, whose tip was located within the layer of the glass beads. At the bottom of the unit, the two cylinders are attached to a stainless steel plate which contains 90 exit holes covered by a nylon filter (11 μm pore size). The bottom of the rotating column is connected to a fixed PTFE slip-ring which also contains 90 exit ports connected to a short section of Tygon tubing (Norton Performance Plastic, Akron, OH, USA). The exit ports are evenly distributed at 4° intervals along the annulus. The column was packed to a height of 41 cm with Superdex 75 PrepGrade. The bed of the glass beads was 2.6 cm high.

The system was additionally equipped with a pump for recycling the aggregates to the feed solution. The collected aggregates were concentrated using a tangential flow laboratory ultrafiltration unit (Labsacle TFF system, Millipore) equipped with three Pellicon XL Biomax 5 membranes (Millipore) with 5000 nominal molecular mass cut off. The total membrane area was 150 cm^2. The concentration was adjusted to approximately 1 mg/ml and recycling rate varied from 0 to 64.5% of the feed flow-rate

Table 1
Experimental conditions of the continuous refolding process

Feature	Condition
Bed height (z)	41 cm
Annulus width	1 cm (13 cm I.D., 15 cm O.D.)
Volume of packed bed (V_{SP})	1800 ml
Rotation rate (ω)	250°/h
Feed flow-rate (F_1)	Approx. 0.31 ml/min (equivalent to a sample volume of 1.5% of packed bed)
Aggregate concentration after ultrafiltration (c_4)	1 mg/ml
Recycling rate (R)	0–65% (0–0.2 ml/min)
Eluent buffer composition	0.1 *M* Tris–HCl, pH 8.5, 20 m*M* $CaCl_2$, 50 m*M* NaCl, 2 m*M* cystine, 2 m*M* cysteine
Eluent flow-rate (F_7)	30 cm/h (1.5 l/h)

$F_1 = 0.31$ ml/min, which was equivalent to 1.5% of column volume. Table 1 summarizes the experimental conditions. The initial concentrations of 6 *M* Gdn·HCl and 50 m*M* DTT in the initial feed solution were diluted to 2.1 *M* and 17.5 m*M* at 65% recycling rate. Under these conditions oxidative refolding of proteins is not possible. The reducing and denaturing power of the buffer system dissolves recycled aggregates into monomeric protein while passing through a reaction loop of $v' = 3$ ml. The elution profile at 280 nm of the 90 exit fractions was detected with a conventional 96-well microplate reader (μ-Quant, Biotek) containing a sample volume of 200 μl per well of each fraction.

4. Results and discussion

Currently, industrial refolding of proteins is mostly achieved by dilution of the denatured protein into an appropriate refolding buffer. Complex refolding reactors including series of tanks have been designed to improve the refolding reaction. The refolding in free solution or in a matrix-assisted process without additional helper proteins is not efficient enough to completely transfer the unfolded protein into the folded form. A certain fraction is always lost as aggregates, especially at higher concentrations. Continuous refolding with recycling of aggregates is a novel concept to improve throughput, yield and consequently productivity. The concept is based on continuous chromatography to separate aggregates, formed during the refolding reaction, and the native protein. The chromatographic refolding process was performed on size-exclusion columns and aggregates were concentrated by ultrafiltration. Bovine α-LA was chosen as model protein, since its refolding characteristics are well known [31,37]. For comparison of refolding by batch dilution, refolding in an SEC column in batch mode and continuous refolding with recycling of aggregates, refolding kinetics were first determined by conventional batch dilution studies. To evaluate the effect of protein concentration on refolding yield, a stock solution of denatured and reduced α-LA was transferred into refolding buffer containing a redox system to promote formation of disulfide bonds. The final protein concentration was between 0.05 and 1.5 mg/ml. After incubation at room temperature for 4 h, samples were analyzed by RP-HPLC and SEC. When the refolded protein was eluted at the same position as the native protein we assumed a completely refolded protein (Fig. 7). To confirm native conformation also CD-spectra of unfolded, refolded and native protein were performed (Fig. 8). At a concentration of 0.05 mg/ml, 95% of refolded protein could be recovered, but decreasing to a yield less than 50% when started with a concentration of 1.5 mg/ml (Fig. 3). To describe the competition between folding and aggregation, the time course of formation of native protein was evaluated at two levels of protein concentrations, 0.05 and 1 mg/ml, respectively (Fig. 4). The amount of refolded protein was plotted against time and approximated with Eq. (3). The estimated values of k_2 (rate constant of formation of native protein) and k_3 (rate constant of aggregation) used for the predictions were 0.02 min^{-1} and 0.054 ml mg^{-1} min^{-1}, respectively. The

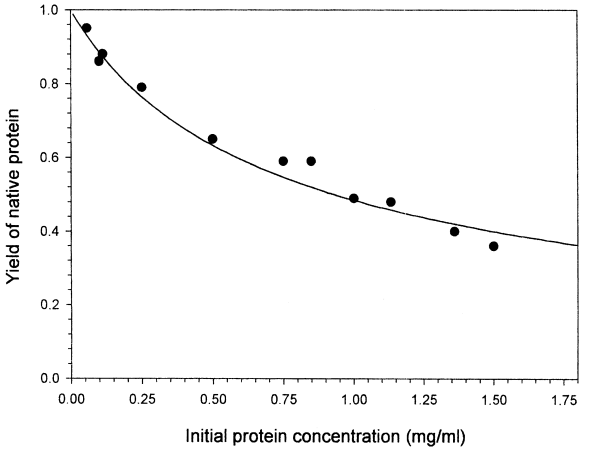

Fig. 3. Refolding yield of α-LA obtained by a 1:25 batch-dilution in 0.1 *M* Tris–HCl, pH 8.5, 50 m*M* NaCl, 10 m*M* $CaCl_2$, 2 m*M* cystine, 2 m*M* cysteine at initial protein concentrations from 0.05 to 1.5 mg/ml (●). Calculated yield of native protein according to Eq. (4) for $k_2 = 0.02$ min^{-1} and $k_2 = 0.054$ ml mg^{-1} min^{-1} assuming a second order reaction for aggregation (—).

oxidative folding rate is in the same range as found by Ewbank and Creighton [37]. The results of refolding by batchwise dilution showed that a buffer including a defined redox potential was appropriate to obtain optimal yield. The reaction equilibrium together with the kinetics do not allow a higher yield under given conditions.

Matrix-assisted refolding using SEC was tested at initial protein concentrations of 1.2, 3.7 and 10 mg/ml. Fully denatured and reduced samples of α-LA were applied onto a Superdex 75 PrepGrade column equilibrated with the same refolding buffer as used for batch dilution studies. A sample chromatogram of such a matrix-assisted refolding by SEC for a feed concentration of 3.7 mg/ml is shown in Fig. 5. The first peak eluting between 30 and 40 ml contained aggregated protein, the second peak (40–50 ml) contained refolded α-LA and the third peak (68–88 ml) contained urea and reducing agents.

Mass balances for all three feed concentrations are presented in Table 2. Since baseline separation between aggregated and native protein fractions could not be achieved, native protein was also detected in the first peak. In comparison to batch dilution studies aggregation was significantly reduced but not inhibited. The refolding yield was not significantly affected by the initial protein concentration even at a feed concentration of 10 mg/ml. On average, 30% of native protein was recovered in the second fraction, whereas the total refolding yield was about 42%. The dilution in the column upon chromatographic separation for the refolded protein fraction was on average ninefold. This is lower by a factor of 2.8 compared to batch dilution. Precipitation or adsorption processes did not occur in the

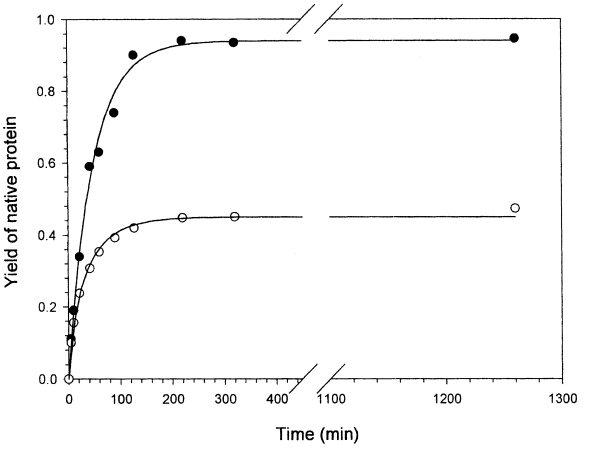

Fig. 4. Time course of formation of native protein at 0.05 mg/ml (●) and 1 mg/ml (○). Calculated formation of native protein according to Eq. (3) for $k_2 = 0.02$ and $k_2 = 0.054$ ml mg^{-1} min^{-1} and $n = 2$ (—).

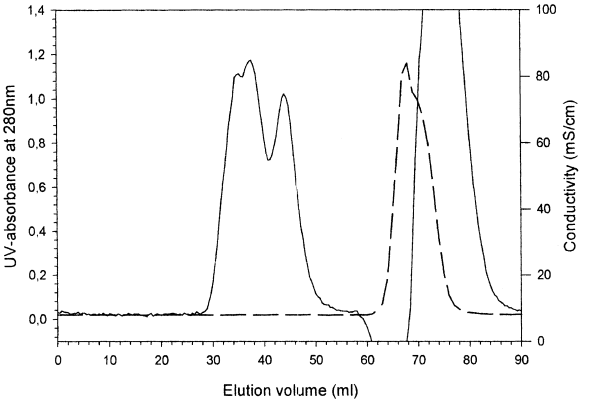

Fig. 5. Refolding of denatured and reduced α-LA (3.7 mg/ml) by size-exclusion chromatography on a Superdex 75 PrepGrade column (1.6 cm I.D. and 37 cm bed height) equilibrated with 0.1 *M* Tris–HCl, pH 8.5, containing 50 m*M* NaCl, 10 m*M* $CaCl_2$, 2 m*M* cystine and 2 m*M* cysteine. Sample volume was 1 ml and linear velocity was 30 cm/h. The solid line represents UV absorbance at 280 nm and the dashed line the conductivity.

Table 2
Refolding of α-LA by discontinuous size-exclusion chromatography

Feed concentration (mg/ml)	Yield of native protein in peak 1 (%)	Yield of native protein in peak 2 (%)	Total mass recovery* (%)
1.2	9	33	102
3.7	6	32	96
10	19	26	105

* Total mass recovery = amount of native protein in peaks 1 and 2 and amount of aggregated protein in peaks 1 and 2.

column during the refolding process, because the mass recovery of α-LA was between 96 and 105%.

The fixed bed operation was transferred to a continuous mode applying annular chromatography. Continuous separation was simply designed by calculation of the angular displacement using Eq. (11). Such an angular velocity was selected to be able to complete the separation within 360°. By this straightforward transformation, eluent streams and productivity are linear comparable to batch chromatography [38]. The same column height and linear velocity of feed and eluent streams were applied. For each peak, the distribution coefficient K and the global mass transfer coefficient k_0a was calculated from fixed bed experiments using Eq. (14) (Table 3). Bed void fraction determined by the blue dextran was $\varepsilon = 0.38$. Eq. (12) was used to predict the theoretical elution profiles at different angular velocities using the experimentally determined distribution coefficient and overall mass transfer coefficient from fixed bed operation results. A rotation rate of 250°/h was applied for continuous refolding experiments. Loading factor Q, superficial velocity u and bed height z were in the same range as in discontinuous chromatographic experiments. The initial concentrations of denatured and reduced protein were 1.2 and 3.8 mg/ml. After the system had reached steady state, the effluent of those exit ports containing the aggregated protein was continuously collected and concentrated to a final concentration of approximately $c_4 = 1$ mg/ml. Concentrated aggregates were recycled to the feed stream, where they were dissolved and allowed to refold again. Samples were drawn and the amount of aggregated and native protein was determined. Fig. 6A shows the UV profiles at 280 nm without recycling and Fig. 6B with recycling of aggregated protein.

Table 3
Equilibrium distribution and mass-transfer coefficients (Superdex 75 PrepGrade, 20 °C) at a protein concentration of 3.7 mg/ml

Fraction	K (–)	k_0a (s^{-1})
Aggregated protein	0.178	0.049
Native protein	0.35	0.158
Salt	1.00	0.226

Table 4 summarizes the experimental results of the continuous refolding experiments. Since also under this conditions baseline separation between aggregates and native protein could not be achieved, refolded α-LA was lost in the aggregated protein fraction eluting from 100 to 200° (Fig. 7). The refolding yield was in the same range as observed in discontinuous SEC refolding experiments. The recovery of refolded protein in both fractions was about 40 to 46%, but only 30% were eluted in the native protein fraction at 200 to 270°. After the system had reached equilibrium, the refolding efficiency at a protein concentration of 3.7 mg/ml was raised from about 32% without recycling to 41% at a recycling rate of 64.5%.

In order to provide further evidence for correct refolding, CD spectroscopy of the refolded protein fraction of P-CAC experiment 4 was employed. Fig. 8 shows a comparison of the CD spectra of the refolded protein fraction, native α-LA standard and the denatured protein. The spectra of the refolded and native proteins were identical, whereas the unfolded protein showed a completely different spectrum. The CD spectra of the refolded and native protein resembled those published by Wu et al. [39]. For further experiments we refrained from measuring CD spectra. We assumed a refolded protein when it was eluted at the position of the native protein in RP-HPLC.

Batchwise separation is simply transformed according to Eq. (11) into a continuous one by multiplying elution time (t) with angular velocity (ω). The elution position is given as angular dis-

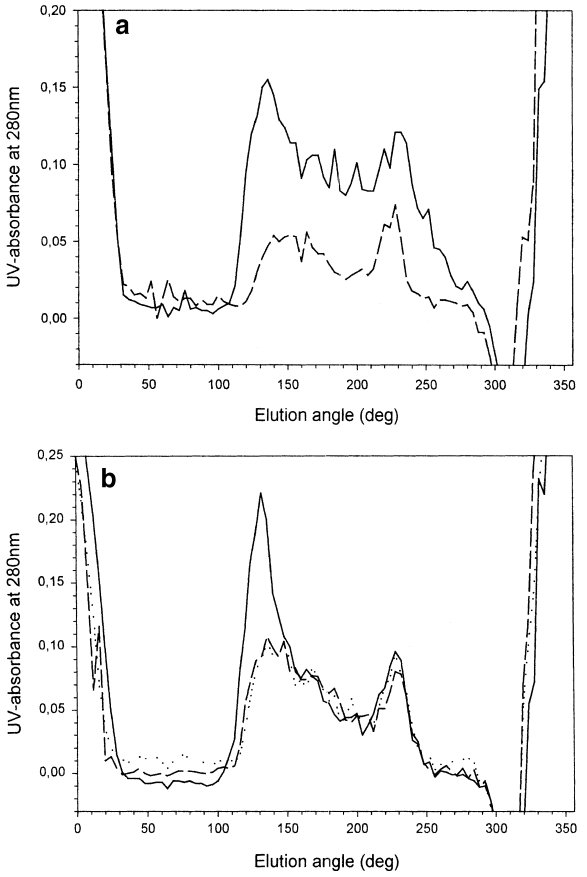

Fig. 6. Continuous refolding of denatured and reduced α-LA by annular chromatography on a Superdex 75 PrepGrade column at 250°/h and 30 cm/h. (A) Refolding without recycling with a concentration of feed solution of 1.26 mg/ml (- - -) and 3.8 mg/ml (—). Feed flow-rate was 0.31 ml/min. (B) Refolding with recycling of the aggregates. Concentration of feed solution was 3.8 mg/ml and aggregate concentration was 1 mg/ml. Recycling rates were 36.6% (—), 53% (— —) and 64.5% (· · ·).

placement (θ). Fig. 9 shows a normalized superimposition of a discontinuous SEC-refolding experiment applying a feed concentration of 3.7 mg/ml at 30 cm/h and the equivalent continuous separation (P-CAC experiment 2). The larger elution volume of the continuous experiment can be explained by additional dispersion effects at the outlet of the feed nozzle caused by the density and viscosity differences between load and eluent buffer. These effects result in zone spreading of the feed before it enters at the top of the packed chromatographic bed. Similar effects have been previously described and considered as major contribution to peak broadening in continuous annular chromatography, when operated under isocratic conditions [40].

The most important criteria to assess a refolding process are yield and productivity. Yield is primarily determined by the kinetics and equilibrium of the refolding reaction. It can be influenced by the refolding conditions, but at given conditions yield is limited by a theoretical upper value. Theoretically throughput and therefore productivity increases with feed concentration, but is also effected by the yield, which decreases with feed concentration due to augmentation of aggregation.

Yield of native protein and productivity of the system at different feed concentrations and different recycling rates, were calculated by Eqs. (5)–(10) assuming a constant refolding yield in the column ($Y_1 = 0.33$). Furthermore the concentration of denatured protein in the feed solution (c_0) was assumed to be to 4 mg/ml, the feed flow-rate (F_1) 0.32 ml/min and the volume of the stationary phase (V_{SP}) 1800 ml. The aggregate concentration (c_4) was varied from 0 to 4 mg/ml and the recycling rate (R) from 0 to 100%. Fig. 10A shows the calculated total yield of refolded protein, Y_2, at steady state conditions. Yield increased with recycling rate reaching a theoretical value of 100 when a certain recycling rate was exceeded.

For example, at $R = 0.7$ and $c_4 = 2$ mg/ml, the total yield of refolded protein, Y_2, is 72%. When the aggregate concentration was 3 mg/ml, a 90% yield could be obtained. When high aggregate concentration is present, recycling is more efficient compared to low aggregate concentration. The productivity decreased with increasing recycling rate R and lower aggregate concentration c_4 (Fig. 10B). The initial concentration of denatured protein is diluted by the recycling stream at lower aggregate concentration, resulting in a reduction of the amount of refolded protein per volume of stationary phase and time.

Further work is required to optimize productivity and yield in our experimental system. The reason of the differences between our experimental set up and theoretical calculations were shortcomings of the design of small-scale continuous ultrafiltration processes. In case of a very valuable product one would optimize the yield by increasing the recycling rate on

Table 4
Continuous refolding with recycling: summary of conditions and experimental results determined

	Experiment number				
	1	2	3	4	5
Concentration of denatured protein, c_0 (mg/ml)	1.259	3.801	3.801	3.811	3.811
Feed flow-rate, F_1 (ml/min)	0.31	0.303	0.326	0.321	0.328
Recycling rate, R (%)	0	0	36.6	64.5	53
Concentration of load, c_1 (mg/ml)	1.259	3.801	2.848	1.808	2.084
Concentration of native protein, c_2 (mg/ml)	0.036	0.062	0.070	0.053	0.058
Concentration of aggregates after ultrafiltration, c_4 (mg/ml)	0	0	1.134	1.010	1.010
Yield of native protein in aggregate fraction (%)	14.6	10.1	12.7	12.9	9.6
Step yield of native protein in monomer fraction, Y_1 (%)	31.2	32.8	27.8	31	30.5
Recovery of native protein with recycling of aggregated fraction, Y_2 (%)	–	–	33.2	41.4	35.5
Total mass recovery (%)	92	97	101.7	100.6	101.8
Productivity (μg ml^{-1} h^{-1})	4.06	12.60	8.59	6.00	6.95

the expense of productivity. In the opposite case when a low cost product has to be processed, recycling would be minimized to improve productivity. Further optimization could be done by using additives in the refolding buffer which suppress aggregation of proteins during the refolding process [41,42]. Urea (up to 2 *M*) and L-arginine (up to 0.5 *M*) in the refolding buffer increase the refolding yield of α-LA significantly (data not shown).

5. Conclusion

Other continuous refolding methods such as the method described by Katoh and Katoh [43] had not included recycling of aggregates. Besides theoretical yield made possible by recycling of aggregates, another advantage may be a lower process volume. This was achieved by matrix-assisted refolding using SEC. A much higher concentration of denatured protein can be processed compared to batch dilution. A further advantage is the continuous operating resulting in a higher of productivity in comparison to conventional batch chromatography. In order to keep the chromatography bed properly working, it must be regenerated. A regeneration solution has to be applied to the packed bed at a position distant enough from the feed inlet position to avoid mixing of the regeneration solution with the feed. Depending on

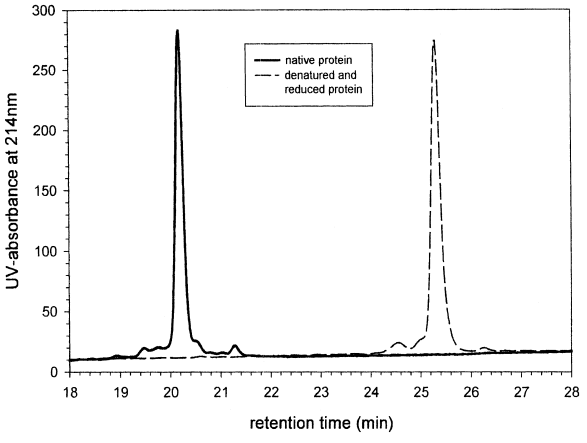

Fig. 7. Reversed-phase HPLC analysis of refolded and denatured α-LA. The chromatograms of native and unfolded and reduced protein were superimposed.

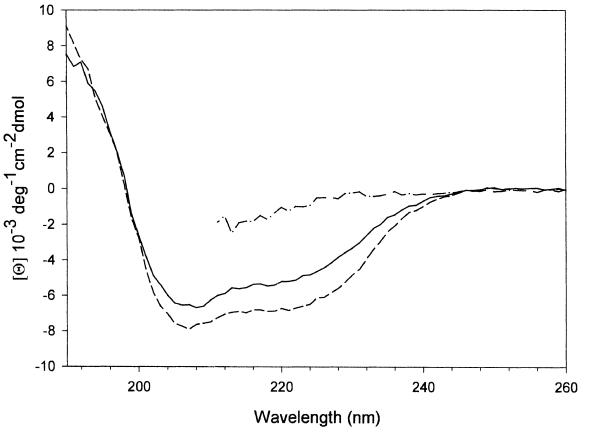

Fig. 8. CD spectroscopic analysis of native (—), refolded (— —) and denatured (· · ·) α-LA.

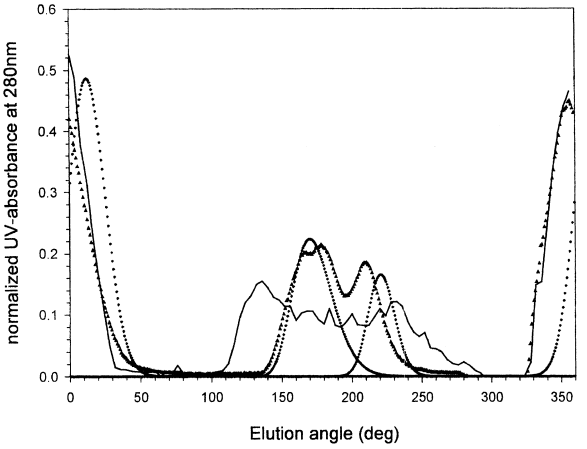

Fig. 9. Comparison of discontinuous (▲) and continuous (—) refolding of α-LA by SEC on Superdex 75 PrepGrade. Discontinuous elution profile was transformed into angular displacement (θ) according to Eq. (1). Protein concentration was 3.8 mg/ml and linear velocity was 30 cm/h. Calculation of theoretical elution profile (•) was done by Eq. (12) with corresponding values for K and k_0a for aggregates, native protein and salt fraction from Table 3.

the chemical properties of the chromatography bed, this solution is either a strong alkaline or acidic solution or a strong denaturant. The regenerating solution must be able to remove fouled layers from the packed bed. In the case of continuous refolding, the chaotropic agents from the feed solution can be used for continuous regeneration.

The most important feature of the continuous matrix-assisted refolding is the stoichiometic conversion of denatured proteins into natives. Low yield and low productivity is often observed in conventional batch refolding by dilution. This continuous refolding reactor overcomes these obstacles.

6. Nomenclature

c	Liquid-phase solute concentration at the column outlet (mg/ml)
c_F	Feed solution concentration (mg/ml)
c_0	Initial concentration of denatured protein (mg/ml)
c_1	Concentration of denatured protein applied onto the column (mg/ml)
c_2	Concentration of native protein (mg/ml)

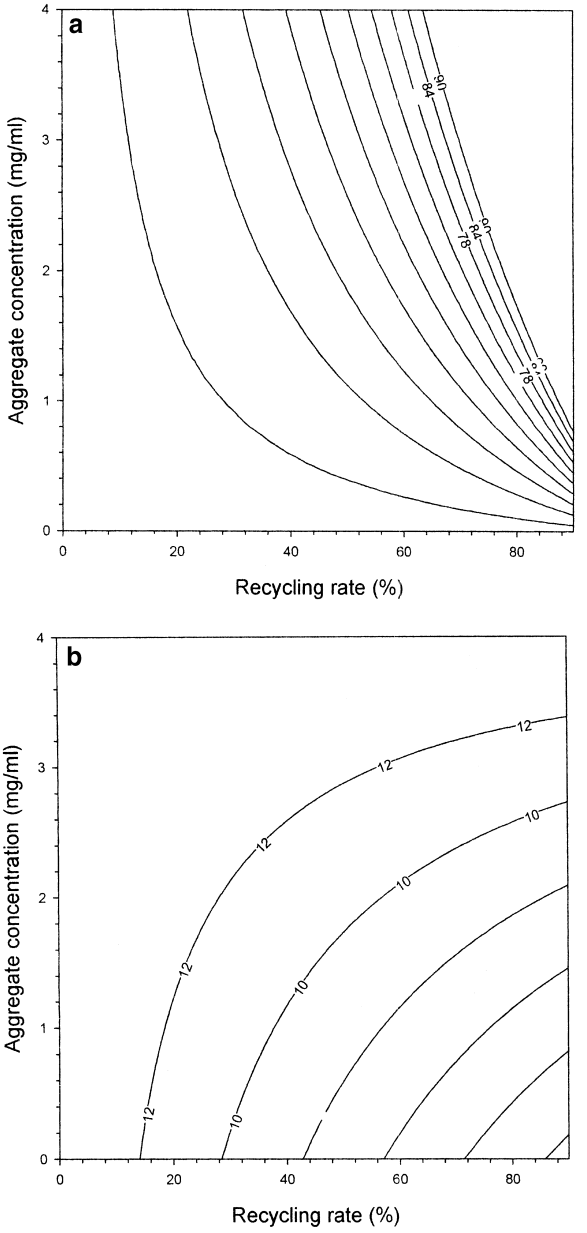

Fig. 10. Theoretical relationship of aggregate concentration (c_4) and recycling rate (R) on the yield (Y_2) of refolded protein (A) and on productivity of the system (B). Calculation was done assuming a constant refolding yield of $Y_1 = 0.33$. The initial concentration of denatured protein was $c_0 = 4$ mg/ml, the feed flow-rate $F_1 = 0.32$ ml/min and stationary phase volume $V_{SP} = 1800$ ml. Concentration of aggregated protein after ultrafiltration varied from 0 to 4 mg/ml and recycling rate from 0 to 100%.

c_3	Concentration of aggregated protein (mg/ml)
c_4	Concentration of aggregates after ultrafiltration (mg/ml)
c_F	Feed solution concentration (mg/ml)
$F_{0\ldots7}$	Flow-rates (ml/min)
K	Distribution coefficient (–)
k_0a	Global mass transfer coefficient (s^{-1})
k_2	Rate constant of folding (min^{-1})
k_3	Rate constant of aggregation (ml mg^{-1} min^{-1})
N	Dimensionless concentration of native protein (–)
n	Reaction order (–)
PR	Productivity (mg/ml min)
Q	Column loading of solute (mg/cm^2)
Q_F	Feed flow-rate (ml/min)
Q_T	Total flow-rate (ml/min)
R	Recycling rate (–)
t	Time (s)
t_R	Peak elution time (s)
$\hat{t}$	Chromatographic time (s)
u	Superficial velocity (cm/s)
U	Concentration of unfolded protein (mg/ml)
U_0	Initial concentration of unfolded protein (mg/ml)
v'	Volume of reaction loop (ml)
v_0	Initial volume of denatured protein (ml)
v_5	Volume of concentrated aggregates (ml)
V_{SP}	Stationary phase volume (ml)
Y_1	Yield of native protein without recycling of aggregates (–)
Y_2	Yield of native protein with recycling of aggregates (–)
z	Column length (cm)
Greeks	
Δ	Peak width at half concentration (s)
θ	Displacement from feed point (°)
ε	Bed void fraction (–)
ω	Rotation rate (°/h)

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