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Recombinant protein purification using gradient-assisted simulated moving bed hydrophobic interaction chromatography. Part I: Selection of chromatographic system and estimation of adsorption isotherms

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ABSTRACT

The design of gradient simulated moving bed (SMB) chromatographic processes requires an appropriate selection of the chromatographic system followed by the determination of adsorption isotherm parameters in the relevant range of mobile phase conditions. The determination of these parameters can be quite difficult for recombinant target proteins present in complex protein mixtures. The first part of this work includes the estimation of adsorption isotherm parameters for streptokinase and a lumped impurity fraction present in an *Escherichia coli* cell lysate for a hydrophobic interaction chromatography (HIC) matrix. Perturbation experiments were carried out using a Butyl Sepharose matrix with purified recombinant protein on buffer equilibrated columns as well as with crude cell lysate saturated columns. The Henry constants estimated for streptokinase were found to exhibit in a wide range a linear dependence on the salt concentration in the mobile phase. These parameters were applied in subsequent investigations to design a simulated moving bed (SMB) process capable to purify in a continuous manner recombinant streptokinase from the *E. coli* cell lysate.

1. Introduction

Continuous simulated moving bed (SMB) chromatography has become an increasingly attractive alternative to batch chromatography for the continuous purification of biomolecules from laboratory up to industrial scale [1–11]. For model based design of such complex processes, an accurate estimation of the adsorption isotherms of the target components is mandatory [12–15]. The specification of the separation problem and the amount of pure substances available to carry out experiments are essential for the selection of a suitable method to determine the adsorption isotherms [16–19].

The separation of recombinant proteins, produced as a soluble intracellular product, from a mixture of host cell proteins offers considerable challenges [20]. In this work recombinant streptokinase, expressed as a soluble intracellular protein in *Escherichia coli*, was considered as a target protein for the continuous chromatographic purification process. Streptokinase, a 47 kDa protein (single polypeptide chain), is a well known FDA-approved thrombolytic agent, widely used for the cost-effective treatment of congestive heart failure [21] and peripheral vascular diseases [22]. Hydrophobic interaction chromatography (HIC) has been used for the separation of recombinant streptokinase from an *E. coli* cell lysate [23]. Fixed-bed batch chromatography as well as expanded-bed chromatography (Streamline™) have been applied with different HIC matrices for streptokinase purification [24]. However, one of the important criteria for designing continuous (SMB) separations is that the matrix must be completely regenerated during each cycle. Having this in mind, we initially screened several HIC matrices in order to characterize their selectivity for the separation of recombinant streptokinase from the host cell protein impurities, their adsorption properties, as well as their regeneration behaviour. The chosen matrix was used for determining the adsorption parameters of the concerned proteins in order to design a suitable SMB chromatographic process for streptokinase purification.

In this first part of the work, we will discuss the estimation of the relevant adsorption equilibrium constants from pulse and perturbation experiments. The influence of both the protein concentration and the salt concentration in the mobile phase will be

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also investigated. In the second part of the work will use these adsorption parameters for designing the continuous purification of recombinant streptokinase exploiting a gradient-assisted SMB chromatographic process using the HIC matrix selected.

2. Perturbation theory

In general, the thermodynamic equilibrium in a chromatographic system at constant temperature is given by the adsorption isotherms [18]. In case of diluted systems and sometimes also in more concentrated solutions, there is a linear relation between the equilibrium concentrations of a component i in the liquid phase, C_i, and in the stationary phase, q_i

$$q_i = H_i C_i \tag{1}$$

where H_i is the Henry constant of component i.

The perturbation method or minor disturbance method is one of the experimental dynamic methods used to determine adsorption equilibrium constants for mixtures [16–18]. It is based first on a step-wise equilibration of the chromatographic column at different saturation concentrations. After equilibration of the column, small pulses of the feed material containing the target component(s) or of pure solvent are injected. The responses (positive or negative) to this perturbation are measured. These responses (retention times or volumes) at various injection concentrations give information about the linear or non-linear course of the adsorption isotherms. A similarity of the retention volumes for different saturation concentrations indicates that the adsorption isotherms are linear. The responses measured on a column equilibrated with the mobile phase (buffer) give the initial slope of the adsorption isotherms.

The theory of the perturbation method is based on the mass balance of the column and the classical equilibrium theory. The coherence condition of the equilibrium theory provides that the applied perturbation will lead to M responses for the M adsorbed components. Hereby, the characteristic retention volumes of the components, $V_{R,i}$, at a certain equilibrium plateau concentration, $C_1^*, C_2^*, \ldots, C_M^*$, can be calculated as follows:

$$V_{R,i}(C^*) = V_0 \left(1 + \left(\frac{1-\varepsilon}{\varepsilon}\right) \left.\frac{dq_i}{dC_i}\right|_{C^*}\right), \quad i = 1, \ldots, M \tag{2}$$

where V_0 is the dead volume, $V_0 = \varepsilon V_{col}$, with V_{col} and ε being the volume and the total porosity of the column, respectively. The information about the competitive nonlinear adsorption isotherms is included in the total derivatives dq_i/dC_i [16–18,25].

For linear non-competitive isotherms, Eq. (2) can be simplified and the retention volumes are related to the Henry constants of the components by the following equation:

$$V_{R,i}(C^*) = V_0 \left(1 + \left(\frac{1-\varepsilon}{\varepsilon}\right) H|_{C^*}\right), \quad i = 1, .., M \tag{3}$$

A matching of the experimentally determined retention volumes, V^{exp}, and the predicted ones, V^{th}, allows to estimate the parameters of the adsorption isotherm models considered [16–18,25].

3. Materials and methods

3.1. Cultivation conditions

E. coli BL21 (DE3) (Invitrogen) carrying the T7 RNA polymerase under the control of the *lac* promoter in the chromosome was used as a host for the production of recombinant streptokinase. The plasmid pRSETB–STK was used as vector for the intracellular expression of the soluble recombinant streptokinase [23]. *E. coli* cells were routinely grown and maintained in Luria–Bertani (LB) medium. Terrific broth (TB) medium [26] (5 g/L glucose as carbon source) was used in the bioreactor for streptokinase production. Streptokinase was produced in substantial quantities by batch cultivations in 10 L (B. Braun) and 100 L bioreactors (Bioengineering) equipped with pH, temperature, antifoam and dissolved oxygen controllers. Air was sparged at a constant rate (0.5 vvm). Dissolved oxygen was maintained at 20% of air saturation by controlling the agitation rate. The pH was maintained at pH 7 using an acid–alkali dosing pump. A 10% seed inoculum was used for the production bioreactors. Production of streptokinase was induced at OD_{600} 4 with 0.1 mM IPTG. The cell pellet from centrifuged cultivation samples was re-suspended in phosphate buffer (pH 7.2), sonicated and analysed by SDS-PAGE to monitor the product formation. Finally the cultivation broth was harvested around four hours after induction, centrifuged and stored as cell pellet at −80 °C.

The final cell density in batch cultivation was around 6 g/L dry cell mass. The recombinant protein produced was around 20% of the total cell protein, as determined by densitometry (SDS-PAGE analysis). Thus, around 600 mg/L soluble recombinant streptokinase was produced in the batch cultivation. The recombinant protein (stored as cell pellet at −80 °C) was further purified for the adsorption experiments. The feed material for each batch purification process (∼10 g biomass, thawed from −80 °C) was disrupted in a high-pressure homogenizer (Constant Cell Disruptin Systems, UK) at 1.17 kbar and 4 °C and then re-suspended in 50 mL of 20 mM phosphate buffer (pH 7.2). The cell lysate was centrifuged at 10 000 rpm (Sorvall RC 6, Thermo Scientific) for 10 minutes and then filtered using 0.45 μm syringe filters. The clarified solution was used as feed material (made by proportionately mixing the filtrate and $(NH_4)_2SO_4$ stock solution) for a batch chromatographic process using a 10 mL Butyl Sepharose column.

3.2. Chromatographic system

3.2.1. Matrix selection

The screening of the hydrophobic media (Hitrap™ HIC Selection KIT, GE Healthcare) was performed with ÄKTAprime™ plus system (GE Healthcare). The criteria used for the selection of the appropriate HIC matrix include: selective adsorption of streptokinase from the total cell lysate, protein binding capacity of the matrix and complete regeneration of the matrix after a batch separation process. Screening experiments were conducted at a flow rate of $\dot{V} = 1$ mL/min and room temperature. After equilibration with loading buffer, the column was overloaded with the sample (cell lysate). Unbound proteins were washed out and bound proteins were eluted by applying either linear or step gradients. The protein-containing fractions (1 mL each) were collected by following the online UV monitor at 280 nm and analysed by SDS-PAGE. All hydrophobic matrices were screened at three different salt concentrations of the equilibration buffer: 0.25 M, 0.5 M, 0.75 M $(NH_4)_2SO_4$. Among the HIC matrices, Butyl Sepharose High Performance (HP) and Phenyl Sepharose HP were found to be the best matrices for the streptokinase purification. Butyl Sepharose was preferred to Phenyl Sepharose due to its higher selectivity and reversible adsorption behaviour for streptokinase (data not shown).

3.2.2. Preparative batch purification of recombinant streptokinase

The preparative chromatographic experiments were conducted in a column packed with Butyl Sepharose HP matrix (V_{col} = 10 mL) equilibrated with 20 mM phosphate buffer (pH 7.2) containing 200 mM $(NH_4)_2S0_4$. Each batch chromatographic purification process was carried out using 6 mL of feed mixture containing around 10 mg/mL recombinant streptokinase.

The experiments were carried out at a constant feed flow rate of $\dot{V} = 2$ mL/min. The feed material (6 mL) was loaded onto

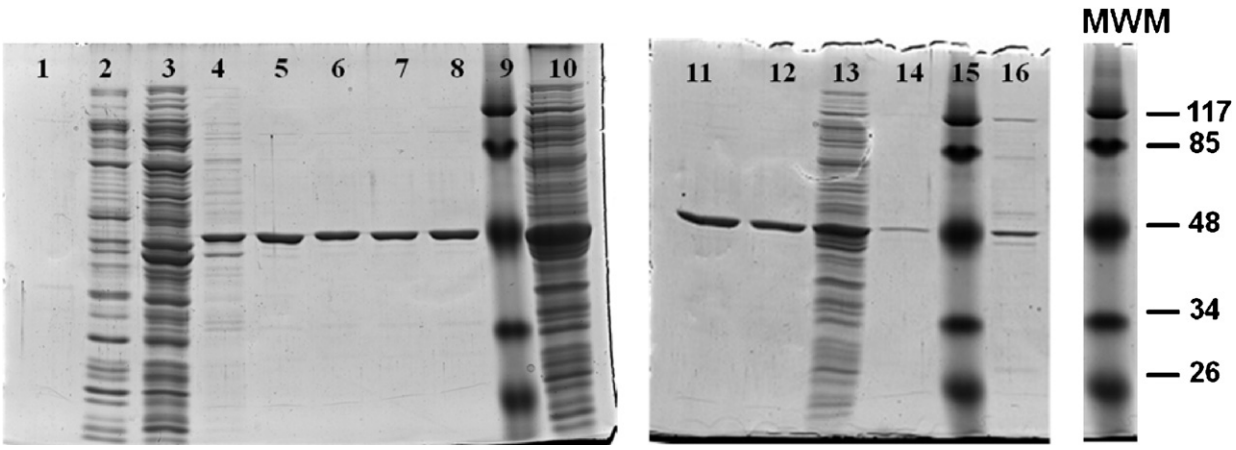

Fig. 1. SDS-PAGE (Coomassie blue staining) of the fractions collected from the preparative batch purification of recombinant streptokinase. Lanes 1–3, unbound fractions; lanes 4–8, 11–12, 25% step gradient elution fractions; lanes 9 and 15, molecular weight marker (MWM); lanes 10 and 13, inlet feed sample; lanes 14 and 16, 100% step gradient elution fractions.

the column and the unbound proteins were washed out with 2 column volumes (CV) of equilibration buffer. The bound streptokinase was selectively eluted by applying a two-step gradient starting from 200 mM $(NH_4)_2SO_4$ in 20 mM phosphate buffer (pH 7.2). The first step was to 25% of 200 mM $(NH_4)_2SO_4$, i.e. 150 mM $(NH_4)_2SO_4$ in 20 mM phosphate buffer (pH 7.2) for 4.5 CV. The second step was to 100% of 20 mM phosphate buffer (pH 7.2) for 6 CV. Fractions of 2 mL were collected by following the UV signal at 280 nm. The streptokinase-containing fractions were analysed by SDS-PAGE (Fig. 1). The analysis showed that only the first 2 fractions after applying the first step of the gradient contains contaminants (Fig. 1, lanes 4 and 5). All other fractions contain predominantly streptokinase (Fig. 1). The average yield of pure recombinant streptokinase in each process (~3 mg, 25% of loaded streptokinase) was collected and pooled in a total volume of 24 mL (12 fractions). The pure protein was further concentrated (100-fold), using a 10 kDa membrane (Millipore). Further, it was used as feed stock for the pulse experiments aiming to determine the Henry constants at various salt concentrations.

3.2.3. Estimation of the adsorption isotherms

Butyl Sepharose HP 1 mL columns (length $L = 2.5$ cm, diameter $d = 0.7$ cm, GE Healthcare) were used for estimation of the adsorption isotherms. The experiments were conducted at room temperature. The number of equilibrium stages of the column, N, were estimated in the conventional manner from linear retention times and peak widths at half heights. The measurements were carried out by analysing the UV_{280} response to 100 μL injections of purified streptokinase with a concentration of 2 mg/mL. A porosity, ε, and a corresponding dead volume, V_0, of the column were estimated from the retention times of the contaminant peaks measured under non-binding conditions. Further, an average plate number was estimated for streptokinase from the variances of the peaks measured at 150 mM, 170 mM and 200 mM $(NH_4)_2SO_4$ in 20 mM phosphate buffer.

The adsorption equilibria were evaluated in two ways: by pulses of different concentrations of purified streptokinase (collected in preliminary studies using repetitive batch chromatographic runs) injected into the columns equilibrated at various salt concentrations (0–200 mM $(NH_4)_2SO_4$); and by perturbations into columns preloaded with cell lysate at 100–200 mM $(NH_4)_2SO_4$. For both types of experiments the injected volumes were $V_{inj} = 100$ μL. For the pulse experiments the concentration of injected purified streptokinase was in the range of 0.06–0.25 mg/mL. For the perturbation experiments the total protein concentration in the cell lysate (containing about 20% streptokinase) was in the range of 0.5–20 mg/mL. The measured retention volumes (peak maxima) of the analytical peaks and of the perturbations peaks were used to calculate the initial slope of the isotherms and to evaluate the linear/non-linear behaviour of the system, respectively. Throughout all experiments multiple fractions (1 mL) were collected and analysed by SDS-PAGE.

3.3. Product analysis

The total protein concentrations were estimated using the Bi-Cinchoninic Acid (BCA) method [29,30]. Presence of streptokinase was determined by 12% SDS gel electrophoresis [27]. A quantitative estimation of the streptokinase concentration was done by SDS-PAGE densitometry (Gel-Pro analyser, Media Cybernetics). Purified streptokinase collected from the preparative chromatography batches was used as an internal standard.

4. Results and discussion

4.1. Estimation of the column parameters

An average porosity and the corresponding dead volume of the 1 mL columns were estimated using the method described above as $\varepsilon = 0.27 \pm 0.02$ and $V_0 = 0.27 \pm 0.02$ mL, respectively. An average number of chromatographic equilibrium stages for streptokinase at the applied flow rate ($\dot{V} = 1.0$ mL/min) and salt concentrations was estimated from several peaks to be $N = 10$.

4.2. Effect of protein and salt concentrations on the retention volume

The results of the pulse experiments conducted with different concentrations (0.06–0.25 mg/mL) of purified streptokinase are illustrated in Fig. 2. It was observed that the retention volumes were independent of the injection concentrations, which indicates linear adsorption behaviour at these concentrations (Fig. 2). The pulse experiments conducted at different salt concentrations show that the retention behaviour of the protein is modulated by the mobile phase salt concentration (Fig. 3). The SDS-PAGE analysis of the collected fractions from both types of experiments shows the presence of a fragment with a molecular weight of 44 kDa along with the 47 kDa streptokinase (Fig. 4) [23,28]. This 44 kDa fragment was not observed in the analysis of purified protein obtained from the preparative chromatographic batch runs. Degradation of streptokinase into fragments

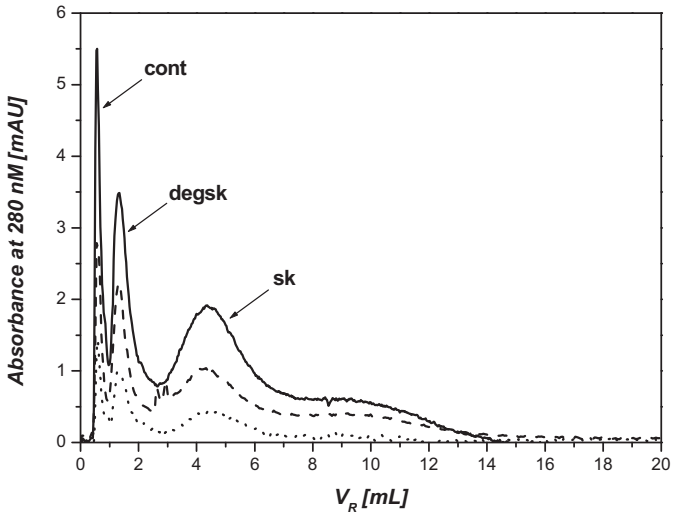

Fig. 2. Pulse experiments – effect of streptokinase concentration. Mobile phase: 20 mM phosphate buffer (pH 7.2) containing 150 mM $(NH_4)_2S0_4$; $\dot{V} = 0.5$ mL/min; V_{inj} = 100 μL; total protein concentrations: 0.25 mg/mL (solid), 0.13 mg/mL (dashed), 0.06 mg/mL (dotted).

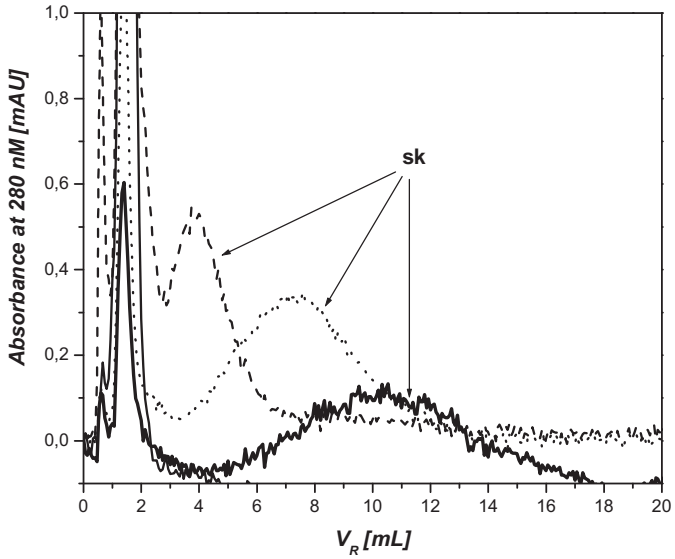

Fig. 3. Pulse experiments – effect of salt concentration. $\dot{V} = 0.5$ mL/min; V_{inj} = 100 μL; total protein concentration 0.2 mg/mL; mobile phase 20 mM phosphate buffer (pH 7.2) with: 0 mM (solid), 150 mM (dashed), 170 mM (dotted), 200 mM (bold line) $(NH_4)_2S0_4$.

occurred when stored at −20 °C without stabilisers. This degradation does not proceed in the intracellular feed mixture containing streptokinase and other cellular impurities [23]. Degraded streptokinase has a lower hydrophobicity in comparison to the native streptokinase and, thus, elutes earlier than native streptokinase (Fig. 4a).

4.3. Determination of the equilibrium constants with pure streptokinase – pulse experiments

Henry's constants for streptokinase (sk) and the degraded streptokinase (deg sk) were estimated using Eq. (3) from the results of the pulse experiments (retention volumes of the peak maxima). The results are summarised in Table 1 and Fig. 5. At lower salt concentration (0–50 mM) of the mobile phase, there is no resolution between the 47 kDa streptokinase and the mixture of 44 kDa degraded streptokinase and cellular impurities. The adsorption equilibrium constants for the 47 kDa streptokinase (sk) increase as the salt concentration in the mobile phase increases. In contrast, these constants remain the same for the 44 kDa polypeptide. At higher salt concentrations of the mobile phase (100–200 mM), streptokinase is clearly better adsorbed than the degraded polypeptide.

Table 1
Henry constants, H_i, determined from pulse experiments at different salt concentrations using Eq. (1).

$(NH_4)_2SO_4$ [mM]	Henry constants (Eq. (1))	
	H_{sk}	$H_{deg\,sk}$
50	2.23	2.23
100	3.69	1.59
150	7.76	1.52
200	11.74	1.57

In the range of salt concentrations, C_{salt}, between 100 mM and 200 mM $(NH_4)_2\ SO_4$, which is of particular interest in the second part of this work, for the Henry constants of the two compounds considered holds a linear dependence on the salt concentration (Fig. 5):

$$H_i(C_{salt}) = a_{1,i} + a_{2,i} * C_{salt}, \quad i = \text{sk, deg sk} \tag{4}$$

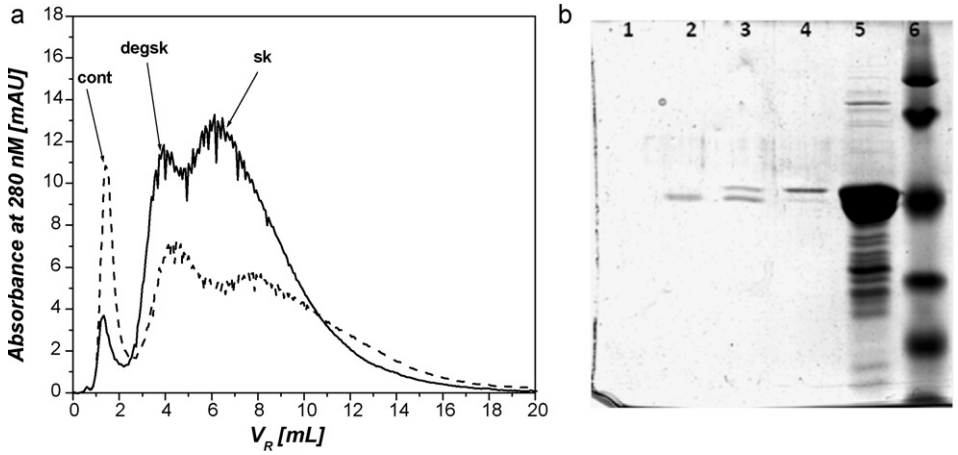

Fig. 4. Analysis of the fractions from the pulse experiments. (a) Mobile phase: 20 mM phosphate buffer (pH 7.2) containing 150 mM $(NH_4)_2S0_4$, total protein concentration 2 mg/mL, fraction volume 1 mL, $\dot{V} = 0.5$ mL/min; V_{inj} = 100 μL (solid); 50 μl (dashed); (b) SDS-PAGE analysis; lane 1 – contaminants; lane 2 – degraded sk; lane 3 – sk + contaminants; lane 4 – sk; lane 5–feed; lane 6 – MWM: 117, 85, 48, 34 and 26 kDa.

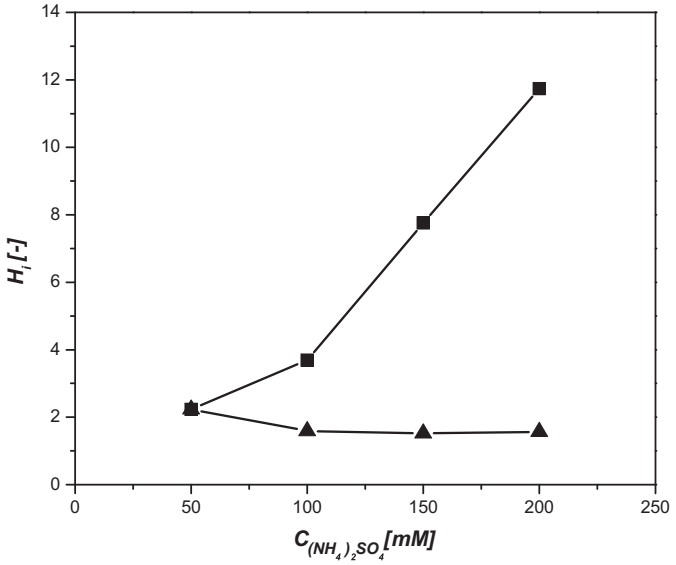

Fig. 5. Henry constants H_i for the streptokinase (squares) and degraded streptokinase + contaminants (triangles) obtained from pulse experiments as a function of the salt concentration.

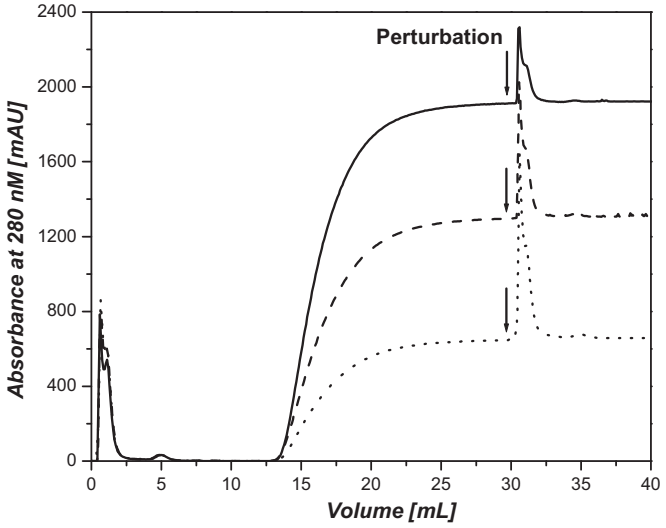

Fig. 6. Perturbation experiment mobile phase: 20 mM phosphate buffer (pH 7.2) containing 150 mM $(NH_4)_2SO_4$, $\dot{V}$ = 1 mL/min; V_{inj} = 100 μL cell lysate with total protein concentration 5.1 mg/mL; fraction volume 1 mL; plateau protein concentrations: 2.55 mg/mL (solid); 1.02 mg/mL (dashed); 0.51 mg/mL (dotted).

Table 2
Parameters of the linear dependence of the Henry constants on the salt concentration (Eq. (4)).

	$a_{1,i}$	$a_{2,i}$ [1/mM]
sk	−4.34	0.081
deg sk	1.59	0.0002

The estimated parameters, $a_{1,i}$ and $a_{2,i}$, are summarized in Table 2. Without loss of accuracy an averaged constant values could be applied for the degraded streptokinase.

4.4. Evaluation of the isotherm shape analysing further perturbation experiments

The highest total protein concentration applied in the pulse experiments was 1 mg/mL. To evaluate possible non-linearities of the adsorption isotherms, experiments were performed on a column preloaded with *E. coli* cell lysate. For these experiments the total protein concentration was varied in the range between 0.5 and 20 mg/mL, thus between 0.1 and 4 mg/mL streptokinase.

Based on the data from the pulse experiments presented before, salt concentrations between 100 and 200 mM $(NH_4)_2SO_4$ were chosen for the perturbation experiments. To perform these experiments the column was equilibrated initially with loading buffer. Then the column was saturated with cell lysate, containing different total protein concentrations. After saturation, a small perturbation was introduced triggered by injecting 100 μl of cell lysate (5.1 mg/mL). The corresponding retention volumes were measured for each plateau. Fig. 6 shows selected results of perturbation experiments carried out at 150 mM $(NH_4)_2SO_4$ and for total protein concentrations in the range between 0.5 and 0.55 mg/mL. The fact that the observed retention volumes do not change significantly in the concentration range covered confirms the presence of linear behaviour. Because of disturbances in the UV signal for protein concentrations above 2.6 mg/mL it was not possible to draw conclusions for higher concentrations.

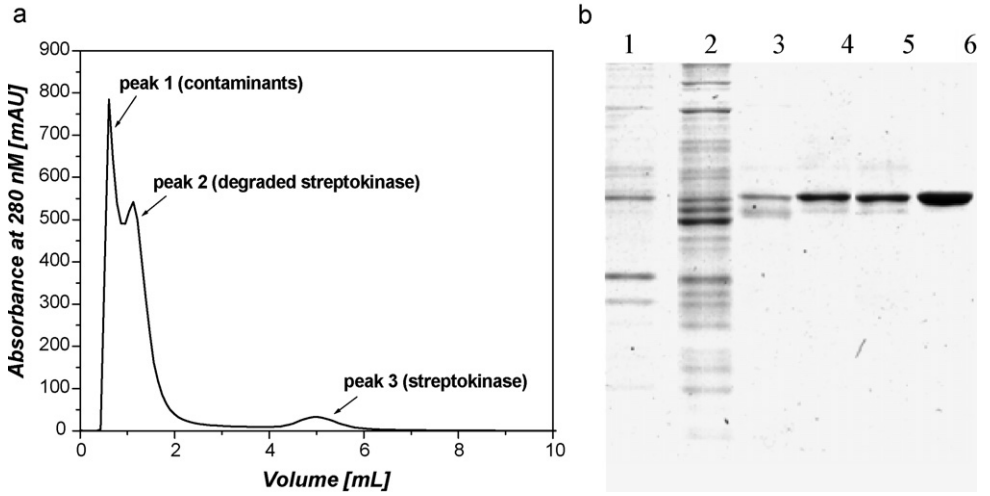

Fig. 7. Analysis of the perturbation experiments. (a) Mobile phase: 20 mM phosphate buffer (pH 7.2) containing 150 mM $(NH_4)_2SO_4$; $\dot{V}$ = 1 mL/min; V_{inj} = 100 μL (cell lysate); total protein concentration 5.1 mg/mL; (b) SDS-PAGE analysis (Coomassie blue staining); lane 1 – peak 1; lanes 2 and 3 – peak 2; lanes 4 and 5 – peak 3; lane 6 – sk standard (in house).

A final validation regarding the elution order of the components was done after collecting and reinjecting fractions from the perturbation plateaus (e.g. Fig. 7a). They were analysed by SDS-PAGE (Fig. 7b). This analysis confirmed that the first two peaks belong to the intracellular impurities and the degraded 44 kDa fragment of streptokinase and the third peak corresponds to the 47 kDa streptokinase. Compared to Fig. 2 an even better separation between streptokinase and the degraded streptokinase is found for the cell lysate samples. Nevertheless for a conservative SMB design the closer Henry-constants obtained from the pulse experiments with the purified streptokinase were applied subsequently.

From the results of the perturbation study it can be justly concluded, that the linear isotherm model (Eq. (1)) holds at least up to protein concentrations of 2.6 mg/mL. The observed dependence allows a simplified design of a continuous chromatographic separation process [2,13,31] described in part II of this work.

5. Conclusions

The determination of thermodynamic data for biomolecules, as e.g. adsorption isotherms, is a difficult and time-consuming process. Simple pulse and perturbation techniques provide useful information. They were applied successfully to estimate the Henry constants at different salt concentrations for streptokinase and its degradation product on a HIC matrix from the total cell lysate mixtures. It was proven that the streptokinase isotherms are linear in a relatively wide concentration range.

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