Human Chymotrypsinogen B Production from *Pichia pastoris* by Integrated Development of Fermentation and Downstream Processing. Part 2. Protein Recovery

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The purification of human chymotrypsinogen B (hCTRB) after expression and secretion by the yeast *Pichia pastoris* is described based on two different approaches using integrated initial recovery. Extraction employing aqueous two-phase systems (ATPS) from poly(ethylene glycol) and sodium sulfate allows direct processing of cell containing yeast suspensions of 50% wet weight. The target protein is obtained partially purified in the top phase while cells and cell debris are partitioned to the bottom phase of the system. hCTRB is further purified by adsorption from the top phase to the cation exchanger SP Sepharose Big Beads and elution in a salt step. The single step isolation of hCTRB is possible by expanded bed adsorption (EBA) using a fluidized cation exchanger (Streamline SP XL). A design strategy is shown taking both target protein binding and stable fluidization of the stationary phase in cell containing suspensions into consideration. For the example of hCTRB isolation from cell containing *P. pastoris* suspensions, a successful use of this strategy is demonstrated. Both initial recovery strategies deliver a product that can be further purified and formulated by ultrafiltration/diafiltration followed by lyophilization, resulting in a homogeneous product. Scale-up to 30−90 L of culture suspension was shown for both methods, resulting in a product of similar quality. Comparing both strategies reveals that the two-step ATPS route is better suited for high cell density cultures, while the single step EBA method is preferred for cultures of moderate cell density. This is due to the fact that application of EBA is restricted to suspensions of 10−12.5% wet weight cell concentration, thus necessitating dilution of the original broth prior to sample application. The data presented show that integrated recovery operations are a valuable alternative to traditional processing for systems that are problematic during initial solid−liquid separation.

Introduction

During development of production processes for recombinant proteins the design of cultivation and purification of the target molecule is often performed independent from each other, thus leading to significant problems at the interface of production and recovery. The primary recovery operations, which are supposed to deliver a clarified and partially fractionated product pool of reduced volume, are particularly sensitive to missing coordination of process development. A bad interfacing of production and first recovery steps may result in additional operations in order to make the feedstock compatible with further processing, which in turn elongates the purification process and also may result in reduced overall yield. To demonstrate the benefits of a close cooperation between cultivation and downstream processing design the production and isolation of human chymotrypsinogen B (hCTRB) by recombinant expression and secretion with the methylotrophic yeast *Pichia pastoris* serves as an example of suitable interfacing of two process parts often treated as independent operations.

The suitability of the expression system for production of the zymogen has been demonstrated in Part 1 of the paper. A thorough optimization of the cultivation process regarding both product yield as well as product integrity led to two different culture strategies. A fed-batch protocol with closed-loop controlled methanol feed resulted in a nondegraded target molecule at reasonable space-time yield. This process has the drawback of very high cell concentration (170 g/L cdw ≈ 50% ww), which may lead to scale-up problems in terms of excessive oxygen consumption and heat generation by the metabolically very active cells. Employing a continuous process the steady-state biomass concentration could be reduced (65 g/L cdw ≈ 20% ww) at a concomitantly 5-fold increased space-time yield. The scope of Part 2 of the series is to demonstrate that the high productivity of the cultivation process also results in a high product recovery at the end of the overall production process. The goal of the integrated process development was to interface cultivation and purification in such a way that an easy

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Table 1. Different Types of Aqueous Two-Phase Extraction Systems

type of ATPS	characteristic phase-forming components	mechanism of phase separation
polymer/polymer	PEG/dextran; PEG/starch	incompatibility
polymer/salt	PEG/potassium phosphate; PEG/sodium sulfate	salting out
nonionic detergent	C12EO5; Triton X-100	thermoseparation
thermoseparating polymer	poly(ethylene oxide)/poly(propylene oxide) block-polymers	thermoseparation

"handshake" at the end of the cultivation process allowed the transfer of the culture broth to the primary recovery operation with as few manipulations as possible.

Primary recovery of proteins from secreting yeasts mostly comprises removal of cells by conventional methods such as centrifugation, as well as dead-end or cross-flow filtration. Yeast cells are comparatively large entities (d_p 3−10 μm) and should thus show a suitably high sedimentation velocity that makes centrifugation the primary method of choice. This has been demonstrated by the large-scale production of baker's yeast. Alternatively plate filter systems or vacuum rotary filtration are used for yeast separation on a technical scale in food and beverage processing. As strain and growth conditions have a decisive influence on the sedimentation behavior as well as on filterability, it has to be investigated, whether *P. pastoris*, in particular following the highly productive cultivation processes with methanol as a carbon source, is compatible with these traditional methods of solid−liquid separation. An initial analysis will characterize the *P. pastoris* suspensions obtained by fed-batch as well as by continuous cultivation with regard to filtration and sedimentation behavior. An increased need for rapid and efficient downstream processing of recombinant proteins has put integrated product recovery, which combines solid−liquid separation with initial fractionation, into the focus of attention. Expanded bed adsorption and extraction using aqueous two-phase systems represent methods that fulfill the criterion of vertical integration within a downstream process by allowing the processing of crude feedstock without prior solid−liquid separation. Both methods will be compared with regard to their potential for recovery of hCTRB directly from the culture broth after different cultivation strategies. As the overall goal is delivery of a pure product, an additional criterion for choosing the primary recovery step is the resulting total number of operations required for obtaining a homogeneous product formulation.

Extraction using aqueous two-phase systems (ATPS) has been studied for technical scale protein recovery in great detail (*1, 2*). This technique is based on the formation of two aqueous phases exceeding a critical concentration of potentially phase-forming components. Such components may either be two hydrophilic polymers as poly(ethylene glycol) and dextran (or starch) or a polymer (mainly poly(ethylene glycol)) and a lyotrophic salt (e.g., phosphate or sulfate salts). Phase separation is induced by incompatibility or a salting out effect. Advanced systems using amphiphilic detergent-type phase components make use of thermoseparating phase properties due to reversible hydratization of polar groups (*3, 4*). Table 1 summarizes the basic principles and characteristic representatives of different aqueous two-phase systems. In all cases the resulting phases contain predominantly water and increased concentrations of one of the phase-forming components. Their aqueous nature in combination with significant physicochemical differences makes them an ideal biocompatible extraction system, which is particularly suited for thermolabile compounds such as proteins. ATPS are well compatible with solids containing feedstock, partitioning of solids to the bottom phase with concomitant enrichment of target proteins in the top phase has been reported for many cases. The major advantage of these systems is their suitability for processing of suspensions of up to 50% wet weight without compromising capacity or resolution (*5*). This fact makes ATPS a prime candidate for direct recovery of hCTRB from *P. pastoris* culture broth after fed-batch cultivation, which are characterized by very high cell concentration. Initial information on partitioning of zymogens from Asenjo et al. *(6)*, who partitioned pure α-chymotrypsinogen A in PEG 4000/potassium phosphate phase systems, was available as a good starting point for further investigations.

Expanded bed adsorption (EBA) has been introduced in order to enable direct adsorptive purification starting from crude (solids containing) feedstock (*7−9*). By fluidizing a suitable stationary phase in the respective feedstock in an upward direction of flow a fluidized bed is formed, whose most important property is a void fraction significantly higher than in a packed adsorbent bed. This increased void fraction allows free passage of solids contained in the feed with concomitant adsorption of the target protein. After removing residual solids and unbound proteins in a wash step the protein of interest can be eluted in a particle free, partially purified pool. EBA has been shown to be governed by the same limitations as protein adsorption in packed beds, as long as a perfectly classified fluidized bed is formed under process conditions (*10−12*). The formation of the perfectly classified fluidized bed, which has been termed "expanded bed" by many authors, can be verified by analysis of residence time distributions, both in model solutions (cell free) as well as in crude (biomass containing) feedstock (*13*). Deviations from the ideal expanded bed were shown to be caused by interactions between suspended solids in the crude feed and the fluidized stationary phase (*14*) whereas a pulse response technique for analysis and minimization of these interactions was presented (*15*). It was shown in subsequent investigations that there is a strict coupling between the quality of fluidization and the performance of protein adsorption starting from crude suspensions (*16*). It is evident that a strategy for designing EBA processes must be suited to discover the global optimum both with regard to maximizing binding of the target protein and to minimizing biomass/adsorbent interactions for sufficient quality of fluidization in turbid feedstock. An additional criterion to be taken into consideration during process design is the extent of fluidization in crude suspensions, as it determines the fluid velocity applicable as well as the requirement for dilution of original culture suspensions in order to reduce viscosity and density. To date literature shows that commercially available media for EBA can be challenged with suspensions of a maximum of 15% wet weight biomass concentration at reasonable fluid velocities of $6-8 \times 10^{-4}$ m/s, making EBA an interesting candidate for isolation of hCTRB from continuously produced *P. pastoris* suspensions characterized by moderate cell concentration. In this work a design approach is presented, which shall serve as a general guideline of how to efficiently design EBA processes for industrial protein purification. By comparing the results of both the ATPS- and EBA-based

initial recovery, a general discussion on the benefits and limitations of integrated primary recovery operations will be initiated.

Materials and Methods

Adsorbents, Equipment, and Process Chemicals. Streamline SP XL is a commercially available stationary phase for EBA produced by Amersham Pharmacia Biotech (Uppsala, Sweden); SP Sepharose Big Beads were obtained from the same manufacturer. Poly(ethylene glycol)s of varying molecular weight were purchased from Degussa-Hüls (Marl, Germany). All other process chemicals were acquired from commercial sources in technical grade. The biomass impulse studies were conducted in a homemade column of 0.02 m i.d. as described elsewhere (*15*). Laboratory- and pilot-scale EBA were performed with commercially available columns of 0.05 m i.d. (Streamline 50) and 0.2 m i.d. (Streamline 200, Amersham Pharmacia Biotech, Uppsala, Sweden). All columns were integrated into a conventional setup for EBA.

Analytics. Product titer of hCTRB was determined by proteolytic activity after activation of the zymogen as described in Part 1. Product purity was checked by SDS−PAGE analysis identical to the procedure outlined in Part 1.

Fluid phase viscosity was measured using a rotational viscosimeter (Rotovisco RV 100, Haake, Berlin, Germany), and fluid phase density was determined using a digital densitometer (Paar, Graz, Austria).

Filtration Studies. Batch filtration experiments were conducted at constant pressure in order to determine the filtration characteristics of the *P. pastoris* suspension. A circular disk cheesecloth filter of 0.0095 m^2 area was used at 20 000−100 000 Pa in a conventional setup. The filtrate flow was recorded over time and plotted as t/V_{filtrate} vs V_{filtrate}. The cake resistance α was calculated from the slope of the linear plot, and cake compressibility was obtained by plotting log α vs the pressure applied (for detailed procedures see Perry's Chemical Engineers' Handbook or standard textbooks on unit operations).

Preparation of Aqueous Two-Phase Systems. For optimization of the extraction yield and purity, systems of 10 g total mass were used. Poly(ethylene glycol) and sodium sulfate were added (by weight) to scaled test tubes in order to result in the required system composition. Original culture suspension was added after adjusting it to the required pH until the total mass of 10 g was obtained. The completed systems were mixed in an overhead shaker for 30 min and subsequently centrifuged for 3 min at 4500 rpm in a laboratory centrifuge. The phases were separated and analyzed for product titer and purity. The overall yield (Y) and concentration factor (C_f) of hCTRB was determined as follows:

$$Y = \frac{C_{\text{top}} \cdot V_{\text{top}}}{C_0 \cdot V_{\text{susp}}} \tag{1}$$

$$C_f = \frac{C_{\text{top}}}{C_0} \tag{2}$$

where C_{top} is the top phase of the resulting system and C_0 refers to the original *P. pastoris* suspension added to the phase system.

On an increased scale the respective amount of phase chemicals required were transferred to suitable containers, and then the appropriate amount of *P. pastoris* suspension was added. The mixture was stirred at 25 °C for 2 h, and phase separation was then performed by gravity settling in the temperature interval between 22 and 26 °C. The top phase was removed by a peristaltic pump for further processing.

Finite Bath Protein Uptake Experiments. Streamline SP XL or SP Sepharose Big Beads was placed on a filter funnel and washed with 20 mM sodium citrate buffer at specified pH values. A defined amount of adsorbent (by mass) was placed into a beaker, and a 4-fold mass of the same buffer was added, resulting in a 1:5 slurry of the stationary phase. Twenty-five milliliters of *P. pastoris* culture supernatant was adjusted to the required pH and conductivity (by dilution or addition of NaCl) and placed into a 50 mL shake flask. After a sample for determination of the initial hCTRB concentration was removed, a defined volume of the adsorbent slurry was transferred to the shake flask thus starting adsorption to the stationary phase. The mixture was incubated overnight at 25 °C until the equilibrium was reached. As a control the same supernatant was incubated without adsorbent. The hCTRB concentration at equilibrium (C_{eq}) was determined, and the equilibrium capacity (Q_{eq}) was determined as follows:

$$Q_{eq} = \frac{(C_0 - C_{eq}) \cdot V_l}{V_s} \tag{3}$$

By plotting Q_{eq} versus C_{eq} the equilibrium isotherm was set up and fitted either to the Langmuir model (eq 4) or a linear isotherm (eq 5):

$$Q_{eq} = \frac{Q_{max} \cdot K_a \cdot C_{eq}}{1 + K_a C_{eq}} \tag{4}$$

$$Q_{eq} = K \cdot C_{eq} \tag{5}$$

Measurement of Protein Breakthrough in Packed Beds. *P. pastoris* culture suspension was clarified and adjusted to pH 4.5 and 10 mS/cm conductivity. Streamline SP was filled into a column of 0.005 m i.d. to a bed length of 0.1 m. The bed was equilibrated with 20 mM sodium citrate buffer of the same pH and conductivity as the sample at 6×10^{-4} m/s fluid velocity. The clarified supernatant was then applied to the column, and samples of the effluent were taken and analyzed for hCTRB concentration. After complete breakthrough of the target protein ($C_{effl} = C_0$) sample application was stopped. The normalized effluent concentration (C_{effl}/C_0) was plotted versus the normalized throughput ($T = (V_{effl} \cdot C_0)/(Q_{eq} \cdot V_s)$ in order to determine the loading capacity. The equilibrium capacity of the stationary phase was determined by integration of the breakthrough curve as described by Yamamoto and Sano *(17)*.

Processing of the Top Phase after ATPS Extraction by Packed Bed Adsorption. SP Sepharose Big Beads were filled into a column of an appropriate diameter to accommodate the amount of adsorbent needed at the respective scale to a total of 0.2 m bed height. Equilibration was performed at $4-5 \times 10^{-4}$ m/s fluid velocity with 20 mM sodium citrate buffer at pH 3.0. After phase equilibration the top phase of an ATPS was separated from the bottom phase and diluted 1:2 with equilibration buffer. After checking pH of the so obtained sample the solution was applied to the packed adsorbent bed at the same fluid velocity as above until a maximum hCTRB challenge of 40 mg/mL was applied. After washing with equilibration buffer the target protein was eluted with the same buffer containing 0.6 M NaCl.

Analysis of Cell/Adsorbent Interaction by the "Biomass Impulse" Method. The respective adsorbent ($L = 0.12$ m) was fluidized in a small-scale column (0.02 m i.d.) at 8.3×10^{-4} m/s in 20 mM sodium citrate buffer at varying pH. Sodium chloride was added to the buffer in order to adjust conductivity, and 40-mL pulses of *P. pastoris* suspension (0.1% wet weight) were injected to the system and analyzed before and after the column by UV/vis absorption at 600 nm. Pulse transmission was measured by comparing the pulse area before and after passage through the column as described elsewhere (*15*). The ratio of pulse area before entering (A_{before}) and after passage through (A_{after}) the column yields the transmission parameter P:

$$P = \frac{A_{\text{before}}}{A_{\text{after}}} \tag{6}$$

Purification of *P. pastoris* Culture Suspension by EBA. A suspension of *P. pastoris* was diluted to 10% wet weight of cells and adjusted to the required pH and conductivity for sample application. Streamline SP XL was filled into a column of appropriate scale for the EBA experiment to a sedimented bed length of 0.22−0.24 m. The bed was equilibrated at 5×10^{-4} m/s with 20 mM sodium citrate buffer adjusted to pH and conductivity as required until a stable phase boundary was formed. After checking bed stability by analyzing RTD after addition of an acetone pulse as described elsewhere (*12*), the cell suspension was applied at the same fluid velocity. Washing was performed with equilibration buffer until all residual cells and unbound proteins were removed from the bed. Elution was performed using 20 mM sodium citrate buffer at pH 4.5 including 1 M NaCl at 4×10^{-4} m/s. CIP of the stationary phase was performed using 1 M NaCl in 1 M NaOH solution followed by deionized water, 25% acetic acid, and again by deionized water at the same fluid velocity. After reequilibration of the bed a second adsorption cycle could be performed. All operations were conducted in an upward direction of flow keeping the bed expanded throughout the experiment.

Final Purification and Formulation of Elution Pools from Adsorptive hCTRB Purification. The elution pools were concentrated by ultrafiltration using a PVDF membrane of 10 000 Da cutoff followed by buffer exchange to 0.001 M HCl. The final product was obtained in solid form after lyophilization.

Results and Discussion

Solid−Liquid Separation. To obtain a clarified supernatant, which can be further processed for isolation and purification of hCTRB, preliminary tests of centrifugation and dead-end filtration were carried out with suspensions of *P. pastoris* after fed-batch cultivation. Sedimentation of a 50% wet weight suspension by gravity resulted in a very high entrainment ($V_{\text{sediment}}/V_{\text{total}} \approx 0.85$) of fluid in the sediment even after settling overnight, thus demonstrating typical behavior of hindered settling. Centrifugation at g-forces obtainable in industrial scale centrifuges ($3000-5000 \times g$) also resulted in significant entrainment, making washing of the sediment a necessity for full recovery of secreted hCTRB. As this will result in an increase of fluid volume to be processed in further steps, this procedure was considered not applicable from a production point of view. Filterability of the suspension was characterized by small-scale batch cake filtration at constant pressure drop using cheese filter cloth. For the 50% wet weight *P. pastoris* suspension, a specific cake resistance of 8×10^{12} to 5×10^{13} kg/m was found for 30 000 to 80 000 Pa pressure drop. The cake formed was highly compressible as indicated by a compressibility factor of 1. These values are representative for a problematic filtration system, therefore an alternative initial recovery strategy involving integrated methods was examined.

Development of an Aqueous Two-Phase Extraction. Based on the initial information on extraction of α-chymotrypsinogen A, a study was performed on the isolation of hCTRB from cells containing *P. pastoris* suspension after fed-batch cultivation. The goal was to partition the target protein to the top phase while extracting cells and cell debris to the bottom phase. The criteria for an optimized process were 100% product yield in the top phase and maximum reduction of solids content in the product phase. The extraction system reported in the literature (*6*) was based on PEG 4000/potassium phosphate at pH 7. These conditions were considered not optimal for hCTRB for two reasons. First, at pH values higher than 4.5 a significant degradation of the zymogen takes place, so all processing should take place at low pH. In addition the cultivation process was operated at pH 3.0, so in order to minimize the manipulations prior to isolation of the target molecule a sodium sulfate based phase system was chosen. Second, the isolation of hCTRB from the top phase of the system after extraction is important. Conventional processing of the top phase comprises back extraction into the salt phase of a second two-phase system with concomitant buffer exchange by ultra/diafiltration before packed bed chromatography can be conducted (*18*). For designing an efficient process the number of subsequent operations should be minimized, therefore a direct processing of the top phase by adsorption/elution chromatography was intended. In this case pressure drop across the bed is the parameter to be observed critically. It is influenced by fluid velocity, length of the bed, and the viscosity of the sample, as well as by the diameter of the adsorbent particle used. In addition the concentration of cells in the sample should be as low as possible in order to avoid blockage due to accumulation of cells on top of the bed. In the studies presented here SP Sepharose Big Beads were employed as a stationary phase, since the increased particle diameter ($d_p = 2 \times 10^{-4}$ m) allows increased sample viscosity without compromising the applicable fluid velocity. Nevertheless, using PEG 4000 as a phase-forming component as described by Asenjo et al. (1994) results in a very viscous top phase that has to be diluted considerably prior to application even to this stationary phase.

As a consequence the development of the phase system was conducted with the aim of optimum yield at a minimum PEG molecular weight. Three sets of experiments were carried out using PEG 1000, PEG 600, and PEG 400 and sodium sulfate at pH 3 starting from *P. pastoris* culture suspension after fed-batch cultivation containing 30−50% wet weight of cells. For all systems investigated a range of concentrations was found where full recovery of hCTRB in the top phase was achieved, with concentration factors varying between 1.5 and 2.5. The windows of operation found are summarized in Table 2. A study was performed with the intention to test whether the partitioning of hCTRB to the top phase could be achieved with a consistency and stability suited for a technical scale process. By using a phase system consisting of 14.5% PEG 600 and 10.5% Na_2SO_4 at pH 3, an average yield of 107% ± 16% and an average C_f of 2 ± 0.4 was obtained for eight independently compiled ex-

Table 2. Window of Operation for Full Recovery of hCTRB in Extraction Systems Investigated

ATPS	weight fraction (%) PEG	weight fraction (%) salt	hCTRB concn in top phase (mg/mL)
PEG 1000/Na_2SO_4, pH 3	14−18	9−12	0.2−0.45
PEG 600/Na_2SO_4, pH 3	12−18	10−12	0.5−0.7
PEG 400/Na_2SO_4, pH 3	20−24	6−10	0.28−0.35

tractions. The specific phase system composition was obtained after optimization of the extraction yield using a modified simplex algorithm as described elsewhere (*19*) with the PEG molecular weight and concentration as well as the salt concentration as variables. The optimum found was very broad and confirmed that successful separation of *P. pastoris* cells and target protein by ATPS extraction can be achieved under a variety of operating conditions.

Further processing of the top phase by packed bed adsorption requires a reasonable pressure drop along the column, which in turn is related to the viscosity of the feed solution. Viscosity of the top phase of a 14.5% PEG 600/10.5% Na_2SO_4 two-phase system was measured to be 8×10^{-3} kg·m^{-1}·s^{-1}; after 1:2 and 1:3 dilution this value dropped to 2.7 and 2×10^{-3} kg·m^{-1}·s^{-1}, respectively. To check the compatibility of these solutions with a packed bed of SP Sepharose Big Beads, the pressure drop was measured during application of the top phase at varying dilutions as a function of fluid velocity. The results shown in Figure 1 demonstrate that after a 1:3 dilution of the top phase the pressure drop characteristics are similar to an aqueous buffer and that even the application of an only 1:2 diluted top phase to a packed bed is no problem.

After securing the mechanical stability of the system the adsorption of hCTRB to the ion exchanger was examined. Figure 2 shows the equilibrium isotherm for binding of hCTRB to SP Sepharose Big Beads at pH 3 starting from a 1:2 diluted top phase of a 14.5% PEG 600/10.5% Na_2SO_4 two-phase system. Fitting a Langmuir isotherm (eq 4) to the experimental data results in a Q_{max} of 89 mg/mL and an association equilibrium constant K_a of 14 mL/mg. The comparatively rectangular shape of the isotherm guarantees that even from a diluted top phase the region of favorable binding equilibrium was reached. Optimization of adsorption and desorption conditions resulted in equilibration of the bed with 20 mM sodium citrate buffer pH 3, titration of the 1:2 diluted top phase to pH 3, and elution of the target protein 0.6 M sodium chloride in equilibration buffer. From hCTRB breakthrough during frontal application of a 1:2 diluted top phase a useable capacity of 50% of the equilibrium capacity could be estimated. Therefore the loading of a packed bed of the cation exchanger with 1:2 diluted top phase was set to maximum 40 mg hCTRB per mL of stationary phase at 0.2 m bed length and 5×10^{-4} m/s fluid velocity.

With this protocol hCTRB from 1.5 l of *P. pastoris* culture broth after fed-batch cultivation was purified. The results clearly demonstrate that an efficient process for isolation of hCTRB protein from cell-containing suspension was established (Figure 3). The target protein purity after the various operations was checked by SDS−PAGE. From the SDS gel shown in Figure 3 it can be seen that after two simple steps a very high purity of the target protein was obtained, allowing direct application of final polishing and formulation steps from the ion exchange eluate.

On the basis of these encouraging results, a scale-up of the method was conducted to 30 L of suspension.

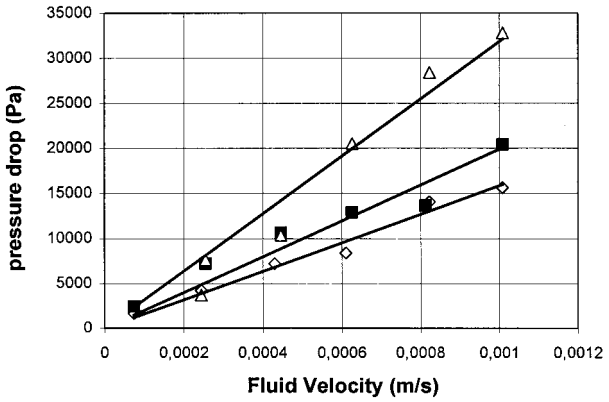

Figure 1. Pressure drop vs fluid velocity for SP Sepharose Big Beads ($L = 0.2$ m) for various mobile phases: (◊) 20 mM sodium citrate buffer pH3; (■) top phase from aqueous two phase system (14.5% PEG 600/10.5% Na_2SO_4) diluted 1:3 with water; (△) top phase from aqueous two phase system (14.5% PEG 600/10.5% Na_2SO_4) diluted 1:2 with water

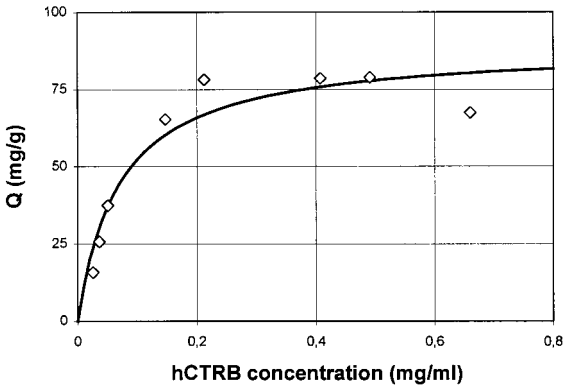

Figure 2. Equilibrium adsorption isotherm for hCTRB in 1:2 diluted top phase of an aqueous two-phase system (14.5% PEG 600/10.5% Na_2SO_4, 75% of *P. pastoris* suspension with 30% wet weight cell concentration) on SP Sepharose Big Beads at pH 3.

Handling of PEG 600 on this scale was problematic because of the fact that at room temperature this compound has a waxlike composition. Therefore the phase system on the larger scale was composed of 20% ww PEG 400 and 10% Na_2SO_4 at pH 3. To have a good solubility of the components in the culture suspension mixing of components had to be performed at 25 °C. Phase separation by gravity settling was performed at the same temperature. It is important to stay within the temperature window of 22−26 °C because the solubility of the salt is reduced at lower temperatures and the phase separation is suboptimal at higher temperature. The hCTRB was isolated from 30 L of *P. pastoris* suspension after fed-batch cultivation containing 50% wet weight of cells and 0.52 mg/mL target protein. hCTRB could be quantitatively extracted to the top phase, while the cells were partitioned to the bottom phase. The top phase was diluted 1:2 and applied to 2 L of SP Sepharose Big Beads under similar conditions (Figure 3). After elution 7.1 g of product were obtained (55% yield) in high purity similar to that in the lab-scale experiment. In summary, it can be concluded that a stable and scaleable process has been developed for the isolation of hCTRB from cell containing suspensions, enabling easy handling of suspensions with very high cell concentration.

Development of an EBA Process. On the basis of the discussion in the Introduction to this paper the following strategy for developing an EBA process is proposed:

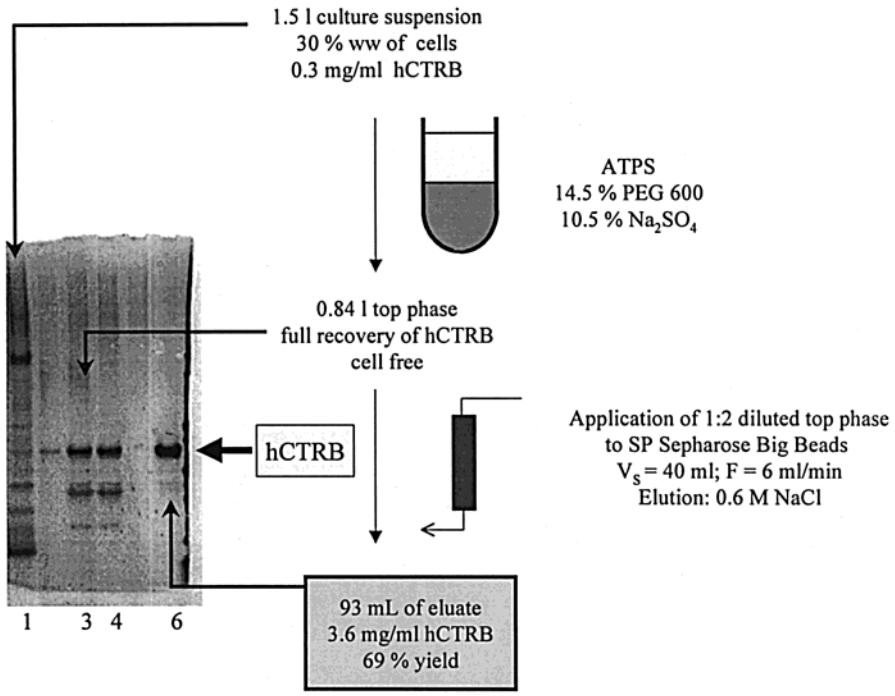

Figure 3. Summary of hCTRB purification by aqueous two-phase extraction followed by adsorption of target protein from top phase to SP Sepharose Big Beads. SDS gel: lane 1, culture supernatant after fed-batch cultivation; lanes 3 and 4, top phase after extraction; lane 6, eluate from SP Sepharose Big Beads

- Optimize binding of target protein to selected adsorbent from clarified supernatant in finite bath experiments; vary fluid-phase conditions such as pH, conductivity, etc. and set up equilibrium isotherm
- Check cell/adsorbent interactions by biomass impulse technique within the same array of operating conditions
- Define window of operation: good static binding capacity, minimized cell/adsorbent interactions
- Measure bed expansion in cell-containing suspension under optimized conditions
- Determine kinetics of protein uptake in packed bed breakthrough experiments under the optimized conditions
- Define elution and CIP protocol
- Perform process evaluation on laboratory scale and scale-up

As chymotrypsinogen is a very basic protein (pI 8.9) adsorption of hCTRB to a fluidized cation exchanger, in our case STREAMLINE SP XL (Amersham Pharmacia Biotech, Uppsala, Sweden), was the method of choice. The most important parameters determining the affinity and capacity of protein ion exchange are pH and the concentration of small ions in the mobile phase. Thus the binding equilibrium of hCTRB to the stationary phase was characterized in finite bath experiments at varying pH and conductivity. An additional criterion, which is very often not taken into consideration to sufficient extent is the protein composition of the sample. Therefore all measurements were performed starting from clarified culture supernatant after fed-batch cultivation of *P. pastoris*. In all cases investigated a linear equilibrium isotherm (eq 5) was found within the range of hCTRB concentration investigated (0.03−0.3 mg/mL). Comparison of the affinity of the target protein toward the stationary phase therefore could be performed using the equilibrium constant K (see Table 3). As expected, the K value is reduced with increasing pH and conductivity. For an easier visualization of the results the equilibrium capacity at 0.2 mg/mL hCTRB concentration is provided. Assuming that during frontal application of *P. pastoris* supernatant approximately 50% of the equilibrium capacity can be loaded onto the bed, Streamline SP XL can be challenged with 30−50 mg hCTRB per mL of adsorbent in a window of operation between pH of 3−4.5 below a critical conductivity of 10 mS/cm.

Table 3. Equilibrium Binding Constant for Adsorption of hCTRB to Streamline SP XL at Varying pH and Conductivity

conductivity (ms/cm)	K			Q_{eq} (mg/mL) at C_{eq} = 0.2 mg/mL		
	pH 3	pH 4	pH 4.5	pH 3	pH4	pH 4.5
8.9	1420	530	390	284	106	78
9.5	450		310	90		62
12.6		370	dnb[a]		74	dnb
16.5	37	dnb	dnb	7.4	dnb	dnb
26.8	19	dnb	dnb	3.8	dnb	dnb

[a] dnb: does not bind.

With this window of operation in mind the compatibility of the fluidized stationary phase with *P. pastoris* cells grown in fed-batch culture was examined using the biomass pulse technique. Figure 4 displays the result of typical experiments, where *P. pastoris* pulses were applied to an expanded bed of Streamline SP XL at 5 mS/cm conductivity and varying pH. As can clearly be seen, below a critical pH value of 4.5 the yeast interacts very strongly with the cation exchanger resulting in almost no cell transmission. At pH 4.5 almost no interaction is found as can be taken from a transmission parameter P of 0.92. As already discussed in the Introduction the major mechanism responsible for binding of cells to ion exchangers is electrostatic interaction of oppositely charged particles. Apparently the surface of *P. pastoris* cells is positively charged at pH values below 4.5, leading to a strong interaction with the sulfonic acid ligands of the adsorbent. In previous papers we have shown that conductivity can be used as a tool to suppress these interactions. Therefore a series of experiments was performed exploiting the mediation of interactions by varying pH and conductivity. The critical value for P,

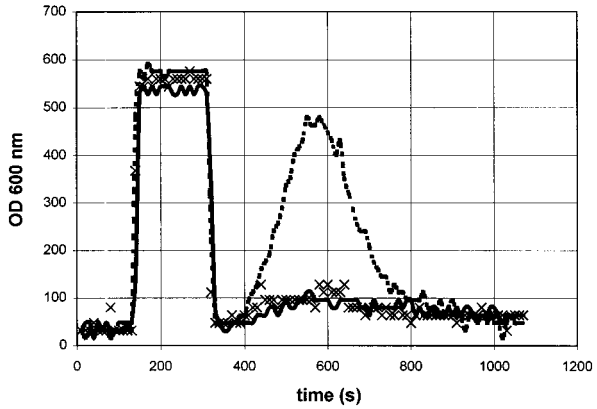

Figure 4. Transmission of *P. pastoris* pulses (40 mL of 0.1% wet weight in 20 mM sodium citrate buffer) through an expanded bed of Streamline SP XL ($L = 0.12$ m, $U = 8.3 \times 10^{-4}$ m/s) at varying pH: (×) pH 3.5, (—) pH 4, (- - -) pH 4.5

which should be exceeded in order to guarantee stable fluidization in terms of a perfectly classified expanded bed in the presence of cells, is 0.95. The results shown in Figure 5 demonstrate that a stable bed expansion in *P. pastoris* suspension can be expected at a pH value above 4.5 at 9.5 mS/cm conductivity. Lower pH and conductivity will result in compromised quality of fluidization, while higher pH and conductivity does not improve the already ideal situation.

By combining the information obtained from the studies on protein adsorption *and* cell/adsorbent interaction, the operating point for direct adsorption of hCTRB from *P. pastoris* suspension can be defined as pH 4.5 and 9.5 mS/cm conductivity. Under these conditions the breakthrough of the target protein to a packed bed of Streamline SP XL was measured in order to determine the loading capacity during frontal application. A typical breakthrough curve, where the normalized effluent concentration X (C/C_0) is plotted versus the normalized throughput T is shown in Figure 6. If the loading capacity for hCTRB is defined as the capacity at 10% breakthrough, then approximately 70% of the equilibrium capacity can be loaded onto a bed of Streamline SP XL. From the early onset of breakthrough it may be concluded that fluid side transport significantly contributes to the overall limitation of sorption performance. To make hCTRB adsorption even more efficient, an increased residence time ($\tau = L/U$ was 158 s in the case reported here) should be used. Reducing the fluid velocity is not advisable because the reduced bed expansion may hamper passage of cells through the bed. For direct adsorption of hCTRB from cell-containing broth the bed length will thus be increased to 0.24 m resulting in a 2.4-fold higher residence time.

The final piece of information missing is the extent of bed expansion under operating conditions. Fluidization studies were conducted in suspensions of varying *P. pastoris* concentration under conditions of full cell transmission at varying fluid velocity. From Figure 7 it can be concluded that at a cell concentration of 10% wet weight a 3-fold bed expansion can be expected at a fluid velocity of 5×10^{-4} m/s. All expansion curves were evaluated according to eq 7, which is derived from the fundamental correlation of Richardson and Zaki (*20*).

$$\frac{L}{L_0} = \frac{1 - (1 - \epsilon_0)}{1 - \left(\frac{U}{U_t}\right)^{1/n}} \tag{7}$$

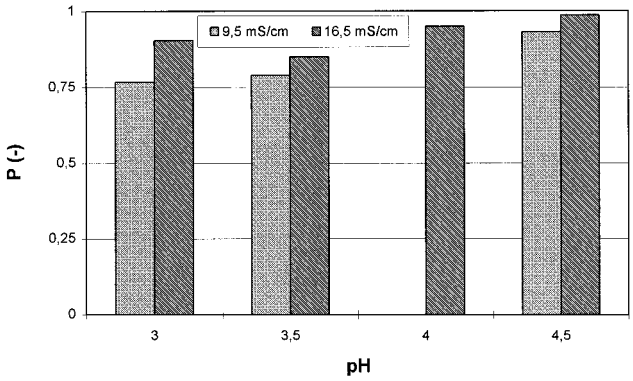

Figure 5. Transmission parameter P for *P. pastoris* pulses (40 mL of 0.1% wet weight in 20 mM sodium citrate buffer) through an expanded bed of Streamline SP XL ($L = 0.12$ m, $U = 8.3 \times 10^{-4}$ m/s) at varying pH and conductivity

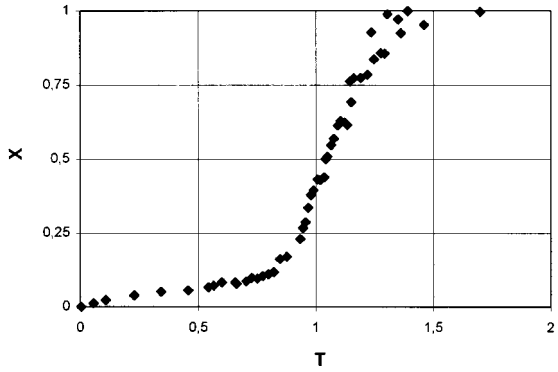

Figure 6. Breakthrough of hCTRB from clarified *P. pastoris* supernatant on a packed bed of Streamline SP XL ($L = 0.098$ m, $U = 6.2 \times 10^{-4}$ m/s, $C_0 = 0.18$ mg/mL)

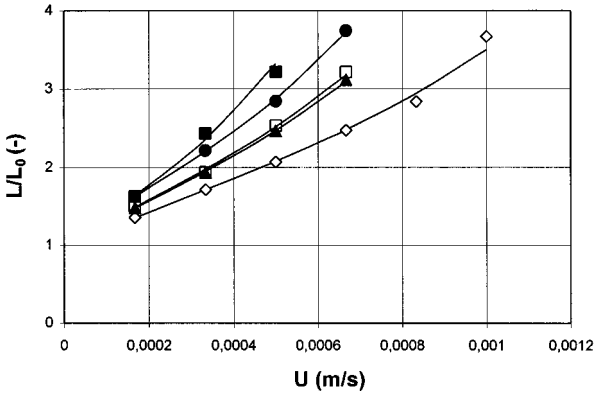

Figure 7. Fluidization of Streamline SP XL in suspensions of *P. pastoris* of varying solids concentration: (◊) cell free supernatant; (▲) 2.6% wet weight; (□) 5.4% wet weight; (●) 7% wet weight; (■) 10% wet weight; (lines) fit to eq 7, parameter values for U_t and n are given in Table 4.

Table 4 summarizes the values for terminal settling velocity U_t and expansion parameter n obtained experimentally. Theoretical values for U_t were calculated according to Stoke's law (eq 8), while n was estimated according to the correlation provided by Al Dibouni and Garside (*21*) (eq 9):

$$U_t = \frac{(\rho_s - \rho_f) \cdot d_p^3 \cdot g}{18 \cdot \eta} \tag{8}$$

$$\frac{5.1 - n}{n - 2.4} = 0{,}1 \cdot Re_t^{0.9} \tag{9}$$

While the values for U_t are reasonably well predicted, the correlation for n just may serve as a trend indicator

Table 4. Bed Expansion of Streamline SP XL during Fluidization in *P. pastoris* Culture Suspension of Varying Cell Concentration

			U_t (m/s)		n	
P. pastoris concn (% wet wt)	fluid viscosity (kg/(m·s))	fluid density (kg/m^3)	expt	calcd	expt	calcd
0	0.00094	997	0.0023598	0.00301	4.7	4.98
2.6	0.00106	1003	0.00179805	0.00259	4.8	5
5.4	0.00121	1008	0.00174379	0.00221	4.8	5.02
7	0.00141	1012	0.00158611	0.00186	5.18	5.04
10	0.00176	1015	0.00117936	0.00147	4.5	5.06

during estimation of the expansion of Streamline SP XL in *P. pastoris* suspension.

With all information at hand the conditions for direct adsorption of hCTRB from *P. pastoris* suspension can be set:

- Application of 10% wet weight *P. pastoris* suspension at pH 4.5 with conductivity adjusted to 10 mS/cm
- Fluid velocity 5×10^{-4} m/s; 0.24 m sedimented bed length
- Loading capacity of Streamline SP XL to maximum 70% of equilibrium capacity as calculated from linear isotherm ($Q_{eq} = 310 \cdot C_0$)
- Load volume: $0.7 Q_{eq} \cdot V_s / C_0$
- After sample application wash with sodium citrate buffer pH 4.5 adjusted to 10 mS/cm conductivity
- Elute in expanded bed mode at 4×10^{-4} m/s fluid velocity with 20 mM sodium citrate buffer pH 4.5 including 1 M NaCl
- CIP in expanded bed mode at 4×10^{-4} m/s fluid velocity using 5 column volumes of a 0.5 M NaOH/1 M NaCl solution

In an initial small-scale experiment 4.8 L of *P. pastoris* suspension after fed-batch cultivation were processed using the 0.05 m i.d. column according to the protocol listed above. After 2.5-fold dilution and adjustment of pH the sample ($V_l = 11.9$ L; $C_0 = 187$ μg/mL) was applied to 0.43 L of Streamline SP XL, resulting in a load of 9% of the theoretical equilibrium capacity. The target protein was obtained in the eluate at 21-fold reduced volume in 80% yield. In an SDS gel only a single band was detected in the elution pool (not shown), thus demonstrating the successful development of an EBA process allowing the direct purification of hCTRB starting from crude *P. pastoris* suspension.

Fine Purification and Formulation. As the eluates from both EBA and cation exchange after ATPS extraction yielded a product of very high purity, it was decided to restrict further purification to buffer exchange and lyophilization. The stability of the zymogen is restricted by degradation, which can be inhibited by storage at pH 3. Therefore concentration by ultrafiltration using a 10 kDa ultrafiltration membrane and subsequent buffer exchange to 0.001 M HCl was chosen in order to stabilize the product after elution from the cation exchanger. HCl was removed by lyophilization of the concentrated pool resulting in 80−100% yield. After redissolving of the lyophilized protein powder in aqueous buffer a single band is detected in SDS−PAGE.

Pilot-Scale EBA Process. Encouraged by the small-scale EBA results an experiment at increased scale was performed under identical conditions. A total of 85 L of *P. pastoris* suspension after continuous cultivation (26% wet weight of cells; $C_0 = 185$ μg/mL) was diluted 2.5-fold and applied to 7.5 L of stationary phase in the 0.2 m i.d. column. The elution pool contained the product 14-fold concentrated in 85% yield and at similar purity as in the small-scale experiment. Ultra/diafiltration to 0.001 M HCl and subsequent lyophilization resulted in a homogeneous product comparable to the small-scale investigations (83% overall yield). The purification was repeated three times with similar results, thus demonstrating that the design rules discussed in the previous chapter in fact provide a sound basis for development of successful and scaleable adsorption of proteins from cell containing broth by EBA. Figure 8 summarizes the pilot-scale results at the example of one specific batch of product.

Conclusions

Conventional methods of primary protein recovery comprise solid−liquid separation followed by volume reduction prior to initial fractionation mostly by adsorption/elution chromatography. In particular the solid−liquid separation step is far from trivial in many cases and may well be the source of significant yield losses and extended process time and effort. The *P. pastoris* expression system described here is a good example for such a situation and is thus well suited for demonstrating the benefits of integrated operations allowing the combination of solid−liquid separation and initial fractionation. ATPS extraction and EBA were chosen as two important candidates from this group of operations. The data presented here nicely illustrate that both methods are well capable of isolating hCTRB from cell-containing suspensions in an efficient manner. Nevertheless significant differences can be found between them. ATPS extraction is characterized by its compatibility with very high cell concentrations in the broth, which is of particular importance during primary recovery of the target molecule from suspensions after fed-batch cultivation resulting in up to 50% wet weight cell concentration. Commercially available stationary phases for EBA require dilution of the feed suspension to maximum 12.5% wet weight, as density and viscosity of high concentration broth reduce the terminal settling velocity of the adsorbent, which in turn leads to unreasonably high bed expansion during sample application (compare eq 7 to eq 9). This is a significant disadvantage of EBA systems, as large amounts of water must not only be added to the feed suspension, they have also to be discharged after passage through the expanded bed. As the effluent contains living recombinant organisms, it has to be inactivated prior to discharge, so the contribution of broth dilution to the overall process cost may be significant in case of high cell density cultures as *P. pastoris* after fed-batch culture. From this point of view ATPS extraction appears to be the superior technology. To allow a fair comparison of both methods, further processing of the hCTRB-containing fractions has to be considered. ATPS extraction directs the target protein to a viscous PEG-rich phase, from which the protein has to be recovered. In the case presented here 1:2 dilution of the top phase allowed application to a packed bed of SP Sepharose Big Beads, which is compatible with feedstock of increased viscosity. The eluate from this step had similar purity as the eluate from the cation exchange EBA, so the ATPS-based strategy requires one additional operation to receive a comparable product, a fact that reduces the advantages of ATPS outlined above. As both ways

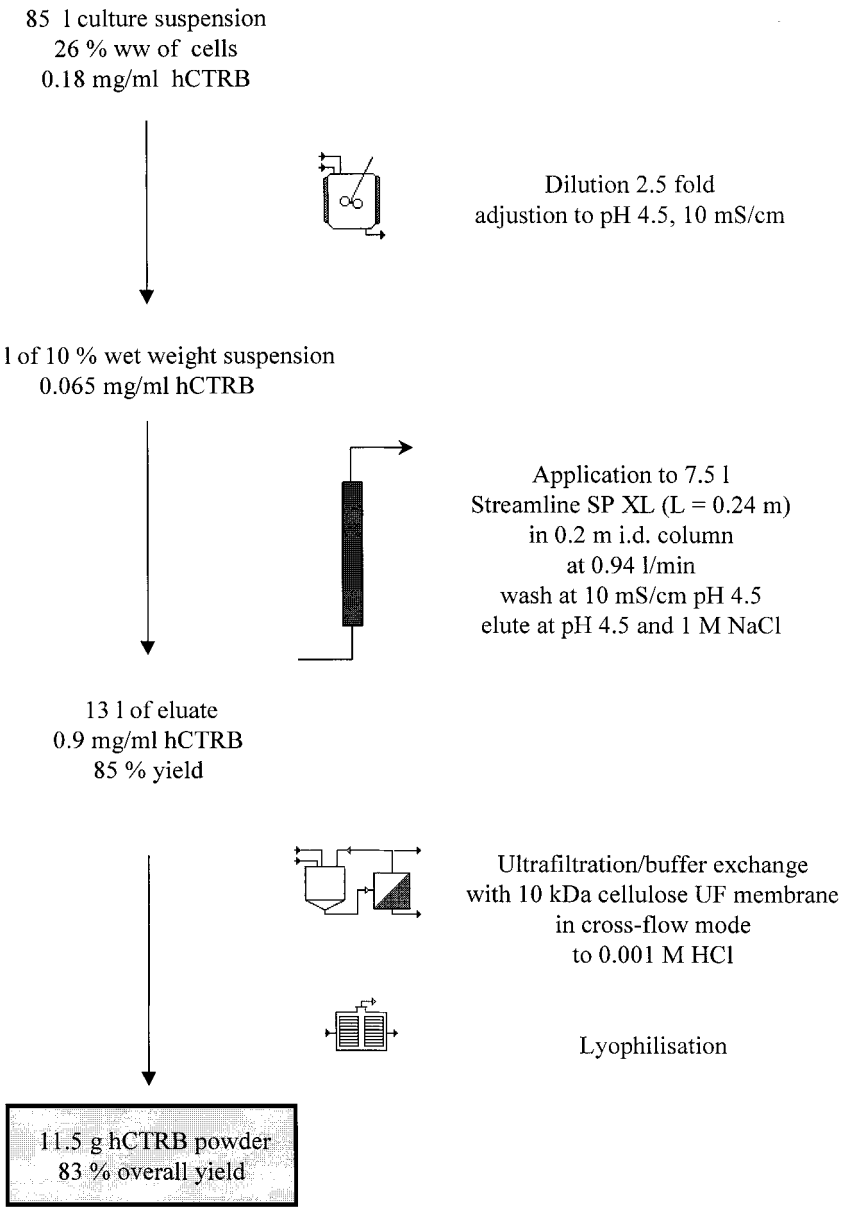

Figure 8. Summary of EBA pilot-scale purification of hCTRB from cell containing *P. pastoris* suspension after continuous cultivation

delivered a clear product solution, which could be further processed by ultrafiltration/diafiltration after simple and fast dead-end filtration, a decision for one of the two integrated strategies should take the cultivation process into account. Continuous production of hCTRB results in cell concentrations in the order of magnitude of 20% wet weight, which requires only a 1:2 dilution prior to EBA. In this case the one-step EBA strategy has distinct advantages and is recommended. High cell density cultures obtained by a fed-batch process call for the ATPS route as in this case 5-fold dilution of the broth prior to EBA is required and introduces an unreasonable increase in overall process and wastewater volume. It should be noted that the points discussed here apply to downstream processing design in general, meaning that the choice of the initial recovery strategy is crucially dependent on the properties of the suspension after cultivation. There is no generally applicable concept for all feedstock, and the integrated recovery route, be it via EBA or ATPS extraction, may be less efficient than conventional solid−liquid separation followed by packed bed adsorption for systems with better filtration characteristics than *P. pastoris*. Integrated processing should be considered as a valuable option for tough solid−liquid separation problems.

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Notation

ATPS	aqueous two-phase system
A_{before}	pulse area before entering expanded bed
A_{after}	pulse area after passage through expanded bed
C	concentration [mg/mL]
C_{top}	concentration in top phase [mg/mL]
C_0	initial concentration [mg/mL]
C_{effl}	concentration in effluent [mg/mL]
C_{eq}	concentration at equilibrium [mg/mL]
cdw	cell dry weight [g/L]
C_f	concentration factor

d_p	particle diameter [m]
g	gravitational constant [9.81 m^2/s]
hCTRB	human Chymotrypsinogen B
K	(linear) equilibrium constant
K_a	association equilibrium constant [mL/mg]
L	sedimented bed height [m]
n	expansion index
P	transmission parameter
Q_{eq}	equilibrium capacity [mg/mL]
Q_{max}	maximum equilibrium capacity [mg/mL]
Re_t	Reynolds number at terminal settling velocity $[U \cdot d_p \cdot \rho_l/\eta]$
T	normalized throughput $[V_{effl} \cdot C_0/(Q_{eq} \cdot V_s)]$
U	fluid velocity [m/s]
V_{effl}	effluent volume [mL]
V_l	fluid volume [mL]
V_s	stationary phase volume [mL]
ww	wet weight [%]
h	fluid viscosity [kg/(m·s)]
ρ_s	solid-phase density [kg/m^3]
ρ_l	fluid phase density [kg/m^3]
t	residence time [s]

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