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Continuous matrix assisted refolding of α-lactalbumin by ion exchange chromatography with recycling of aggregates combined with ultradiafiltration

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Abstract

Continuous matrix assisted refolding (MAR) can be achieved on a solid support by using a continuous chromatographic system. Recycling the aggregate fraction, simultaneously formed during a refolding reaction, can further increase the refolding yield. Due to the nature of this reaction, aggregates are the main reason for a refolding yield below stoichiometric conversion. A preparative continuous annular chromatographic system (P-CAC) equipped with an ion exchange resin was used to continuously refold the model protein α-lactalbumin. For this purpose, this protein was denatured, reduced and adsorbed on the ion exchange resin. Elution was performed with or without redox reagents in the buffer system permitting fast formation of the native disulfide bonds. In the case redox reagents were present, the protein refolds then during its residence time on the matrix. However, aggregate formation is also increased and refolding yields are lower. Tightly bound aggregates were removed from the column by 2 M guanidinium hydrochloride. In order to increase the system yield, this aggregate fraction was recycled after lowering the conductivity by ultradiafiltration and adjustment of the protein concentration by dilution. For on-column refolding, recycling of aggregates at a recycling rate of 0.17 increased the system yield from 25% to 30%. An algorithm was developed to show interdependencies of the single influencing parameters. The operability of the system was demonstrated but limitations due to instability of the P-CAC, especially inhomogeneous flow and peak wobbling, have to be considered.

Keywords: Annular chromatography; Steady state refolding; On-column refolding; Matrix assisted refolding; Ion exchange chromatography

1. Introduction

At first glance, continuous bioprocesses seem to be more complex and therefore require more effort to be established. However, in most cases, major benefits pay off augmented development time and resources. Advantages of continuous bioprocesses compared to batch bioprocesses include reduced space required for storage of the feed and product solutions and lower residence time of the product in the process facility. These features of continuous processes are worth thinking of scaling a batch process to a continuous one [1]. Recombinant protein production is often effected by continuous fermentation of *E. coli*, yeast and mammalian cells, such as CHO and BK cells. Subsequent capture for product isolation and ensuing product purification is mostly achieved by chromatography in batch mode. Therefore, the concept of a continuous bioprocess is interrupted. By coupling a continuous chromatographic process with continuous fermentation, the harvested broth has not to be stored, dwell times of the chromatographic unit can be reduced, sanitation of the column has not to be effected as often as in batch mode and throughput is in general higher for continuous chromatographic systems. This approach was used for fermentation and isolation for blood coagulation factor VIII [1].

Continuous chromatographic systems have already been developed early including simulated moving bed (SMB) [2] in 1961, carousel chromatography [3] in 1961 and continuous annular chromatography (CAC) [4], first published in 1949. The separation of binary systems in counterflow direction

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by a true moving bed can be simulated in SMB technology. An assembly of columns is connected to buffers and to the feed solution. By switching interconnecting valves in distinct time intervals, the moving of the chromatographic bed is simulated. The technology has been successfully applied for separation of enatiomers [5], amino acids, sugars, hydrocarbons and even proteins. In carousel chromatography, multiple batch columns are arranged in a circle. The columns are rotated like a carousel and distributors on the top and bottom of the columns are used to direct the respective flows. Annular chromatography is termed annular because the bed is filled in an annulus between an inner cylinder and an outer glass jacket. The cylindrical assembly rotates whereas the sample solution and the buffers are applied on the top of the bed through fixed nozzles. Separation occurs in crossflow direction, the whole chromatographic bed is used because the sample migrates not only in horizontal direction but also in vertical direction. In contrast to SMB, multicomponent separation can be achieved. Secondly, the set-up is less complicated, as switching times of the valves and connections of buffer pumps, valves and columns drop out. In CAC, only step or isocratic elution can be performed. CAC systems are commercially available and have been used for preparative purification of sugars, metal ions, amino acids and proteins. The system was further improved by the possibility of applying a slight pressure, it was then termed pressurized annular chromatography (P-CAC). P-CAC has been operated with different chromatographic techniques for protein purification. Size exclusion chromatography (SEC) was first studied by Sisson et al. [6] and later applied, e.g. for purification of recombinant green fluorescence protein [7], removal of IgG aggregates from the monomer [8,9] and purification of lipase [10]. Ion exchange was used for purification of blood coagulation factor VIII [1], factor IX [11] and antibodies [12]. A model to predict elution behavior in P-CAC was developed from ion exchange chromatographic experiments [13,14].

In some biotechnology processes, recombinant protein can be directly captured from fermentation supernatants under the precondition that the product is secreted. Bacterial host cells often deposit the product in the cytoplasm as insoluble protein aggregates, termed inclusion bodies. Purification requires previous dissolution with chaotropic agents and subsequent refolding to obtain native protein conformation and biological activity. As this step suffers from low yield, optimization of the refolding procedure is a challenging task and has been the reason for development of alternative techniques. One of the most promising methodologies is refolding on a solid support, termed matrix assisted refolding (MAR) [15–18]. With this methodology, high yield of the native product and reduction of process times and buffer volumes can be realized. Using an ion exchange resin with usually high binding capacities as a matrix, a large volume of dilute feed solutions can be processed [19] in a batch operation. Advantages of both, continuous processes and matrix assisted refolding are elucidated.

A further improvement of a refolding procedure is a combination of both techniques. Hence, continuous matrix assisted refolding was designed using a P-CAC system equipped with an ion exchange sorbent. Other continuous refolding approaches are fed batch addition of denatured protein to a folding solution [20] or development of a ceramic flow type reactor [21]. In these examples, simultaneous product purification and refolding is not achieved. During a refolding reaction, a certain fraction of protein is always lost due to formation of aggregates. Therefore, refolding yields are often considerably low. These aggregates can be separated from the monomer with size exclusion chromatography. Recycling of this aggregate fraction increases the overall yield. The disadvantages of using size exclusion chromatography are the limited feed volume that can be loaded on the column for sufficient resolution and the low flow velocity. Therefore, throughput in size exclusion chromatography is low.

In a previous paper, we described continuous matrix assisted refolding by size exclusion chromatography with recycling of the aggregates formed during the refolding reaction in the column. These aggregates are separated from the native monomer due to their higher molecular mass. The aggregate fraction was than concentrated by a lab scale UDF unit and recycled at various rates. Due to isocratic elution of the aggregate fraction, recycling could be performed after protein concentration with an ultrafiltration unit without further conditioning. As mentioned earlier in SEC, the feed volume should not exceed 5% of the total column volume [22]. Thus, the feed stream of the P-CAC system operated in SEC mode was only 1.4% of the sum of all streams. In contrast, in ion exchange chromatography (IEX), the feed volume is not a limiting factor. The feed volume often exceeds the total column volume. This is considered as a major advantage of IEX over SEC for continuous refolding of proteins. Even extremely dilute solutions can be efficiently processed with a small column compared to SEC. Ion exchange media have in general high binding capacities and usually a high flow velocity can be applied. Matrix assisted refolding on ion exchange resins in continuous mode with aggregate recycling combines all advantages of the single methodologies. Here, we describe continuous refolding of a model protein on an ion exchanger with recycling of the aggregates.

2. Theory

An algorithm was developed to calculate the set-up for continuous refolding with annular chromatography in combination with diafiltration. Operation variables are highly interdependent. Only few parameters have to be estimated experimentally. Some values are given through the operability of the system. The set-up of the refolding unit is shown schematically in Fig. 1. The main parts are the P-CAC unit (A) and the diafiltration unit (B). The operation of this continuous refolding process consists of three phases. The first phase is the time interval prior to first elution of the regener-

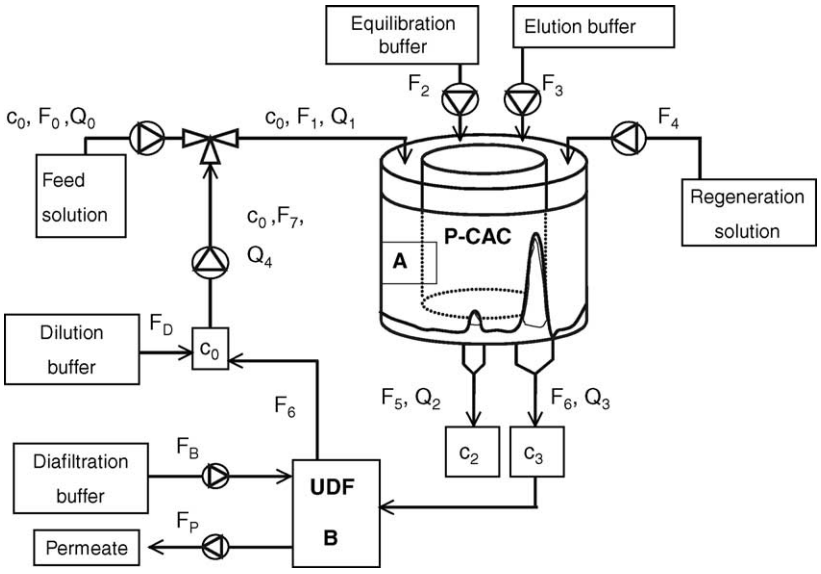

Fig. 1. Experimental set-up for continuous MAR with a pressurized continuous annular chromatographic system (A) and recycling of aggregates in combination with an ultradiafiltration device (B). F_0–F_7: flows; c_0–c_3: protein concentrations in the various sections of the system. For explanations, see text.

ate; the second phase is a transition phase between elution of regenerate and reaching the steady state when the regenerate is recycled. The third phase starts upon establishment of steady state. When starting the operation, the initial protein solution with concentration c_0 is pumped at a certain volume flow rate F_0 to the P-CAC, yielding a mass flow of $c_0F_0 = Q_0$. In the second phase, the protein concentration of the regenerate that is then the recycle solution is adjusted to the same protein concentration as the initial solution. The recycle solution is mixed at a volume flow rate F_7 with the initial solution that is pumped at a volume flow rate of F_0 to the system. The resulting volume flow rate of the feed solution is F_1:

$$F_1 = F_7 + F_0 \tag{1}$$

The mass flow of the feed stream is thus $F_1c_0 = Q_1$. The recycling rate is defined as:

$$R = \frac{F_7}{F_1} = \frac{F_1 - F_0}{F_1} \tag{2}$$

In case of no recycling, the mass flow of the feed stream (Q_1) is the mass flow of the initial stream (Q_0). The column is equilibrated with equilibration buffer at a volume flow rate F_2, elution is performed at a flow velocity of F_3 and the column is regenerated at a regeneration flow velocity of F_4. The native protein fraction elutes at a certain flow F_5 with a protein concentration c_2, that gives a mass flow $F_5c_2 = Q_2$, whereas the regenerate elutes with a flow velocity of F_6 and a protein concentration c_3. The mass flow of the regenerate is $F_6c_3 = Q_3$. The mass balance of the system is:

$$Q_1 = Q_2 + Q_3 \tag{3}$$

The yield (Y_1) of the chromatographic process is:

$$Y_1 = \frac{Q_2}{Q_1} \tag{4}$$

The mass flow of the regenerate Q_3 was recycled. Prior to reloading, it has to be diafiltrated and the protein concentration has to be adjusted. The mass flow of the recycling solution after diafiltration and adjustment of the protein concentration is Q_4. Recycling of aggregates reduces the mass of protein that has to be provided initially. To express the benefit of recycling, a system yield is defined. It relates the mass flow of the recovered native protein to the to the mass flow of the initial solution:

$$Y_0 = \frac{Q_2}{Q_0} \tag{5}$$

For evaluation of the system, not only the yield of native product is of interest, but also total recovery of the protein in all exit streams of the P-CAC system must be considered to judge if protein is accumulated in the matrix. Additionally, recovery is a measure of protein loss during the process due to protein precipitation and experimental errors during manual collection of the eluate. It is defined as follows:

$$r = \frac{Q_2 + Q_3}{Q_1} \tag{6}$$

A detailed drawing of the ultradiafiltration process with the respective streams is shown in Fig. 2. The regenerate stream Q_3 is pumped to the UDF unit and diafiltrated against a buffer solution that keeps the protein in the denatured and reduced state but has a conductivity low enough to permit reloading to the ion exchanger. The conductivity of the feed stream is a key parameter and the extent of diafiltration and subsequent dilution are determined by this parameter. The protein regenerate elutes in higher concentration than

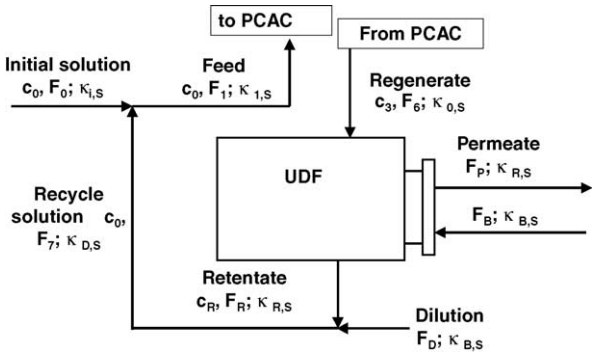

Fig. 2. Detailed drawing of the ultradiafiltration (UDF) process. c_0–c_3: protein concentration; F_P, F_B, F_D, F_R: flow of permeate, diafiltration buffer, dilution buffer and retentate flow; F_1, F_0, F_6, F_7: flow of initial solution, feed solution, regenerate and flow of the recycling solution; $\kappa_{0,S}$, $\kappa_{R,S}$, $\kappa_{B,S}$, $\kappa_{D,S}$, $\kappa_{i,S}$, $\kappa_{1,S}$: conductivity of regenerate, retentate, dilution buffer, which is also diafiltration buffer, recycle solution, initial solution and feed solution.

the feed solution due to the adsorptive properties of the ion exchanger. Thus, after diafiltration the protein solution is adjusted to the initial feed concentration by dilution with the same buffer as used for diafiltration. The recycle solution is pumped at the flow rate F_7 to the feed stream. It was found that molarity of the guanidine hydrochloride (Gdn-HCl) solution is linearly related to its conductivity for a molarity up to 2 M. Therefore, volume flow rates and their conductivity during the UDF process can be equated as follows:

$$F_6\kappa_{0,S} + F_B\kappa_{B,S} = F_P\kappa_{R,S} + F_R\kappa_{R,S} \quad (7)$$

where F_6 is the flow of the regenerate, $\kappa_{0,S}$ is its conductivity, F_B is the flow of the diafiltration buffer and $\kappa_{B,S}$ the respective conductivity (Fig. 2). F_P and F_R are the flow of the permeate and retentate and $\kappa_{R,S}$ is their conductivity. During diafiltration, the permeate flow equals the diafiltration buffer flow. The retentate is continuously pumped out of the reservoir of the diafiltration unit. The system is balanced if the retentate flow is the same as the regenerate flow, F_6:

$$F_B = F_P \quad \text{and} \quad F_6 = F_R \quad (8)$$

After diafiltration, the retentate is continuously diluted with diafiltration buffer in order to adjust the protein concentration. Again, volume flow rates and respective conductivities can be equated by the following expression:

$$F_R\kappa_{R,S} + F_D\kappa_{B,S} = F_7\kappa_{D,S} \quad (9)$$

$\kappa_{D,S}$ is the conductivity of the final solution that is recycled and F_D is the volume flow rate that is used for dilution of the retentate.

The recycle solution is mixed with initial protein solution yielding another equilibrium:

$$F_0\kappa_{i,S} + F_7\kappa_{D,S} = F_1\kappa_{1,S} \quad (10)$$

$\kappa_{i,S}$ is the conductivity of the initial solution, and $\kappa_{1,S}$ is the conductivity of the feed solution. Both values are given.

Two preconditions arise to operate the system continuously. First, the conductivity of the recycling solution has to be lowered to a value that still permits adsorption. Second, the protein concentration in the recycling solution must be adjusted to be the same as the initial feed solution in order to maintain a steady state and thus to be able to develop an algorithm. The algorithm is valid if the following assumptions apply. At the time the continuous chromatography process is in steady state, the mass flow of the native protein as well as the one of the regenerate are constant. The conductivity in the regenerate stream is constant.

In the following, it is described how to calculate the respective parameters for continuous matrix assisted refolding with recycling of the aggregate fraction. A short overview about the order how to calculate the properties of the respective streams is given in Fig. 3. The process yield of the refolding reaction must be determined from earlier batch column experiments and then the mass flow of the native protein can be calculated from Eq. (4). For a recovery of 100%, the mass balance of Eq. (3) then gives the mass flow of the regenerate. The flow of the regenerate is one parameter that has to be determined experimentally. The number of fractions containing the regenerate determines the entire volume. The number of fractions containing the regenerate is thus dependent on the base width of the regenerate peak. The protein concentration in the regenerate, c_R, is derived from the mass flow Q_3 and the volume flow rate of the regenerate, F_6. The regenerate is pumped with F_6 to the UDF unit. It is provided that the diafiltration membranes have a rejection value (Rj) of 1 that is defined in Eq. (11) [23]:

$$\mathrm{Rj} = 1 - \frac{c_P}{c_R} \quad (11)$$

c_P is the protein concentration in the permeate and c_R is the protein concentration in the retentate, so c_3 equals c_R. Eq. (11) should demonstrate that this assumption is only true in case no protein is found in the permeate. To maintain constant volume in the reservoir of the UDF unit, the retentate is continuously pumped out with the flow F_R that is the same as the regenerate flow, F_6 (see Eq. (7)). This solution is then diluted with the same buffer as used for diafiltration. The ratio of c_3 to c_0 determines the dilution factor, DF:

$$\mathrm{DF} = \frac{c_3}{c_0} = \frac{F_7}{F_R} \quad (12)$$

It further determines the ratio of the resulting recycle stream (F_7) to the retentate stream (F_R), as the recycle stream is defined as follows:

$$F_7 = F_D + F_R \quad (13)$$

F_D is the diafiltration buffer volume flow rate. Combining Eq. (12) with Eq. (13) gives an expression for F_D:

$$F_D = F_R(\mathrm{DF} - 1) \quad (14)$$

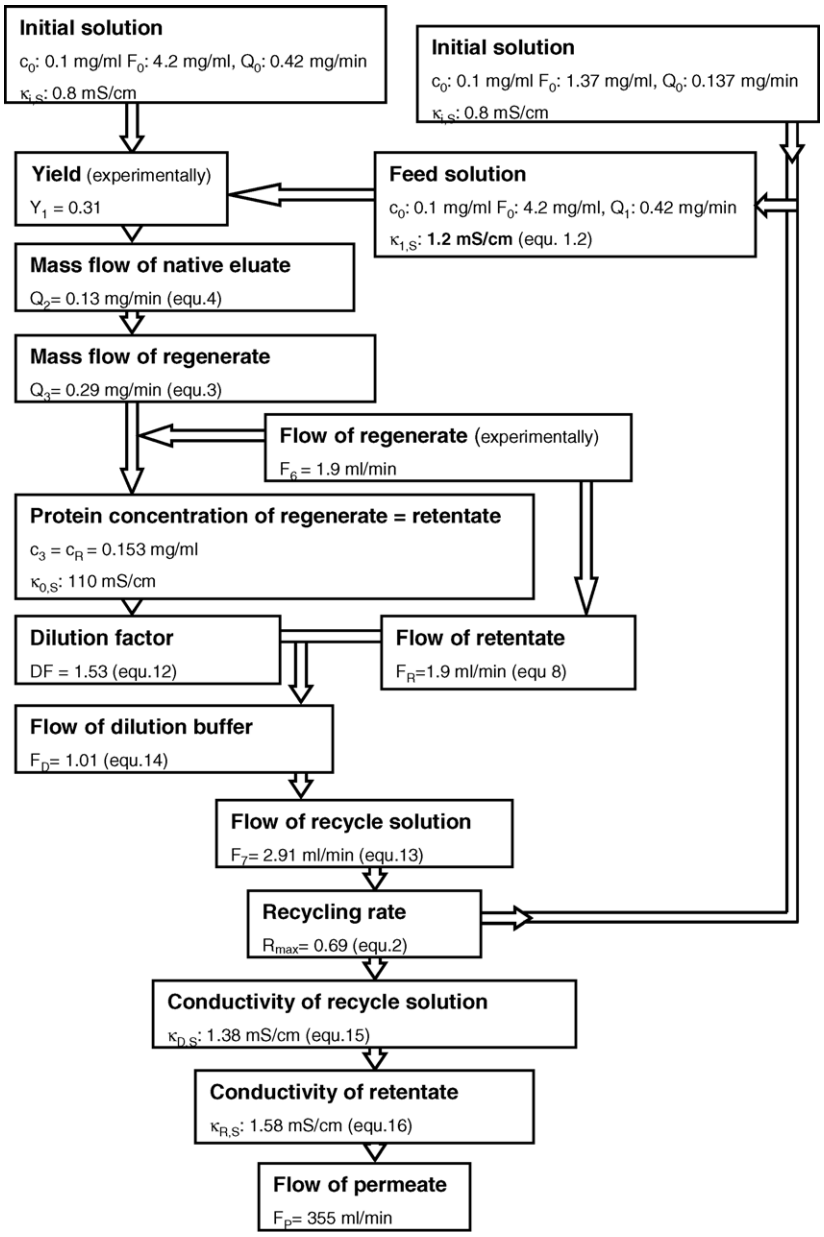

Fig. 3. Flow chart of how respective parameters were calculated for an intended experiment and results of the cited equations.

F_7 is the recycling flow for a maximal recycling rate. The recycling rate can be calculated from Eq. (1). The maximum possible conductivity of the feed still permitting protein binding on the resin is a specific value ($\kappa_{1,S}$). The resulting conductivity of the recycle solution ($\kappa_{D,S}$) is obtained by combination of Eqs. (1), (2) and (12) and subsequent rearrangement:

$$\kappa_{D,S} = \frac{\kappa_{1,S} - (1 - R)\kappa_{i,S}}{R} \tag{15}$$

After the maximum conductivity of the recycling solution ($\kappa_{D,S}$) is calculated, the conductivity of the retentate ($\kappa_{R,S}$) that has to be achieved by diafiltration is determined by the dilution factor, DF, defined in Eq. (12). Inserting Eqs. (12) and (14) into Eq. (9) and rearrangement, the conductivity of the retentate can be calculated:

$$\kappa_{R,S} = \kappa_{B,S} + DF(\kappa_{D,S} - \kappa_{B,S}) \tag{16}$$

The task is to design such an UDF process in order to fulfill this requirement. According to the mass balance of Eq. (7) the permeate flow can be calculated:

$$F_P = \frac{F_R(\kappa_{0,S} - \kappa_{R,S})}{\kappa_{R,S} - \kappa_{B,S}} \tag{17}$$

The maximal recycling rate is obtained when the whole regenerate is recycled. The maximal recycling rate is:

$$R_{max} = \frac{F_6}{F_1} DF \tag{18}$$

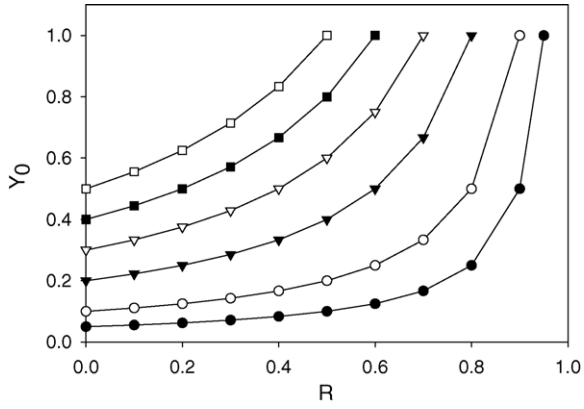

Fig. 4. Dependency of the system yield on recycling rate according to Eq. (19) for different process yields: (●) $Y_1 = 0.05$; (○) $Y_1 = 0.1$; (▼) $Y_1 = 0.2$; (▽) $Y_1 = 0.3$; (■) $Y_1 = 0.4$; (□) $Y_1 = 0.5$.

The system yield (Y_0) depends on the recycling rate (R) and the process yield (Y_1). The relationship is described by:

$$Y_0 = \frac{Y_1}{1 - R} \tag{19}$$

Under the precondition that the protein concentration of the recycle solution is the same as the one of the feed solution, the recycling rate is:

$$R = \frac{Q_3}{Q_1} \tag{20}$$

As Q_3 is always smaller than Q_1, R can never be 1 and thus Eq. (20) can always be solved. The increase in system yield (Y_0) with recycling rate for various values of Y_1 is shown in Fig. 4.

3. Materials and methods

3.1. Protein and chemicals

Ca^{2+} depleted bovine α-lactalbumin and monothioglycerol were from Sigma–Aldrich (Vienna, Austria). Guanidine hydrochloride was purchased from Fluka (Buchs, Switzerland). Urea ultra pure grade was from Amresco (Ohio, USA). Dithiothreitol was from AppliChem (Darmstadt, Germany). Cysteine, cystine, Tris–HCl, acetonitrile HPLC grade and trifluoracetic acid (TFA) were from Merck (Darmstadt, Germany). NaCl was obtained from Salinen Austria (Austria). DEAE Sepharose was purchased from Amersham Biosciences (Uppsala, Sweden).

3.2. Analysis of the folding conformation

The folding conformation of α-lactalbumin was analyzed by reversed phase (RP) HPLC. A Vydac C4 214TP54 column (4.6 mm I.D. × 250 mm) was used. Eluent A was deionized and 0.22 μm filtered water, supplemented with 5% acetonitrile and 0.1% TFA, eluent B was acetonitrile supplemented with 0.1% TFA. Different folding forms of the protein were eluted with a linear gradient from 34% to 45% eluent B in 20 min, regeneration of the column was effected by a step gradient to 90% B. The detection wavelength was 214 nm and the flow rate was 1 ml/min.

3.3. Equipment

The continuous annular chromatograph was from Prior Separation Technology (Götzis, Austria). The main eluent was applied with a P-6000 pump, the feed and the elution buffer were pumped with a P-500 pump and the regeneration solution was applied to the column with a P-50 pump. All pumps were from Amersham Biosciences (Uppsala, Sweden). For protein HPLC analysis of the concentration and conformation, a LC 1100 HPLC system was used (Agilent Technologies). For diafiltration of the regenerate, a tangential flow laboratory ultrafiltration unit (Labscale TFF system, Millipore) was used. The system was equipped with three Pellicon XL Biomax PLCGC membranes with a nominal molecular mass cut off of 10 kDa.

3.4. Continuous matrix assisted refolding

Experimental conditions of MAR on a batch column were scaled to the continuous column by keeping the superficial velocity u and the column bed height constant. Column volumes concerning equilibration, feed, wash out, elution and regeneration were the same as for the batch column. Conditions from the batch column were transferred to the continuous column by the relationship:

$$t = \omega\Theta \tag{21}$$

where t is the time in a batch chromatography, ω is the angular velocity and Θ is the angle at that either buffers are applied at the top or elution of the respective stream occurs at the bottom. Buffers were designed either to enable protein refolding in the eluate or on the column, respectively, as described in [19]. Conditions such as protein concentration, volumetric flow velocity and bed height were chosen in respect to operability of the P-CAC unit in lab scale. A denatured and reduced protein solution of α-lactalbumin was prepared by dissolution of the lyophilized protein in a buffer containing 8 M urea, 20 mM Tris–HCl and 50 mM monothioglycerol, pH 8, with a conductivity of 0.8 mS/cm.

The protein concentration was between 0.94 and 0.1 mg/ml. As a chromatographic gel, DEAE Sepharose was used. The slurry was poured into the annulus and the gel was settled by a flow velocity of 95 cm/h. The outer diameter of the cylinder was 15 cm and the inner diameter was 13 cm, giving an annulus width of 1 cm. The bed height was 11.3 or 8.4 cm giving a bed volume of 550 or 370 ml, respectively. The equilibration buffer was 20 mM Tris–HCl, pH 8, supplemented with 2 M urea. Conductivity of this buffer was 1.5 mS/cm. The elution buffer was the same as equilibration

buffer but containing additionally 0.5 M NaCl. 2 mM cysteine and 2 mM cystine were added to the equilibration and elution buffers to accelerate the refolding reaction and refold the protein on the column. This ensures that already completely folded protein elutes. As a regeneration solution, 2 M Gdn-HCl and 50 mM monothioglycerol in 20 mM Tris–HCl, pH 8, was used. The composition of this regeneration solution was sufficient to regenerate the column. This was determined by scaled down batch column refolding experiment. Additionally, its lower conductivity than a 6 M Gdn-HCl solution facilitates the subsequent dialfiltration.

The sample was applied at a flow velocity of 4.2 ml/min and an angle of 0°. Elution was effected at a volumetric flow of 2.1 ml/min and was applied at 126°. The regeneration solution was applied at a volumetric flow of 1.4 ml/min and at 214°. The flow of the main eluent was 22 ml/min, so the total flow was 29.7 ml/min or 40 cm/h. The angular velocity was 84°/h. The bottom of the cylinder was fixed to a ground plate with 90 exit holes every 4°. The eluate was collected in 90 PET tubes through nylon tubes fixed to the exit holes. These 90 PET tubes can be easily exchanged by a special annular rig. All 90 fractions were analyzed offline by measuring the UV adsorption at 280 nm in a two-channel spectrophotometer and by checking the conductivity. As urea and monothioglycerol have unspecific UV adsorption, the protein content of selected fractions was roughly determined with a Bradford assay. The fractions containing protein as determined by this assay were further analyzed by reversed phase chromatography for determination of the folding conformation and the exact protein content.

As aggregates may be present as well in native fractions, the total protein content in theses fractions was determined by denaturing and reducing the sample again in order to dissolve the aggregates and allowing quantification by RP-HPLC. The difference in mass of native protein and mass of total protein is designated as mass of aggregates.

3.5. Diafiltration and recycling of the regenerate

Due to the experimental limitations in laboratory scale, diafiltration of the regenerate was carried out in batch mode. According to batch column experiments, the protein elutes already at a slight increase in conductivity. Therefore, the maximal value for the conductivity of the feed stream should be 1.2 mS/cm. The conductivity of the feed stream is dependent on the conductivity of the initial solution, that is given, and on the conductivity of the recycling solution. The conductivity of the recycling solution is in turn dependent on the conductivity of the retentate.

The conductivity of the retentate that has to be attained was calculated according to Eq. (16). Prior to pooling the regenerate fractions, the protein content of each single regenerate fraction was analyzed by RP-HPLC. In order to minimize the volume that has to be diafiltrated, only fractions with a protein concentration greater than 0.05 mg/ml were pooled and directed to the UDF unit.

The solution was diafiltrated against a 20 mM Tris–HCl buffer, pH 8, containing 4 M urea and 50 mM monothioglycerol, having a conductivity of 1 mS/cm. At a denaturant concentration of 4 M urea, the protein is kept in its denatured and reduced state. The permeate was measured by collecting the permeate into a measure.

After diafiltration the conductivity was checked and the protein concentration in the retentate and in the permeate was determined. A mass balance was calculated. The protein concentration of the diafiltrated regenerate solution (e.g. the retentate) was adjusted to the initial protein solution by dilution with diafiltration buffer. This recycling solution was then pumped through a branch connection to the initial feed stream. The pumping speed of the recycling solution as well as of the initial solution was adjusted to the recycling rate R.

4. Results and discussion

Promising results from previous experiments of matrix assisted refolding with ion exchange columns in a batch mode encouraged us to transfer this methodology to a continuous process. Performance of several matrices has been investigated [19]. DEAE Sepharose showed highest dynamic binding capacity for denatured and reduced model protein and in general, high yield of native protein was obtained. DEAE Sepharose was selected for continuous operation. In this paper, continuous refolding of a α-lactalbumin with continuous IEX is described.

Continuous refolding was performed in a system composed of an annular chromatography system and an ultra-diafiltration system. The fraction of denatured protein was collected at the bottom of the column, diafiltrated, diluted and recycled into the feed stream. A schematic sketch of the systems, including notation of the various streams is shown in Fig. 1.

The most critical part is the adjustment of the protein concentration, the Gdn-HCl concentration—expressed as conductivity—and the volumetric flow rate of the recycle stream. For conversion of Gdn-HCl concentration to conductivity, a calibration curve was established with solutions of defined molarity of Gdn-HCl, dissolved in 20 mM Tris–HCl, pH 8. A linear relationship between molarity of Gdn-HCl and conductivity was determined for low Gdn-HCl concentration only. The conversion factor of conductivity measured in mS/cm to salt molarity was determined to be 0.018.

There is an interdependency of load (Q_0), eluate stream of the denatured protein (Q_3), extend of diafiltration (F_B), and extend of dilution (F_D) prior to recycling.

After starting the operation, a transition period has to be awaited until steady state conditions are reached. Steady state should be reached usually after one rotation. The transition phase is composed of the time period until denatured protein can be collected at the outlet of the P-CAC system and the time period until the system is again in steady state when the denatured protein is recycled. This transition phase is

extremely difficult to calculate. As complete modelling was not the scope of this work, we only considered steady state conditions.

First, the experimental conditions of the continuous refolding experiment with recycling of aggregates were calculated according to the Eqs. (1)–(18) derived in the theory section. The complexity of the system is generated through the essential conditioning of the regenerate stream prior to recycling. The Gdn-HCl concentration must be lowered by diafiltration and the protein concentration is adjusted by dilution. Additionally, dilution contributes also to a decrease in conductivity of the final recycling stream, therefore the extent of diafiltration is reduced. Similar effects are due to mixing initial solution of low conductivity with the recycle solution.

A schematic drawing of the various process streams involved in conditioning the regenerate stream prior to recycling and their notation in respect of protein concentration and conductivity is shown in Fig. 2. A flow diagram how the various operating parameters were determined—either experimentally derived or calculated—is shown in Fig. 3. Chromatographic conditions and parameters of the respective flows were scaled from previous batch column experiments that in turn were designed in consideration of operability of the P-CAC system in the laboratory. For a particular case, the various process streams were calculated assuming a concentration of denatured protein in the initial solution of 0.1 mg/ml. The volumetric flow of this solution was chosen to be 4.2 ml/min. The upper limit of conductivity in the feed solution was 1.2 mS/cm. The conductivity of the initial solution was measured to be 0.8 mS/cm.

When the operation is started, the P-CAC is loaded with a mass flow of Q_0, which is 0.42 mg/min in this case. Assuming that after first recycling with a maximal recycling rate R_{max}, steady state conditions are reached, the volumetric flow rate of the initial solution must be decreased to 1.37 ml/min according to Eq. (1). Since protein concentration is adjusted, the mass flow rate is also decreased by the same factor. In order to estimate the mass flow of the refolded protein (Q_2), the yield (Y_1) of the matrix assisted refolding process must be known. Y_1 is difficult to predict and has to be determined experimentally for the given system. It has to be noticed that the process yield is depended on the protein concentration in the feed, the column volume and the column dimensions. In batch dilution experiments, the refolding yield can be calculated when the folding kinetics and the kinetic constants are known. Determination of the folding kinetics during matrix assisted refolding is not straightforward. Ligands and matrix may certainly have an influence on folding kinetics. It is not investigated whether the protein refolds in the adsorbed state or during elution. The process yield is viewed as a stoichiometric conversion of denatured protein to native one and is assumed to be constant. The native protein fraction eluting from the column is completely folded. In this example, process yield was assumed to be 0.31. Knowing the yield of the MAR process, the mass flow of the refolded protein is calculated by Eq. (4). Under steady state conditions, it does not further change, even when recycling is started, because the mass flow of the initial solution is decreased accordingly and the mass flow of the feed solution is kept constant. With a yield of 0.31, the mass flow of the refolded protein is 0.13 mg/min. Then, the mass flow of the regenerate (Q_3) is calculated according to Eq. (3) and is 0.29 mg/min in this case. For reasons of simplicity, we did not integrate a complete model of elution behavior of the respective protein fractions from the P-CAC column. Therefore, the volumetric flow rate of the regenerate stream (F_6) has to be determined from previous experiments. F_6 was 1.9 ml/min in the experiment carried out under the conditions assumed for this theoretical consideration. After the P-CAC has reached steady state, the mass flow Q_3 is constant. The protein concentration of the regenerate c_3 is calculated from Q_3 and F_6 and is thus 0.153 mg/ml. The conductivity of the regenerate, $\kappa_{0,S}$ equals the conductivity of the solution used for regeneration, which is 110 mS/cm and was measured prior to the start of the experiment. Since the diafiltration step is assumed to have complete retention, mass flow of the regenerate (Q_3) equals the mass flow of the retentate (Q_R), which is continuously pumped out of the UDF reservoir at a volumetric flow F_R that must be the same as F_6 (see Eq. (8)). So Q_R is also 0.29 mg/min and F_R is 1.9 ml/min. Therefore, also the protein concentration in the retentate c_R is the same as c_3, namely 0.153 mg/ml. c_R determines the dilution factor DF according to Eq. (12). Consequently, the volumetric flow of the dilution buffer, F_D can be calculated from Eq. (14). The dilution factor was calculated to be 1.53 and thus F_D is 1.01 ml/min. The conductivity of the dilution buffer was measured to be 1 mS/cm. From the volumetric flow of the dilution buffer, F_D, and the volumetric flow of the retentate, F_R, the volumetric flow of the recycle solution F_7 is calculated from Eq. (13) for maximal recycling. It is 0.29 ml/min for this set-up. With F_7 and a selected F_1 the recycling rate R is, according to Eq. (2), 0.69. As the protein concentration of the recycle solution was adjusted to the same protein concentration as the initial solution, $c_0 = 0.1$ mg/ml, the mass flow of the recycle solution Q_4 is therefore 0.29 mg/min. The conductivity of the recycling solution, $\kappa_{D,S}$ is determined by the recycling rate, and calculated according to Eq. (15). $\kappa_{D,S}$ is 1.38 mS/cm. The conductivity $\kappa_{R,S}$ that has to be reached by diafiltration is dependent on the dilution factor and can be calculated from Eq. (16). $\kappa_{R,S}$ was determined to be 1.58 mS/cm. The threshold value of the conductivity of the retentate determines in turn the performance of the UDF process. This is indicated by the volumetric flow of the permeate, F_P. F_P can be calculated from Eq. (17) and has to be 355 ml/min. For maximal recycling, the system yield, Y_0, is 1 according to Eq. (19) and Fig. 4. The outcome of the theoretical calculations was that because of the big difference in conductivity between the regenerate solution and the retentate compared to the difference in conductivity between the retentate and the diafiltration buffer, the permeate flow has to exceed the recycling flow to a great amount. The required permeate flow could not be achieved with the UDF unit used. The permeate flow can be increased by enlargement of the

Table 1
Parameters and results of four different continuous matrix assisted refolding experiments

	Experiment number			
	1	2	3	4
Redox potential in buffers	None	None	Cysteine/cystine	Cysteine/cystine
Bed volume	550	550	370	370
Feed protein concentration, c_1 (mg/ml)	0.09	0.94	0.1	0.097
Feed mass flow, Q_1 (mg/min)	0.38	3.95	0.42	0.393
Recycling rate, R	0	0	0	0.17
Process yield, $Y_1 \pm$ SD (%)	81 ± 14	46 ± 6	25 ± 2	24 ± 1
Number of samplings	6	7	7	3
Recovery, r (%)	91 ± 14	56 ± 6	91 ± 14	89 ± 23
Native mass flow, Q_2 (mg/min)	0.31	1.81	0.104	0.104
System yield, Y_0 (%)	$=Y_1$	$=Y_1$	$=Y_1$	30
Refolding time, t (h)	8.7	8.7	1.7	1.7

membrane surface, but required membranes were not available and could not be operated. Therefore, the diafiltration process was done in batch mode. However, it should not make a big difference concerning yield and recovery whether the recycle solution is continuously prepared or the recycle solution is continuously pumped out of a reservoir containing an already diafiltrated and adjusted protein solution. Hereby, it is demonstrated that all respective parameters can be calculated from the derived equations. Furthermore, the interdependency of the single values of the respective streams is elucidated. Then four different experiments of continuous MAR by ion exchange chromatography were compared. The most important parameters and results are summarized in Table 1. Two different strategies have been applied: The refolding process can either be completed after elution of the protein or during adsorption and subsequent residence time on the column. Whether the protein refolds in the eluate or on the column is solely dependent on the addition of redox reagents. For a buffer system devoid of 2 mM cysteine and 2 mM cystine, the protein is not completely refolded after elution and the chromatographic process serves to remove the denaturing and reducing agents. In this case, no aggregates were formed but it takes a certain time interval (t_P) until the refolding reaction is completed in the eluate. Addition of the redox reagents in the eluate increases the process yield. The refolding time after elution is dependent on the protein concentration of the eluate. For these experiments, the protein concentration is typically 1–10 mg/ml and consequently, the refolding time was estimated to be approximately 7 h. If the running buffers are supplemented with 2 mM cysteine and 2 mM cystine, then the refolding reaction is accelerated and the protein elutes in two peaks, a first containing refolded protein and a second representing the redissolved aggregate fraction. The native protein is completely folded after elution and refolding time can be assumed to be similar to the residence time. In the first experiment, refolding was completed in the eluate and therefore no redox reagents were added to running buffers. The protein concentration of a denatured and reduced solution of α-lactalbumin that was applied to the continuous chromatograph was 0.09 mg/ml. The redox potential was then adjusted in the eluate. In a second experiment, a protein solution with higher concentration (0.94 mg/ml) was applied, and refolding was also completed in the eluate. In a third experiment, refolding of a protein solution of similar concentration as used in experiment 1 was refolded during chromatography and in the last experiment, the aggregate fraction of the third experiment was recycled. In Fig. 5, a chromatogram of experiment 3 is shown. High UV adsorption at 280 nm of the flow through caused by non-retarded compounds of the feed solution (solid line) requires protein determination by the Bradford method for a quick screen. The first protein peak corresponds to native protein, the second protein peak is the regenerate, which contains the dissolved aggregates. Since a continuous monitoring of all different streams is not possible, samples from all 90 outlet ports were taken at several time intervals (see number of samplings in Table 1). From these samples, UV absorbance at 280 nm, conductivity and protein concentration by the Bradford method were determined. As the elution angle and thus the number of outlet fraction for native and unfolded protein was either estimated from batch column experiments or similar continuous chromatographic experiments, the Bradford assay was performed with each fraction corresponding to the respective elution angle, which was from 148° to 184° (fractions 37–46) for the native protein and from 232° to 276° (fractions 58–69) for denatured protein. Every fourth fraction of the remaining ones was analyzed to check for protein content. Those fractions containing a high protein concentration as measured by the Bradford method were further analyzed for native and unfolded protein by RP-HPLC. Standard deviation for recovery and process yield (Y_1) was calculated in order to evaluate the stability of the system. Except deviation of recovery in experiment 4, which was high because only three samplings could have been withdrawn, deviation was below 15% that is acceptable for this complex system. The reason why only three samplings could have been withdrawn was that as mentioned earlier, UDF had to be performed offline and therefore, only a limited amount of recycling solution was available. The amount was sufficient for one rotation and—as then steady state was reached—withdrawal

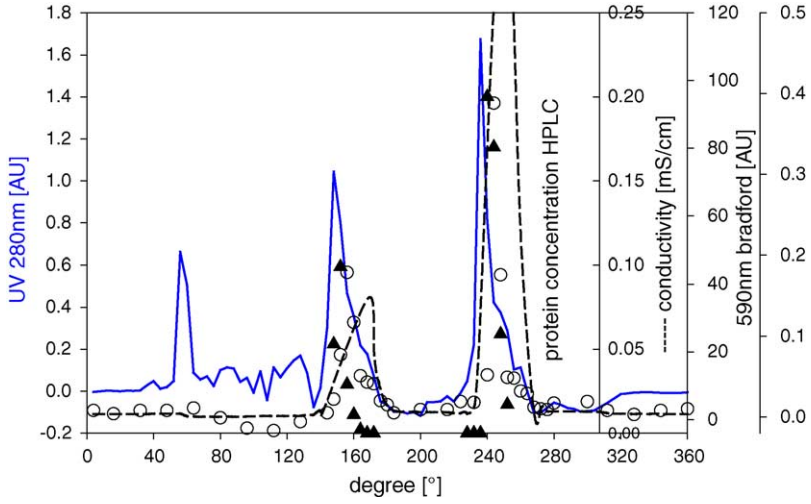

Fig. 5. Representative chromatogram of a refolding experiment with annular chromatography. UV adsorption and conductivity of all 90 fractions were measured offline and the chromatogram was reconstructed by connection of the data points. (—) UV adsorption at 280 nm; (- - -) conductivity (mS/cm); (◯) adsorption at 590 nm after addition of Bradford reagent; (▲) protein concentration (mg/ml).

of three samplings. Recovery and yield were low for experiment 2 because the protein concentration in the feed stream was 1 mg/ml causing protein precipitation and fouling on the top of the column bed. High local protein concentration on the sorbent may be the reason for precipitation. The column had to be repacked after experiment 2, so the bed volume was different for the subsequent experiments. As the ratio of bed volume to mass of protein loaded was large, the column volume is not a critical parameter. However, approximately the same dimensions should be maintained for comparative reasons. A recycling rate of 0.17 in experiment 4 was chosen so that the volume of recycling solution is still enough until the system was in a steady state. Thus, sampling was possible only three times. In Figs. 6 and 7, a mass balance of experiment 3 is shown. Fig. 7 can be compared with Fig. 3 in order to find deviations from theoretical considerations. The system was started with a feed stream that is the initial solution because recycling has not started yet. The mass flow of the native protein eluting in the first peak was 0.13 mg/ml or 30.5% of the initial load. Approximately 20% of the native fraction was determined to be aggregates, formed after elution. Formation of aggregates in the native eluate was not considered in the theory. Therefore, the native protein stream was reduced to 0.104 mg/ml, giving a yield of 25%. The mass flow of the regenerate was determined to be 0.251 mg/ml, which is 60% of the initial load. Recovery was thus 91%. Each single fraction of the regenerate was analyzed by RP-HPLC. Unexpectedly, native protein was found in the first eluting fractions of the second peak, which is indicated by Q_{2b} in Fig. 6. This might be due to dissolution of the aggregate fraction and refolding during migration in the column and subsequent refolding in the eluate, as the concentration of the chaotrop is low in these early fractions. The mass of the native protein in the regenerate could not be neglected. It was approximately 13% of the initial load. Fractions containing native or denatured protein varied from sampling to sampling. Fig. 8 shows mass of native protein represented in light gray bars and mass of total protein represented in dark gray bars in the regenerate fractions 58–63 corresponding to an elution angle between 232° and 252° for seven samplings during one continuous run. Mass of total protein is the sum of native protein and coexisting aggregates. It is determined by RP-HPLC after denaturing and reduction of the sample, as aggregates cannot be quantified directly. Content of aggregates was estimated by subtraction of native protein from the total protein.

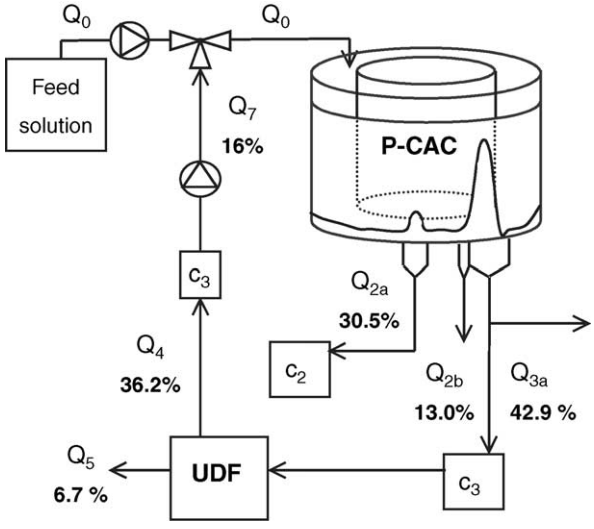

Fig. 6. Mass balance of a continuous MAR experiment with recycling of aggregates. Q_0, Q_1, Q_2, Q_3, and Q_7 are the mass flow of the initial protein solution, the feed solution, the native protein the regenerate and the recycling solution. Q_4 and Q_5 are the protein mass in the retentate and permeate, respectively. Numbers represent the proportion in respect to the amount loaded.

Fig. 8 demonstrates the instability of the system. The number of protein containing fractions varies, the mass of protein in each fraction varies, the ratio of native to total protein varies and the fraction containing most of the protein varied for each sampling. This makes automated sampling difficult and is a severe problem when continuous processing is desired. The phenomenon is called peak wobbling and has been described earlier [1]. Each single fraction has to be analyzed twice, one time for determination of native protein, and one time denatured and reduced for determination of aggregate content, prior to pooling. Peak wobbling is mostly due to inhomogeneous flow that is in turn due to an inhomogeneously packed bed. Regenerate fractions containing native protein were not pooled to the regenerate solution. They could not be added on the yield because concentration of chaotrop was higher than in the fractions elution in the first peak. The mass flow of the pooled regenerate fractions, Q_{3a} was 0.18 mg/min. Q_{3b} is the mass flow of regenerate fractions of low protein concentration and was not pooled. Q_{3b} was 0.016 mg/min. The regenerate was collected for 176 min and the resulting volume was 149 ml having a conductivity of 79 mS/cm. Protein concentration was 0.216 mg/ml. The solution was diafiltrated for 84 min to a final conductivity of 2.6 mS/cm. Due to limited volume of the recycling solution, a low recycling rate was envisaged and therefore, the conductivity of the recycling solution was low enough. The retentate could not be completely recovered due to the hold up volume of the UDF unit, which was approximately 7 ml. Protein was also found in the permeate the amount was approximately 11% from the regenerate. This was unexpected as ultradiafiltration membranes with a 10 kDa molecular weight cut off were used and the protein having a molecular mass of 14 kDa. Rejection was lower than one caused by a different protein conformation when the protein is unfolded. This is explained by the molecular shape of

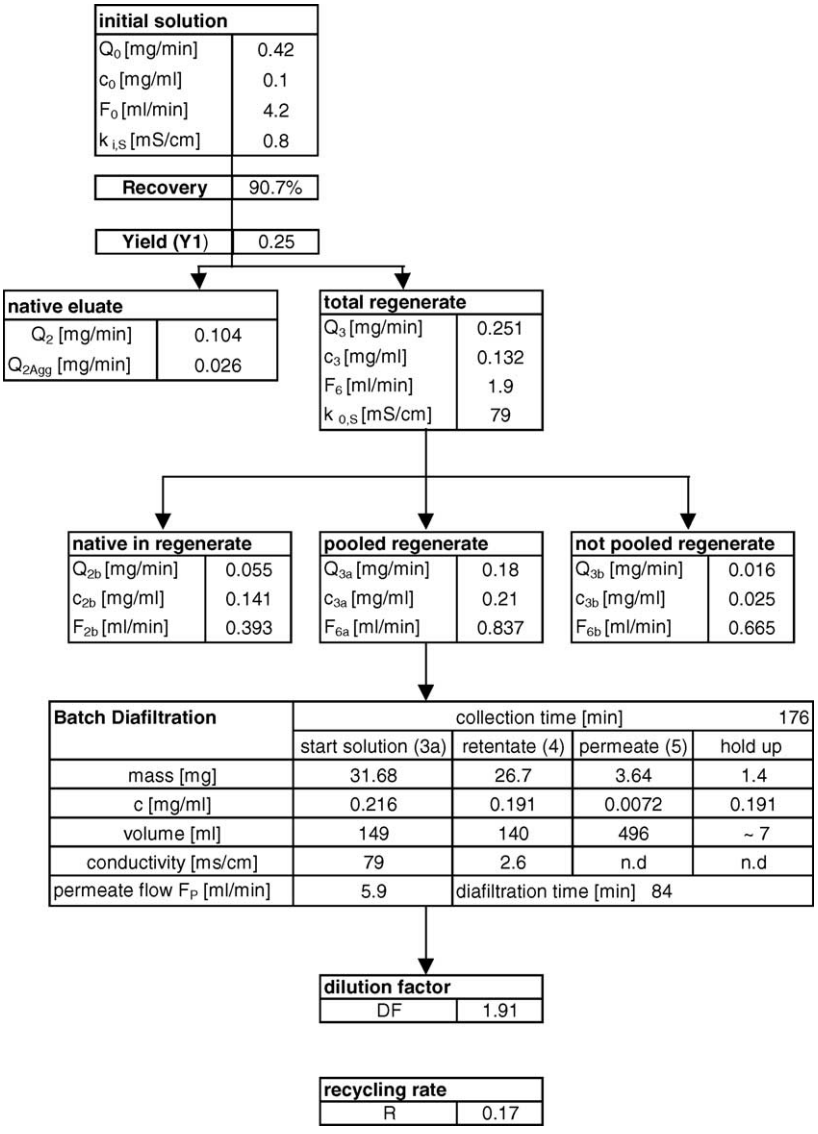

Fig. 7. Experimental data of MAR with recycling of aggregates for a solution considered in theoretical considerations.

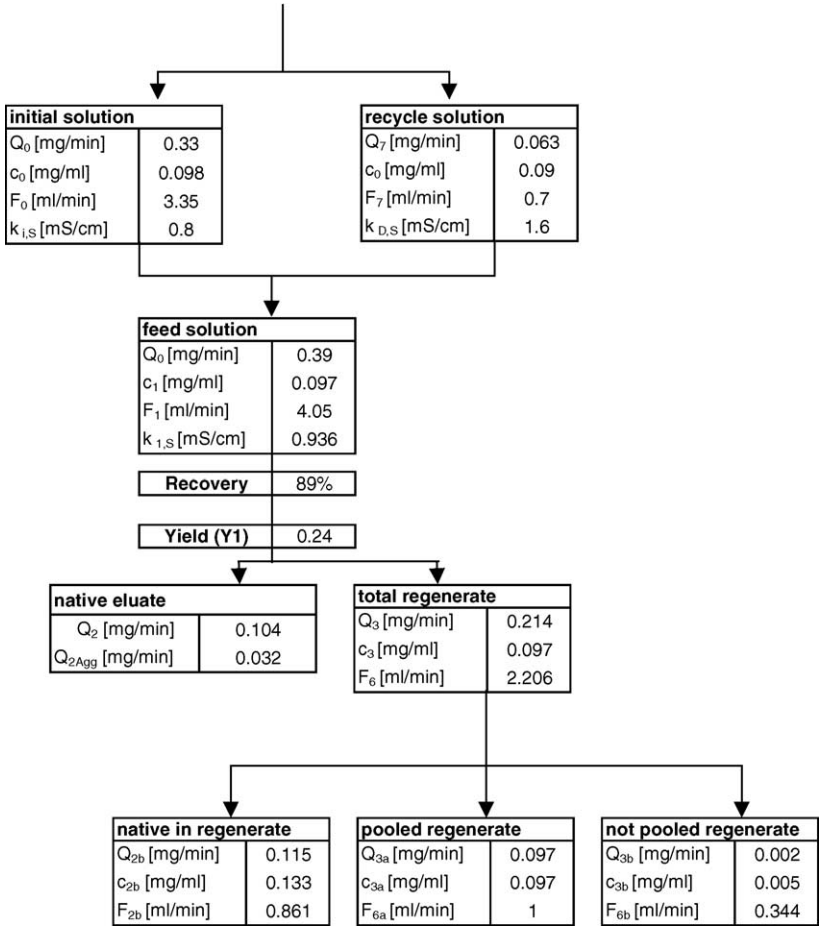

Fig. 7. (*Continued*).

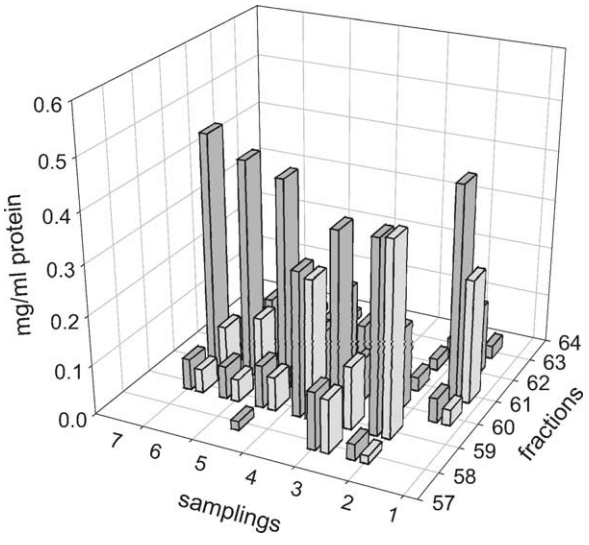

Fig. 8. Distribution of native and total protein in the regenerate fractions for seven subsequent samplings. Light gray bars: native protein (mg/ml); dark gray bars: total protein (mg/ml).

proteins in unfolded state. It is a linear molecule and thus a small fraction may enter the pores of the membrane, whereas completely folded proteins are retained. So, the final protein concentration in the retentate was 0.19 mg/ml. Therefore, the dilution factor was 1.9. The final volume of the recycle solution was 266 ml. The final protein concentration prior to recycling was 0.09 mg/ml, the deviation from a theoretical concentration of 0.1 mg/ml is due to protein degradation. The recycling rate was 0.17 because then more than one rotation of the system could be performed with this recycling solution. Sampling was started after one rotation because then it was ensure to collect recycled protein, but therefore, only three representative samplings could have been taken. During recycling, the initial solution was pumped at a flow velocity of 3.35 ml/min, protein concentration was approximately 0.1 mg/ml. The recycling solution was pumped at the respective flow of 0.7 mg/ml, giving a mass flow of 0.063 mg/ml. The resulting feed mass flow was thus 0.39 mg/ml. Yield from this feed was again 24%, the recovery was about 90%. The aggregate fraction in the native eluate increased to 23.5%. Mass flow of the regenerate was lower due to lower protein load. Flow of the regenerate stream was higher because of peak broadening of the regeneration peak. The elution angle was

broader and consequently, more fractions with lower protein concentration have to be pooled. The amount of native protein in the regenerate increased. Mass flow Q_{2b} was 0.055 mg/min without recycling but increased to 0.115 mg/min with recycling. This was rather due to an instable process than to the fact that protein is recycled. The regenerate was not again diafiltrated because of emerging instability and an expected low volume of recycling solution (three samplings only) and thus the inability to take representative samplings. Results of the recycling experiment are listed in Table 1 for experiment 4 and Fig. 7. The system yield could be increased to 30% under these conditions. Fig. 7 can be compared to Fig. 3 as the same starting conditions were used. Experimental results deviated from the theoretical considerations. First, a recovery of 100% was assumed in theory, which could not be obtained in the experiments. Secondly, the regenerate that could be recycled was less in the experiment than in theory because of unexpected native protein in the regenerate and not pooled side fractions of the regenerate. Third, the recycling rate was not maximal in order to have enough recycle solution for one rotation. Next, a steady state could not be obtained because of the shift of protein containing fractions and their protein concentration. As already mentioned continuous diafiltration is a very critical step. It was demonstrated that continuous refolding of α-lactalbumin with an ion exchange resin is possible.

5. Conclusion

A methodology for continuous refolding with an annular chromatographic system using an ion exchange sorbent is presented. The experiment is designed so that eluting protein is completely folded and concomitantly formed aggregates are separated because of stronger adsorption. This fraction is recycled after diafiltration, and therefore the overall yield is increased. However, peak wobbling and operation in lab scale did not allow to operate the process in a total continuous way. A mathematical description of the set-up is provided to show interdependencies of the single parameters and a scenario is calculated with parameters similar to the experimental one. It was demonstrated that a refolding yield up to 100% is possible in theory.

6. Nomenclature

c_0	concentration of the initial protein solution (mg/ml)
c_1	concentration of the feed protein solution (mg/ml)
c_2	concentration of the native protein solution (mg/ml)
c_3	concentration of the regenerated protein solution (mg/ml)
DF	dilution factor
F_0	flow velocity of the initial protein solution (ml/min)
F_1	flow velocity of the feed protein solution (ml/min)
F_5	flow velocity of the native protein solution (ml/min)
F_6	flow velocity of the regenerated protein solution (ml/min)
F_7	flow velocity of the recycling solution (ml/min)
F_D	dilution buffer flow (ml/min)
F_B	diafiltration buffer flow (ml/min)
F_P	permeate flow (ml/min)
F_R	retentate flow (ml/min)
Q_0	mass flow of the initial protein solution (mg/min)
Q_1	mass flow of the feed protein solution (mg/min)
Q_2	mass flow of the native protein solution (mg/min)
Q_3	mass flow of the regenerated protein solution (mg/ml)
r	recovery
R	recycling rate
Rj	rejection
t	time (min)
u	chromatographic velocity (cm/h)
Y_0	system yield
Y_1	process yield

Greek letters

Θ	elution angle (°)
$\kappa_{0,S}$	conductivity of the regenerate (mS/cm)
$\kappa_{1,S}$	conductivity of the feed protein solution (mS/cm)
$\kappa_{B,S}$	conductivity of the dilution and diafiltration buffer (mS/cm)
$\kappa_{D,S}$	conductivity of the recycling solution (mS/cm)
$\kappa_{i,S}$	conductivity of the initial protein solution (mS/cm)
$\kappa_{R,S}$	conductivity of the retentate and at the same time of the permeate (mS/cm)
ω	angular velocity (°/min)

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