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Abstract: The isolation and purification of Tat protein from bacterial lysate using avidin-biotin interaction in microfiltration membranes have been reported in the literature. To increase the efficacy of the technique, improvements in flux, Tat separation efficiency, and processing time are essential. In the current research work a pre-filtration step was introduced to remove unwanted high molecular weight proteins and other impurities from feed prior to affinity membrane separation. Significant enhancement in flux and separation efficiency of Tat was observed. Processing time was also reduced significantly. For example, with UF pretreatment step the total Tat recovery was around four times higher (with processing time 25% lower) than that observed with the untreated feed.

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The quality of purified Tat was analyzed by SDS-PAGE, Western Blot, and biotin analysis. Flux behavior in affinity separation was described by model equations.

Keywords: Affinity separation, avidin, biotin, tat, separation efficiency, flux, processing time

INTRODUCTION

Affinity based separation in membranes has opened a new horizon for selective isolation and purification of proteins from mixtures. The fact that affinity based separation involves very specific interaction based on biological properties, makes it attractive among the biotechnologists. It has the ability to obtain superior quality of purified protein in a substantially lower processing time than the conventional column chromatographic separation. Membrane based processes also have economic advantages in terms of polymer support platform and scale-up. The above mentioned advantages are primarily due to hydrodynamic and site accessibility benefits available for membrane based processes over column chromatography (1–6).

Affinity membrane separation, also popularly known as recognition based membrane separation or membrane chromatography, utilizes very specific affinity between a ligand and the target protein (or tagged target protein). The membrane is first functionalized to attach active groups and then the appropriate ligand is incorporated in the membrane structure by reacting with the active groups. The membrane-incorporated ligand then selectively separates the target protein from the mixture (7–13). Microfiltration (MF) membranes are most widely used for affinity separations because of their wide pore structure, which allows easy access of the target protein to the ligands under convective flow condition. The impurities in this type of separation arise from the non-specific protein-protein and protein-membrane interactions, which cause adsorption of unwanted protein molecules in the membrane structure. These types of interactions also lead to fouling in the membrane structure causing decrease in the permeate flux (10, 14).

Decrease in permeate flux due to fouling in membrane structure is the biggest challenge encountered while processing protein solutions through membranes. The mechanism of fouling in membrane structure has been described by many researchers (15–18). Also, the effects of different process conditions and membrane materials on fouling have been studied (15, 19–21). The decline in flux due to fouling is governed by adsorption/deposition of protein molecules on the external surface and/or pores of the membranes. Fouling is most commonly described by any of the following four universal blocking models (17, 18).

(a) In the standard blocking model, it is assumed that reduction in effective radii occurs due to adsorption of molecules in pore walls,

(b) in the complete blocking model, each molecule is assumed to block a pore,
(c) in the intermediate blocking model, some molecules are assumed to block the pores and some attached to the nonporous external surface,
(d) finally, in the cake filtration model, a protein layer (cake) is assumed to cover the external membrane surface.

Studies have also been carried out on fouling mechanisms developed by combination of more than one blocking mechanism (22).

Tat protein is a regulatory protein of Human Immunodeficiency Virus (HIV) type 1 and is very important for AIDS related vaccine and drug development research (23–25). For vaccine development and generation of neutralization antibodies, access to antigenic sites on monomeric Tat is necessary as it is functional in the monomeric form only, not in the dimeric or polymeric forms (26). However, isolation of monomeric Tat is not trivial as it has a high tendency to form polymers due to cysteine rich regions, it binds with RNAse because of basic sequences, and also binds with different cellular proteins (9, 10, 27).

The motivation of membrane separation of Tat came from the fact that conventional packed-bead column chromatography was not able to isolate pure monomeric Tat protein from a complex mixture of proteins (Bacterial Lysate, BL) containing genetically engineered biotinylated Tat. As an alternative, we have reported the affinity based membrane technique to separate Tat from BL using avidin-biotin interaction in MF membranes (9, 10). It was established that pure monomeric Tat protein could be isolated using the membrane based technique. However, it has also been observed that to make the technique more efficient, an increase in separation efficiency of Tat, an increase in permeate flux, and a decrease in processing time are essential. The separation efficiency of Tat could be increased by enhancing the accessibility of the immobilized avidin sites. Permeate flux, and hence processing time could be improved by reducing the fouling. All of these matters were governed by the presence of unwanted high molecular weight proteins and other impurities (cellular debris, etc.) in the BL. Therefore, in the current research work a pre-filtration step was introduced to remove the unwanted high molecular weight proteins and other impurities from BL prior to the affinity separation. Two types of pre-filtration were studied; Ultrafiltration (UF) and Microfiltration (MF). Pre-filtered BL feed containing biotinylated Tat was then subjected to affinity based membrane separation.

The main objective of the current research work was to study the effects of pre-filtration on the separation efficiency, the permeate flux, and the processing time for affinity membrane separation of Tat from BL. The comparative study between the pre-filtered and unfiltered affinity separation was also carried out. The flux decline during affinity separation is described by model equations and the calculated flux values are compared with the experimental values for different situations. The quality of purified Tat protein

obtained from this research was compared with that obtained by packed-bead column chromatography by several analytical techniques.

EXPERIMENTAL METHODS

Equipment and Materials

All experiments were carried out in the stirred membrane cell purchased from the Millipore Corporation (Product number XFUF07601). The anhydride functionalized nylon based MF membranes (Immunodyne) used in all affinity separation experiments were purchased from the Pall Corporation. The pore size of the membranes was 0.45 μm and the thickness was 165 μm. 100 KDa MWCO regenerated cellulose membranes used for UF and hydrophilized PVDF membranes (Pore size 0.1 μm) used for MF were purchased from Millipore Corp.

The Biotin Quantification Kit was purchased from Pierce Biotechnology. The restriction protease Factor X_a was purchased from Roche Diagnostics. The reagents for Bradford protein assay were purchased from the Bio-Rad Laboratories. Tetrameric avidin (MW 67000) and all other chemicals were purchased from the Sigma Corporation. The bacterial lysate (BL) supernatant was prepared in the Department of Anatomy and Neurobiology, the University of Kentucky.

Analytical Protocols

All spectrophotometric measurements were done by UV-VIS Spectrophotometer (Varian, Cary 300). The total protein present in a solution was measured by the well-known Bradford Protein Assay technique (29) using BSA as the standard. Avidin quantification in the feed and permeate was also done by the Bradford Protein Assay technique. All streams of affinity separations were treated for desalting and endotoxin removal prior to SDS-PAGE and Western Blot analysis. SDS-PAGE was performed using 15% polyacrylamide gel under reducing environment. For Western Blot analysis monoclonal anti-Tat Ab and goat anti-mouse IgG were used as primary and secondary antibodies, respectively. Biotinylated Tat protein present in different streams of membrane separation was quantified by measuring the biotin content using HABA (2-(4′-Hydroxyazobenzene) Benzoic Acid)-avidin complex method (30, 31). A detail of this analytical procedure is mentioned below.

Biotin Analysis

HABA-avidin complex method works on the ability of binding of HABA with avidin to give an absorption band at 500 nm. The absorption decreases proportionately as biotin is added to the solution to displace HABA. By

measuring the decrease in absorbance at 500 nm, amount of biotin in different solutions was measured. Then amount of Tat protein was calculated considering 1:1 molar ratio of biotin to Tat. Accuracy of the analysis was tested by adding a known quantity of biotinylated-BSA in different BL solutions. Results were accurate within $\pm 5\%$.

Pre-Filtration

Details of preparation of BL supernatant have been reported in article (9). In brief, the biotin tag was attached to Tat (Tat to biotin molar ratio was 1:1) by bridging with a fusion protein using gene fusion technology while cloning in E.Coli. vector. After expressing Tat protein in the bacterial cell, the cells were lysed to obtain a fermentation broth containing biotinylated Tat along with other unwanted cellular proteins and impurities. It was then centrifuged to remove some of the heavy impurities to obtain BL supernatant (9, 28). BL supernatant contained exposed biotinylated Tat, which were available for binding with avidin. It also contained biotinylated Tat, which were entangled within other protein aggregates due to non-specific interactions. Pre-treatment (dilution and addition of chemicals) of the BL supernatant was necessary to disentangle Tat from those protein aggregates in order to maximize the exposed biotinylated Tat (10). Hence, the feed for pre-filtration was prepared by diluting BL supernatant 30 times with 0.5 M NaCl. The following enzyme and chemicals were added while diluting BL supernatant: RNAse to reduce formation of Tat-RNA complex; dithiothreitol (DTT) to avoid oxidation of proteins, and phenylmethylsulphonylfluoride (PMSF) as protease inhibitor (10). The diluted BL supernatant was then subjected to UF in one case and MF in other.

All pre-filtration experiments were carried out at 25°C with constant stirring at 300 rpm. The trans-membrane pressure for UF was 1.36 bar (20 psi) and MF was 0.34 bar (5 psi). Pressure in the cell was maintained by N_2 gas. To study the effect of pre-filtration on BL, biotin analysis and total protein analysis were carried out on the pre-filtration feed and the permeate. Since, the pre-filtration feed (i.e. diluted BL supernatant) contained significant amount of impurities, the direct measurement of biotin was erroneous. In order to get accurate measure of total biotin, the feed was digested with 10% by volume of 6 N HCl at 120°C for 12 hrs until all proteins hydrolyze, but the biotin structure remains same. Then, biotin analysis was carried out as mentioned earlier to obtain total biotin (exposed + entangled) present. For pre-filtration permeate, both direct analysis of biotin and analysis of biotin from the digested fraction of permeate were carried out. SDS-PAGE images were obtained to observe the effect of pre-filtration on BL feed. Characteristic flux decline was also studied for pre-filtration experiments. The permeate of the pre-filtration experiment was used directly as BL feed (UF BL feed and MF BL feed) in an affinity separation experiment.

Affinity Separation of Tat

Tat protein was separated from BL feed using avidin-biotin affinity interaction in 4-stack Immunodyne membranes system. 4-stack membranes were used to increase the loading of avidin, so that a satisfactory amount of Tat could be separated. Avidin was immobilized by permeating in 0.05 M Na_2HPO_4 buffer at room temperature and 0.34 bar (5 psi) pressure with constant stirring at 300 rpm. Amine groups of avidin molecules formed an amide bond with the anhydride groups present in Immunodyne membranes and became covalently attached to it (10). Details of the separation of Tat from BL using the affinity based membrane separation have been given elsewhere (9, 10). In brief, the pre-filtered (UF BL or MF BL) or unfiltered BL feed (UNF BL) containing the genetically engineered biotinylated Tat was permeated through avidin immobilized 4-stack Immunodyne membranes at 0.34 bar (5 psi) pressure. Biotinylated Tat interacted with avidin and captured from the mixture as shown in Fig. 1. In order to remove the non-specifically adsorbed proteins from the system, it was washed subsequently with wash buffer 1 (WB1, containing PMSF and DTT) and wash buffer 2 (WB2, containing DTT only) until no traces of protein were determined in washing the permeate by Bradford protein assay. The system was then preconditioned for cleavage by permeating the cleavage buffer (CB, containing DTT only). After that, biotin analysis was carried out on different streams to quantify biotinylated Tat. Then the material balance was done on biotinylated Tat present in different streams to determine the amount of Tat separated by membrane based affinity separation. From that the separation efficiency was calculated as discussed later in sub-section on Separation Efficiency. The purified Tat was then cleaved along the fusion protein-tat bond (Fig. 1) by Factor X_a in cleavage buffer (containing DTT). Due to specific cleavage location of restriction protease Factor X_a, purified Tat (known as Tat eluate) ideally should not contain any biotin. But, for column chromatographically purified Tat eluate, biotin impurities have been identified by the HABA-avidin complex analysis. Hence the same analysis was carried out for membrane purified Tat eluate. SDS-PAGE and Western Blot analysis were also carried out to check the purity of the membrane purified Tat and compare with column chromatographically purified Tat.

RESULTS AND DISCUSSIONS

Pre-filtration

The results of pre-filtration experiments are summarized in Table 1 and Figs. 2–3. Diluted BL supernatant i.e. the feed to the pre-filtration step contained 1100 μg/ml of total protein among which 3 wt% was total

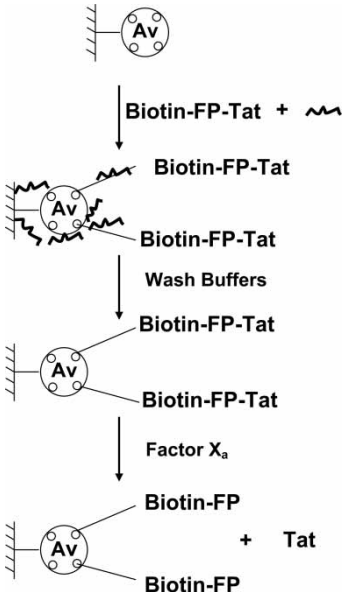

Figure 1. Schematic of affinity separation of Tat protein from bacterial lysate using avidin-biotin interaction in Immunodyne membranes. Biotin-Fusion protein-Tat (Biotin-FP-Tat) is selectively picked up by immobilized avidin (Av) to form Tat-FP-Biotin-Av. Tat is then isolated by cleaving the Tat-FP bond with restriction protease Factor X_a. Non-specifically adsorbed proteins ($\sim$) are removed by wash buffers.

(exposed + entangled) biotinylated-Tat protein. It can be observed from Table 1 that UF was able to remove 85 ± 5 wt% of the total proteins (unwanted proteins) from the diluted BL supernatant. Thus, producing UF BL feed (i.e. UF permeate) containing lowest concentration (155 ± 5 μg/ml) of total protein. Whereas, MF has removed 57 ± 5 wt% of the feed protein, producing MF BL feed (i.e. MF permeate) containing 465 ± 10 μg/ml of total protein. Due to higher rejection of proteins by the UF membrane, it was associated with higher flux decline compare to MF. Fouling in UF and MF can be observed from the flux decline curves shown in Fig. 2. Severe flux drop was observed right from the beginning of UF as proteins started depositing on the external surface of the membrane, blocking some of the pores, and then eventually formed a cake with flux decline almost flattened. Since significant quantities of proteins were rejected by the UF membrane, the gel polarization effect for UF may also cause additional flux decline even though a high rate of stirring was used. For MF, fouling was gradual compared to UF as initially the protein adsorbed mainly inside the pores. Then it also started depositing on the external surface due to formation of aggregates and eventually ended up forming a cake.

Table 1. Effect of pre-filtration (UF and MF) on BL feed. Concentration of total protein in pre-filtration feed = 1100 μg/ml. Concentration of total biotin (exposed + entangled) in pre-filtration feed = 3.8×10^{-3} μmole/ml (i.e. 32 μg/ml Tat)

Pre-Filtration	Protein removed from system, wt%	Conc. of total protein in permeate, μg/ml	Conc. of total biotin in permeate, μmole/ml (μg Tat/ml)	Conc. of exposed biotin in permeate, μmole/ml (μg Tat/ml)
UF	85 ± 5	155 ± 5	3.0×10^{-3} (25)	2.2×10^{-3} (18)
MF	57 ± 5	465 ± 10	3.2×10^{-3} (27)	2.5×10^{-3} (21)

UF: 100 KDa MWCO regenerated cellulose membrane, ΔP = 1.36 bar.
MF: 0.1 μm pore size hydrophilized PVDF membrane, ΔP = 0.34 bar.

The biotin analysis data presented in Table 1 reveal some interesting phenomenon. It can be observed that there is significant difference in concentration of total biotin (obtained from biotin analysis of digested fraction) and exposed biotin (obtained from direct biotin analysis) for both UF and MF permeate. This proves that although the pre-filtration step was able to remove unwanted proteins from BL feed it was not able to disentangle all biotinylated-Tat from non-specific aggregates. There also exists a difference between the concentration of biotin in pre-filtration feed and permeate for both UF and MF, which shows that some of the biotinylated-Tat proteins were inevitably rejected by the membrane due to entanglement in non-specific

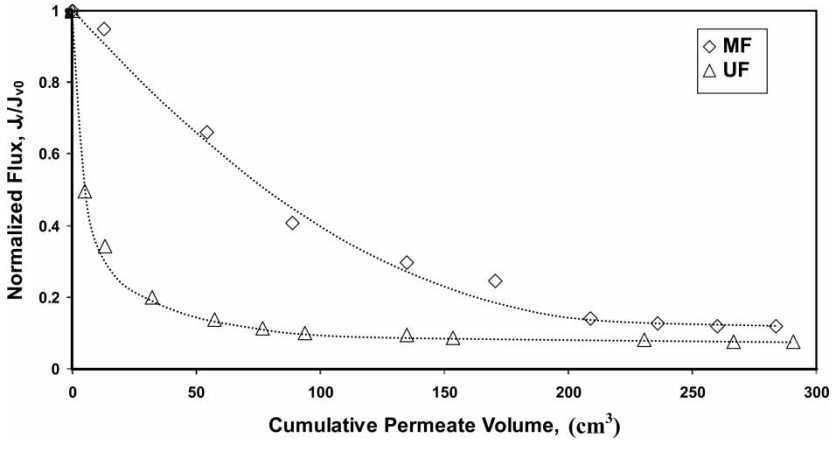

Figure 2. Normalized flux vs cumulative volume for pre-filtration experiments. UF: 100 KDa MWCO regenerated cellulose membrane, ΔP = 1.36 bar, Permeability = 4×10^{-3} cm^3/cm^2-s-bar. MF: 0.1 μm pore size hydrophilized PVDF membrane, ΔP = 0.34 bar, Permeability = 20×10^{-3} cm^3/cm^2-s-bar. Dotted curves represent trend lines for experimental data. Volume of pre-filtration feeds = 300 cm^3.

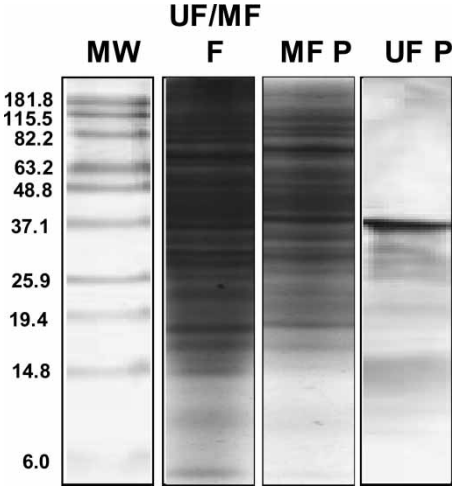

Figure 3. SDS-PAGE images of feed and permeate streams of pre-filtration experiments prior to affinity separation. Total protein concentration in pre-filtration feed (UF/MF F) = 1100 μg/ml, in microfiltration permeate (MF P) = 465 ± 10 μg/ml, and in ultrafiltration permeate (UF P) = 155 ± 5 μg/ml.

protein aggregates and adsorption to membranes. The concentration of exposed biotin was 2.2×10^{-3} and 2.5×10^{-3} μmole/ml for UF and MF permeate, respectively. Since, the exposed biotinylated-Tat was available for binding with avidin, those biotin analysis values were used for further calculations.

Figure 3 shows SDS-PAGE images of process streams before and after pre-filtration. It can be observed from the figure that the MF membrane was only functional to partially reject the high molecular weight proteins as the corresponding bands still exist in lesser intensity. In contrast, the UF membrane, due to its smaller pore size, was able to reject all of the proteins above 35 KDa molecular weight. Although UF membrane was 100 KDa MWCO, the reason of rejecting proteins above 35 KDa was probably the formation of larger size protein aggregates and non-uniform pore size distribution. Both MF and UF were able to completely reject the other impurities (cell debris, etc.).

Affinity Separation

Separation Efficiency

It has been reported (10) that for random covalent immobilization of fixed amount of avidin sites in Immunodyne membranes, the maximum possible

avidin: biotin capture ratio is equal to 1:2 (molar ratio). It is also known that biotin: Tat is 1:1 (molar ratio). Based on these, the separation efficiency of Tat is defined here as

$$\text{Separation Efficiency} = \frac{\text{Actual amount of biotin capture}}{\text{Maximum possible capture of biotin}}$$

$$= \frac{\text{Actual amount of biotinylated Tat capture}}{\text{Tat capture based on avidin to biotinylated Tat ratio of 0.5}}$$

The results of the affinity separation are summarized in Table 2. The total biotinylated-Tat processed for all the three cases was 0.55 μmoles of biotin (i.e. 4585 μg of biotinylated-Tat). For UNF BL, only 0.032 ± 0.004 μmoles of biotin (i.e. 270 ± 30 μg of biotinylated-Tat) was captured, whereas for UF BL and MF BL, 0.1262 ± 0.004 μmoles of biotin (i.e. 1050 ± 30 μg of biotinylated-Tat) and 0.092 ± 0.002 μmoles of biotin (i.e. 750 ± 15 μg of biotinylated-Tat) were captured, respectively. However, it is important to note that the maximum possible capture of biotin by the immobilized avidin (avidin immobilized = 0.09 μmoles or 6000 μg) was 0.18 μmoles (i.e. 1500 μg of biotinylated-Tat) for all of the three cases. Hence, irrespective of nature of the feed, under existing conditions, biotin capture could not be more than 0.18 μmoles. So, the separation efficiency was calculated based on 0.18 μmoles of biotin. UF BL feed has the highest separation efficiency (70 ± 2) followed by MF BL feed (50 ± 1), and UNF BL feed has the lowest separation efficiency (18 ± 2). Lower separation efficiency of Tat for UNF BL feeds was due to lower accessibility of avidin sites by biotinylated Tat. Accessibility depends on both blockage of avidin sites and steric hindrance offered to biotin moieties by unwanted proteins. Since, the amount and the molecular weight of unwanted proteins present was the highest in UNF BL feed, accessibility was

Table 2. Results of affinity separation of Tat by 4-stack avidin-immobilized Immunodyne membranes at 0.34 bar. For all three cases of affinity separation, amount of biotinylated-Tat processed = 0.55 μmoles of biotin (i.e. 4585 μg of biotinylated-Tat), maximum possible capture of biotinylated-Tat = 0.18 μmoles of biotin (i.e. 1500 μg of biotinylated-Tat), and avidin immobilized in 4-stack membranes = 0.09 μmoles (i.e. 6000 μg)

Type of BL feed	Amount of biotinylated-Tat separated (μmole of biotin)	Separation efficiency (%)
UNF BL	0.032 ± 0.004	18 ± 2
MF BL	0.09 ± 0.002	50 ± 1
UF BL	0.126 ± 0.004	70 ± 2

UNF BL = Unfiltered BL, UF BL = Ultrafiltered BL, MF BL = Microfiltered BL.

lowest. On the other hand, the lowest amount of unwanted proteins as well as lower molecular weight proteins were present for UF BL feed compared to others. Hence, it has the highest accessibility. It is a challenging research opportunity to optimize the amount of biotinylated-Tat to be processed so that maximum possible separation efficiency could be achieved in minimum processing time. It is also worth mentioning here that theoretically all of the Tat could be picked up from feed by increasing the avidin sites i.e. by increasing area of membrane or by increasing the number of membranes in stack.

The quality of the purified Tat eluates was analyzed by SDS-PAGE, Western Blot, and Biotin analyses. SDS-PAGE and Western Blot analysis images of membrane purified Tat eluates are presented in Figs. 4(a) and (b), respectively, along with column chromatographically purified Tat eluate. A distinct band near 13–14 KDa can be observed in SDS-PAGE images of all membrane purified Tat eluates along with few additional bands for minor impurities. Whereas, several other bands can be observed due to the presence of a substantial amount of impurities for the column chromatographically purified Tat eluate. It is also worth mentioning here that a minor amount of high molecular weight impurities present could be removed using an additional membrane purification step, if required. The band for Tat (molecular wt. of monomer is $\sim$8335 Da) was obtained at 13–14 kDa due to strong interaction between SDS and the highly basic polypeptide sequence present in Tat. From the Western Blot analysis it was evident that all membrane purified Tat eluates (with or without pre-filtration) provided identical monomeric Tat band. Hence, in Fig. 4(b), the Western Blot image of the Tat eluate obtained from MF BL feed is given, which shows that the membrane based process was able to isolate monomeric Tat protein (image showing a single band for the monomer). On the contrary, Tat isolated by column chromatography was polymeric in nature, as shown in Fig. 4(b), and thus may not be suitable for various medical uses. Biotin analysis results also proved the presence of impurities in the form of biotin for column chromatographically purified Tat. It was determined that the column purified Tat contains impurities equivalent to 17 mole% (0.5 wt%) of biotin, which was substantially high. On the other hand, biotin was not detected (lower limit of detection was found to be 4 mole% of biotin) for membrane purified Tat. It is speculated that the biotin containing impurities might be uncleaved biotin-fusion protein-Tat or biotin-fusion protein or free biotin.

Flux Decline and Modeling

Simplified mathematical models are presented to describe the flux decline due to fouling during the affinity separation only. Two mechanisms of flux decline are considered. In the first one, fouling is assumed to occur both

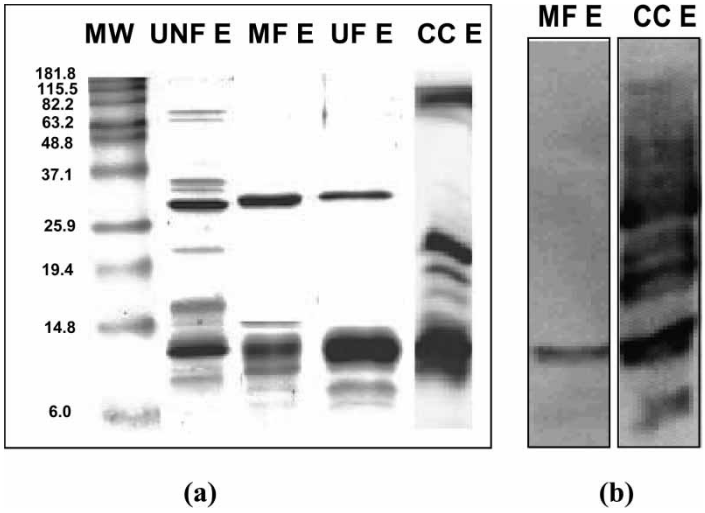

Figure 4. (a) SDS-PAGE and (b) Western Blot images of purified Tat protein eluates obtained by affinity separation. UNF E = Tat Eluate from Unfiltered BL feed, UF E = Tat Eluate from UF BL feed, MF E = Tat Eluate from MF BL feed, CC E = Tat Eluate from Column Chromatography. Western Blot images of Tat eluates from all membrane based separation are identical. Hence only one is shown in the figure.

inside the pores and the external surface of the membrane. This model, denoted here as the "Combined Model," is considered to be applicable for the protein solutions of higher concentration, where the chance of formation of aggregates is significant i.e. for UNF BL feed. In the other model, external surface adsorption is assumed to be negligible and flux decline is described by the adsorption of protein molecules only inside the pores of the membrane thereby reducing the pore diameter. This mechanism of fouling is popularly known as "Standard Blocking" (15) and is considered to be applicable for solutions containing comparatively lower concentration of proteins i.e. for UF BL and MF BL feeds. For both the models it is assumed that the membranes consist of equal numbers of uniform, cylindrical pores, and adsorbed protein molecules form a uniform layer of thickness, δ, within all of the pores. It is also assumed that in 4-stack membranes system, the pores are aligned coaxially on top of each other to form a continuous cylindrical channel of length equal to 4 times the length of a single pore.

The schematic of the membrane pore within which balance is achieved to formulate flux decline due to protein adsorption is shown in Figs. 5(a) and (b). For the combined model it is considered that as the protein molecules permeate through the membrane, some of them adsorb on the pore walls reducing the pore radius from r_0 to r at time t and some of them adsorb on

the external surface of the membrane (Fig. 5a). From protein mass balance, the amount of total adsorption at any time interval can be written as the summation of the amount of pore adsorption and the amount of external surface adsorption as

$$dM_t = N_P(-2\pi r L\rho)\, dr + dM_S \tag{1}$$

where, N_P = number of pores in the avidin-immobilized membrane, which is estimated from pure water flow rate experiment using Hagen-Poiseuille's equation (32) as

$$N_P = \sqrt{\frac{8\mu_w l \varepsilon Q_w A_m}{\pi^2 r_0^6 \Delta P}} = 2 \times 10^9 \tag{2}$$

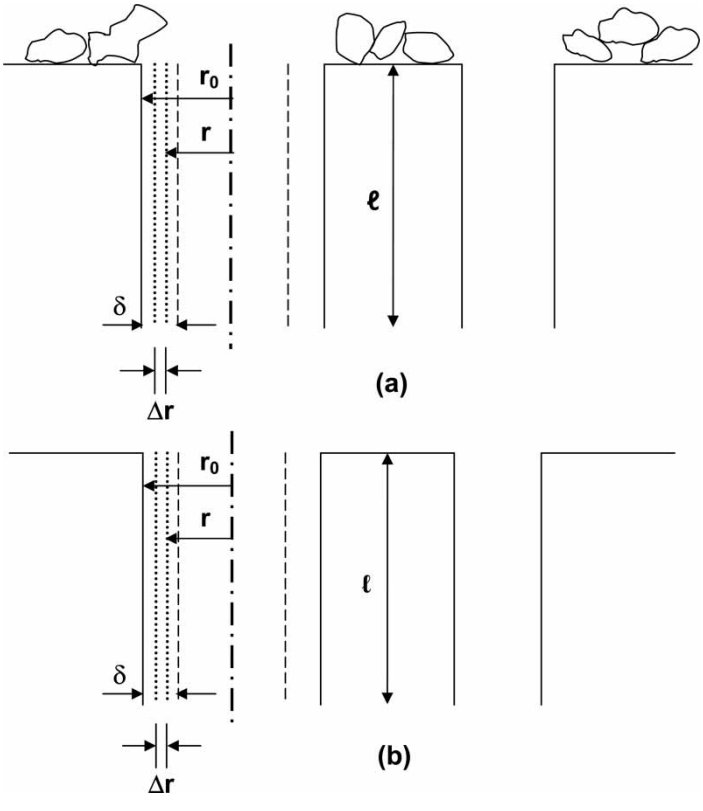

Figure 5. Schematic of adsorption of protein (a) both within the pores and on the external surface of the membranes: Combined Model, (b) only within the pores of the membranes: Standard Model. The thickness of adsorbed protein layer formed at a distance r from the center of pore at any time interval Δt is Δr.

μ_w = viscosity of water = 0.01 gm/cm/s, ℓ = length of a pore = 165×10^{-4} cm, $\varepsilon = 0.32$ (from water adsorption experiment), Q_w = permeate water flow rate = 5.6 cm^3/min = 0.093 cm^3/s, A_m = external surface area of membrane = 33.2 cm^2, r_0 = radius of avidin-immobilized membrane pore = 215×10^{-7} cm(10), $\Delta P = 0.34$ bar = 340000 dyne/cm^2.

L = Total length of a cylindrical channel in 4-stack membranes, $4\ell = 4 \times 165 \times 10^{-4}$ cm

ρ = mass of protein adsorbed in pores per unit volume of adsorbed layer in pore, $\mu g/cm^3$

M_S = mass of protein adsorbed on the external surface of membrane at t, μg

M_T = mass of total protein adsorbed in membrane (external surface + pore) at t, μg

Equation 1 can be integrated with the initial conditions; at $t = 0$, $r = r_0$, $M_S = 0$, $M_T = 0$, to obtain

$$\pi N_P L \rho r_0^2 \left[1 - \left(\frac{r}{r_0}\right)^2\right] = M_T - M_S \tag{3}$$

Hagen-Poiseuille's equation is used to substitute the ratio r/r_0 with the ratio of permeate volumetric fluxes (J_v/J_{v0}) in equation (3). After that, rearranging the resulting equation, an expression for the normalized permeate flux is obtained as

$$\frac{J_v}{J_{v0}} = \left[1 - \left(\frac{M_T - M_S}{\pi N_P L \rho r_0^2}\right)\right]^2 \tag{4}$$

where, J_v = permeate flux at any time t, cm^3/cm^2-s; J_{v0} = initial permeate flux (i.e. at $t = 0$), cm^3/cm^2-s; and J_v/J_{v0} = normalized permeate flux.

Determination of M_T and M_S are necessary to calculate permeate flux. For that, equation (4) is treated as a semi-empirical model and M_T and M_S are obtained by studying the kinetics of total protein and surface protein adsorption, respectively.

The total protein adsorption data are obtained from the affinity separation experiment of UNF BL feed by measuring the concentration of total protein in permeates at different time. It is observed that the amount of adsorbed protein eventually approaches equilibrium. Suki et al. had studied the adsorption kinetics of BSA and observed similar kind of equilibrium adsorption (16). Considering same type of kinetics of adsorption is valid in our case, the rate of adsorption is defined following Suki et al. as:

$$\frac{dM_T}{dt} = K_T\left(M_T^* - M_T\right) \tag{5}$$

where, $M_T^* =$ final amount of total protein adsorbed, μg, and $K_T =$ rate constant of the total adsorption, min^{-1}.

Integrating equation (5) with the initial condition; at $t = 0$ $M_T = 0$, one can obtain

$$M_T = M_T^*[1 - e^{-K_T t}] \tag{6}$$

The experimental values of M_T are plotted in Fig. 6 as a function of time and fitted with equation (6). The best fitting value of the total adsorption rate constant, K_T, is determined to be $0.034\ min^{-1}$, while the value of M_T^* (15000 μg) was known from the material balance on total protein present in different streams of affinity separation of UNF BL feed.

In order to study the kinetics of surface adsorption, a separate batch experiment (no flow condition) was carried out with an avidin-immobilized Immunodyne membrane and UNF BL feed of protein concentration equal to that in affinity separation. The amount of adsorbed protein was determined at different time intervals. Then, similar a procedure as that for total adsorption is followed to obtain

$$M_S = M_S^*[1 - e^{-K_S t}] \tag{7}$$

where, $M_S^* =$ final amount of protein adsorbed on surface = 1400 μg (from material balance on total protein present in different streams of batch experiment), and $K_S =$ rate constant of the surface adsorption = $0.04\ min^{-1}$ (best fitting value obtained from Fig. (6).

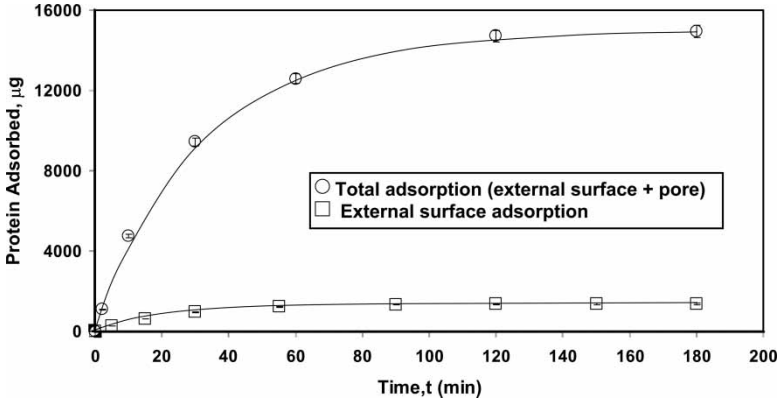

Figure 6. Kinetics of total protein adsorption for unfiltered BL feed. The solid curves represent the best fitted values according to equation 6 for total adsorption and according to equation 7 for surface adsorption, respectively.

The value of ρ is obtained by defining it as

$$\rho = \frac{\left(M_T^* - M_s^*\right)}{N_p 2\pi_0 \delta L} = 1.3 \times 10^5 \mu g/cm^3$$

where, δ = calculated thickness of the protein layer adsorbed within the pores = 61 nm. This value was determined by measuring pure water flow rates through the virgin and fouled membrane and applying Hagen-Poiseuille's equation (10).

Knowing M_T and M_S from equations (6) and (7), respectively, normalized flux for UNF BL can be calculated from equation (4) as a function of time.

To calculate the permeate flux of comparatively dilute solutions of protein, equation (1) can be simplified assuming negligible external surface adsorption i.e. the last term of right hand side of equation (1) is assumed to be zero. The simplified equation can be written as

$$N_P(-2\pi rL)dr = dM_t/\rho = \text{change in volume of adsorbed pore layer.}$$

The change in volume of the adsorbed pore layer, shown in Fig. 5(b), can be correlated with the volume of the adsorbed protein as done by Bowen et al. (15) as

$$N_P(-2\pi rL)dr = XdV \tag{8}$$

where, X = Volume of total protein adsorbed per unit volume of permeate
V = Volume of permeate collected at t = t, cm^3.

Integrating equation (8) with the condition; at V = 0, r = r_0, and then substituting r/r_0 with J_v/J_{v0}, an expression for the normalized permeate flux is obtained as

$$\frac{J_v}{J_{v0}} = [1 - (K_X V)]^2 \tag{9}$$

where, $K_X = X/LA_0$, cm^{-3}; A_0 = Initial pore area = $N_P \pi r_0^2$, cm^2.

Integrating equation (9) by substituting $J_v = (dV/dt)/A_m$ and rearranging gives

$$\frac{1}{V} = \frac{1}{J_{v0} A_m t} + K_X \tag{10}$$

Substituting the value of V from equation 10 to 9 and rearranging gives

$$\frac{J_v}{J_{v0}} = \frac{1}{[1 + (K_X J_{v0} A_m t)]^2} \tag{11}$$

Equation 11 is used to calculate the normalized permeate flux of UF BL and MF BL feeds. It is also used to calculate the permeate flux of UNF BL feed so that a comparison can be made between the applicability of two models for UNF BL feed. K_X values for all three cases are obtained from the Y-axis intercept of straight line fit (not shown in this article) of respective affinity

separation experimental data ($R^2 > 0.98$) according to equation (10). The K_X values are 0.0064, 0.0032 and 0.0006 cm^{-3} for UNF BL, MF BL, and UF BL, respectively. These values are then used in equation (11) to calculate normalized flux (J_v/J_{v0}) values as a function of time. The combined model calculated J_v/J_{v0} values for UNF BL feed as well as the standard model calculated J_v/J_{v0} values for UNF BL, MF BL, and UF BL feed are plotted in Fig. 7 as a function of V. The experimental normalized flux values are also plotted in the same figure for comparison. For all of the affinity separation experiments, J_v/J_{v0} declines with V, however, the extent and nature of the decline are different for different cases. Flux decline for the UNF BL feed was maximum because of the fouling (adsorption of protein) caused by feed containing highest protein concentration. This was followed by MF BL feed and UF BL feed. It was determined that total protein adsorption at the end of each experiment were 15000, 3000, and 2000 μg for UNF BL, MF BL, and UF BL feeds, respectively. Similarly, due to very high rate of fouling the flux decline curve for UNF BL feed is steeper than the others.

It is also observed from Fig. 7 that the standard model calculated J_v/J_{v0} values match quite well with the experimental values of UF and MF BL feeds. This proves that the standard blocking mechanism was sufficient to explain fouling for the UF and MF BL feeds. However, for the UNF BL feed the standard blocking mechanism did not provide J_v/J_{v0} values accurately. The deviation between the experimental and the standard model calculated values was due to surface adsorption of proteins in addition to pore adsorption for the UNF BL feed. It can be observed from Fig. 7 that the combined model

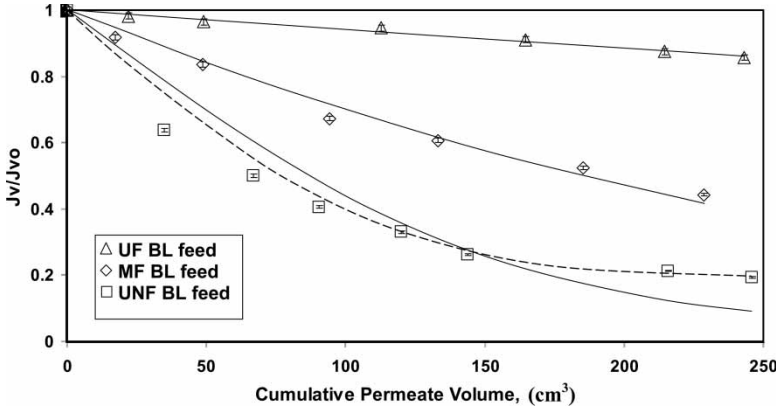

Figure 7. Normalized flux vs cumulative permeate volume for the affinity separation of different BL feeds through avidin-immobilized 4-stack Immunodyne membranes at 0.34 bar. The solid curves represent the Standard Blocking Model calculated values according to equation 11, whereas, the broken curve represents the Combined Model calculated values according to equation 4. UNF BL = Unfiltered BL, UF BL = Ultrafiltered BL, MF BL = Microfiltered BL.

calculated values describe the experimental flux decline for the UNF BL feed more accurately than the standard model. The model calculated values of UF BL, MF BL and UNF BL have indicated that a pre-filtration step was able to restrict the fouling in affinity separation only within the pore structure. The deviation noticed in the model predicted and experimental values for UNF BL feed in the initial phase of affinity separation was probably due to blockage of some of the pores that was not accounted for in the combined model.

Processing Time

Pre-filtration has a critical impact on the processing time of not only the affinity separation, but also the overall process, i.e. including the washing and pre-conditioning for cleavage steps. A comparison of processing time for the various steps of the membrane separation is given in Fig. 8 for different BL feeds. It can be observed from Fig. 8 that a pre-filtration step was able to substantially reduce the overall processing time. Although the pre-filtration step itself has incurred significant time, it has dramatically reduced the processing time for affinity separation, two washing cycles, and the pre-conditioning by cleavage buffer. Time required for affinity separation of pre-filtered feed was less due to overall higher flux (lower fouling). Similarly, time for subsequent wash and cleavage buffers was less due to higher fluxes for the pre-filtered cases. Time for washing cycles was also less because a lower amount of wash buffers were sufficient to remove the

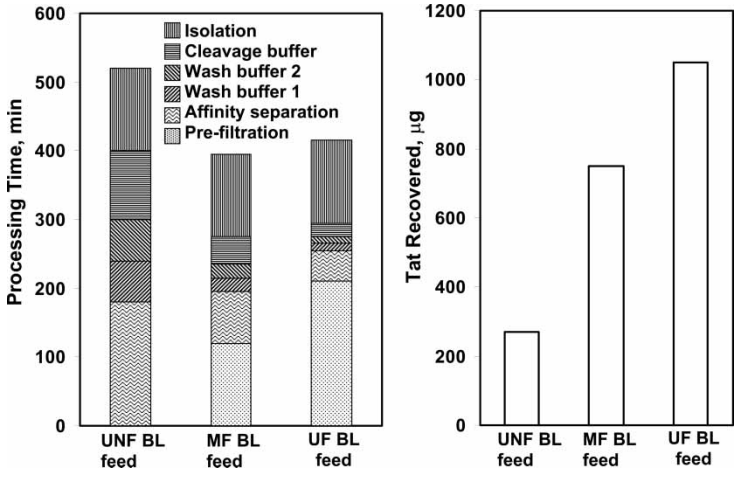

Figure 8. Comparison of total processing time and final amount of Tat recovered for unfiltered BL feed (UNF BL), microfiltered BL feed (MF BL) and ultrafiltered BL feed (UF BL) in separation and purification of Tat from BL. For all cases, external membrane area = 33.2 cm^2, volume of BL feed = 250 cm^3 with 95% permeate water recovery; maximum possible Tat recovery = 1500 μg.

non-specifically adsorbed proteins. However, the isolation of Tat by Factor X_a required the same amount of time for all the three cases as it was dependent on the kinetics of cleavage by the protease. It is also important to notice in Fig. 8 that the amount of Tat recovered was significantly higher for UF BL, followed by MF BL, whereas, it was substantially lower for UNF BL. This shows that pre-filtration step has significantly improved the processing time as well as the extent of Tat recovery.

CONCLUSIONS

The pre-filtration step was able to remove high molecular weight proteins and other impurities from BL feed containing biotinylated-Tat. Accessibility of immobilized avidin sites, and hence the separation efficiency of biotinylated-Tat protein was enhanced for the pre-filtered BL feed compared to unfiltered BL feed. Significant improvement was also observed in the flux decline behavior of the pre-filtered BL feed due to reduced fouling. The processing time was also reduced for the pre-filtered BL feed. However, further studies need to be carried out on the optimization of the amount of Tat to be processed to achieve maximum possible separation efficiency in minimum processing time. The pre-filtered BL feed has also allowed the analysis of biotinylated-Tat content of different process streams of affinity separation directly by HABA-avidin complex method. All the membrane isolated Tat (pre-filtered as well as unfiltered) was pure and monomeric in nature compared to the polymeric nature of Tat obtained by conventional packed-bead column chromatography. Under the experimental conditions mentioned in this research work, a standard blocking model was sufficient to predict the flux decline due to fouling for the pre-filtered BL feeds in affinity separation. However, a combined model containing adsorption on both the external surface and pores of the membrane was necessary to predict fouling behavior of the unfiltered BL feed.

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