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Use of Microfiltration as First Step in Recovery of Protein A From Fermentation Broth

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Abstract

The flux and transmission of protein A during microfiltration have been studied. We studied the performance of two commercial membranes: one made of nylon (Pall Ultipore $Nylon_{66}$, 0.2 μm) and one of polyether sulfone (Pall Omega, 0.16 μm). The $Nylon_{66}$ membrane had by far the best transmission of protein A although a previous study showed that bovine serum albumin (BSA), often used to characterize membranes, had much better transmission through the Omega membrane. The membrane manufacturer also states that the Omega membrane is the best membrane for this kind of application because it is a low-protein-binding membrane. The lower transmission of the Omega membrane for protein A was assumed to be owing to its smaller pores and higher charge density in combination with the larger Stokes radius for protein A. When the pH was lowered, the $Nylon_{66}$ membrane still had the higher transmission. It can thus be concluded that a membrane that is found suitable for the recovery process of one protein is not always the best choice for the recovery process for other proteins even though the membrane is low protein binding.

Index Entries: Protein A; microfiltration; transmission; critical flux; filter cake.

Introduction

Microfiltration is very common in the food and pharmaceutical industry for the separation of microorganisms from the fermentation broth containing the product or for sterile filtration of heat-sensitive liquids. For these applications, crossflow filtration is often used.

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One problem associated with membrane filtration of fermentation broths is that a filter cake of microorganisms often forms on the membrane surface. This filter cake reduces the membrane performance; the flux is often reduced and the retention can be altered since the filter cake often works as a depth filter. The reduction in flux results in an increase in filtration time, and the membrane has to be cleaned more frequently, which leads to increased cost for cleaning chemicals. In addition, the retentive properties of the filter cake are often a major problem when the products are expensive proteins or other macromolecules, because even a small loss decreases the profit.

However, the filter cake is not the only feature that affects the retention. Several studies have shown that a cell-free solution of protein may alter the flux and retention of microfiltration membranes even if the pore radius of the membranes is on the order of 10 times larger than the Stokes radius for the protein molecules *(1–3)*. In these cases, it is believed that the flux is reduced owing to adsorption of proteins on the membrane surface or in the membrane pores, and/or to the formation of protein aggregates that are so large that they cannot pass through the membrane and thus form a filter cake on the membrane *(4,5)*. The transmission of proteins through microfiltration membranes has not been as fully investigated as the decline in flux. However, Heinemann et al. *(1)*, Palecek et al. *(6)*, and Nakamura and Matsumoto *(7)* have conducted studies, on the influence of ionic strength and pH. Unfortunately, the results of these studies are not in accordance with each other owing to different operating conditions, such as pH and ionic strength, and different pretreatment of the samples.

In the present study, the transmission of protein A during crossflow microfiltration was investigated to optimize the recovery of protein A from an industrial fermentation broth. Protein A is used as ligand in the affinity chromatography used for the recovery of immunoglobulin G. The transmission of protein A in two different membranes was investigated: a polyether sulfone membrane (Omega, 0.16 μm) and a nylon membrane (Ultipore $Nylon_{66}$, 0.2 μm). The Omega membrane was chosen because it was claimed by the manufacturer to be a low-protein-binding membrane. It also showed good results for bovine serum albumin (BSA) in a previous study *(8)*. The $Nylon_{66}$ membrane was chosen because it has shown high transmission for protein A *(9)*. The transmission and the critical flux of whole broth was studied. To be able to investigate the transmission of protein A through the membranes without interference from the microorganisms, a fermentation broth from which the microorganisms had been removed was also studied. The cell-free solution was also used to study the influence of pH on the flux and transmission.

Materials and Methods

Protein A Solution

The protein A solution was an industrially produced fermentation broth. Protein A has a molecular weight of 42,000 g/mol, a p*I* at pH 4.86–

5.15, and a Stokes radius of 5.0 nm *(10,11)*. The microorganisms were removed from the broth using a Prostak module (Millipore, Bedford, MA) equipped with a Durapore polyvinyl difluoride membrane (Millipore). The cell-free broth was frozen for storage. After the broth had been thawed for use in experiments, the pH was adjusted with HCl and NaOH.

Membranes and Membrane Filtration

Two different polymeric membranes were used: a polyether sulfone membrane (Omega, 0.16 µm) and a nylon membrane (Ultipore $Nylon_{66}$, 0.2 µm), both from Pall (East Hills, NY). The pore sizes are the nominal pore sizes given by the membrane manufacturer. According to information from the manufacturer, the $Nylon_{66}$ membrane is uncharged at about pH 6.0, negatively charged at higher pH values, and positively charged at lower pH values, and the Omega membrane is uncharged or slightly negatively charged at all pH values used in our investigation. This was also confirmed in an earlier investigation by Paulsson *(12)*. However, Paulsson *(12)* found that the p*I* of the $Nylon_{66}$ membrane (the pH at which the membrane net charge is zero) was somewhat lower, occurring at pH 5.5.

Experiments with whole broth and cell-free broth were performed in a plate-and-frame module with a membrane area of 0.09 m^2 (Centramate; Pall). The pressure at the feed inlet was kept constant at 1.0 bar. The permeate flow was controlled by a permeate pump. The flux was increased stepwise, 5 $L/(m^2 \cdot h)$ at a time, in critical flux experiments using fermentation broth with microorganisms (whole broth). In the experiments in which no microorganisms were present (cell-free broth), the pressure was kept constant at 14.7 $L/(m^2 \cdot h)$. Both retentate and permeate were returned to the feed tank to keep the feed concentration constant during the whole filtration process. The temperature was 24°C.

The effect of pH on the microorganism-free fermentation broth was investigated using a crossflow module equipped with a circular, flat membrane with an area of 0.00196 m^2. The transmembrane pressure was 0.05 bar, the temperature was 24°C, and the crossflow velocity was 0.2 m/s. Both retentate and permeate were recycled to the feed tank.

New membranes were used in all cases. Before use the membranes were cleaned. First the membranes were rinsed with deionized water, then treated with 8 g/L of NaOH at 45°C for 30 min. The cleaning solution was rinsed out of the system with deionized water.

Analysis

The concentration of protein A was analyzed using high-performance liquid chromatography in a Zorbax column (Agilent, Palo Alto, CA) with a carrier fluid of 1.0 *M* ammonium sulfate, 0.05 *M* potassium phosphate (pH 7.2)/70% acetonitrile, 0.1% trifluoroacetic acid (80/20) at a flow rate of 0.85 mL/min.

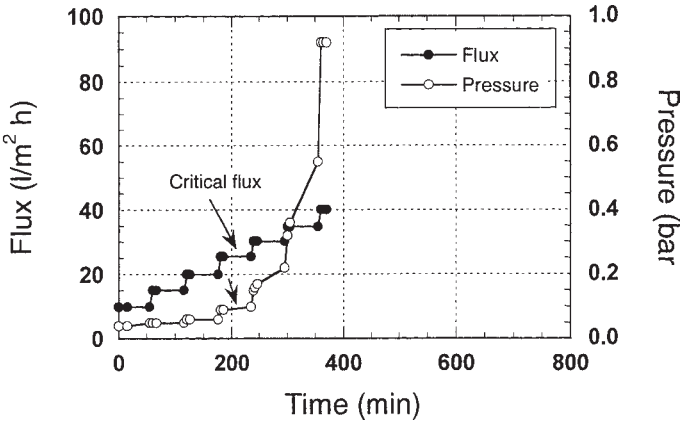

Fig. 1. Stepwise increase in flux and transmembrane pressure for determination of critical flux for $Nylon_{66}$ membrane using whole broth. The pH was about 6.7 and the temperature was 24°C.

Results

The observed transmission, *T*, of a solute through a membrane is defined as follows:

$$T = C_P / C_0 \quad (1)$$

in which *CP* is the concentration of the solute in the permeate and *Cb* is the concentration in the bulk solution at the feed side of the membrane.

Whole Broth

The critical flux of a membrane is the flux at which a filter cake is formed on the membrane *(13–20)*. It was hypothesized that the capacity of the membrane could be increased if the separation could be performed below the critical flux and that the transmission of protein A would be close to 100% since the protein should not be retained because there would not be any filter cake built on the membrane surface. Thus, the first task was to find the critical flux. It was found using a method in which the flux and the transmembrane pressure are increased stepwise. At a certain flux, the pressure continues to increase even though the flux is constant and the critical flux has been reached.

In a previous investigation using a shear-enhanced membrane module (the DMF module from Pall), the critical flux of the $Nylon_{66}$ membrane was found at 150 L/(m²·h) *(9)*. The critical flux of the $Nylon_{66}$ membrane in the Centramate module was markedly lower, only 25 L/(m²·h) (*see* Fig. 1). The flux and the transmembrane pressure at which the transmembrane pressure starts to increase at constant flux is marked by arrows in Fig. 1. The lower critical flux in the Centramate module is most likely

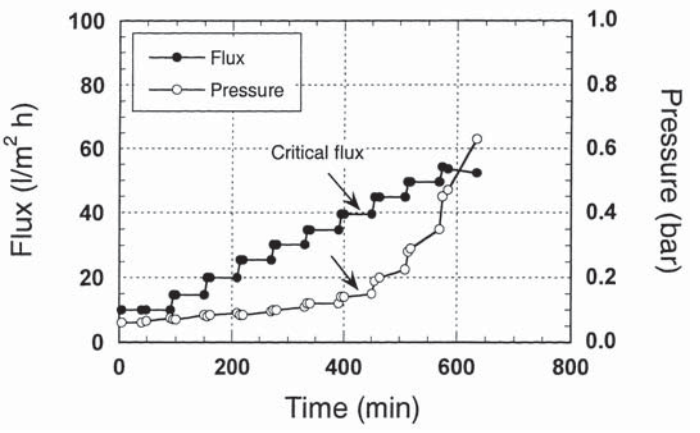

Fig. 2. Stepwise increase in flux and transmembrane pressure for determination of critical flux for Omega membrane using whole broth. The pH was about 6.7 and the temperature was 24°C.

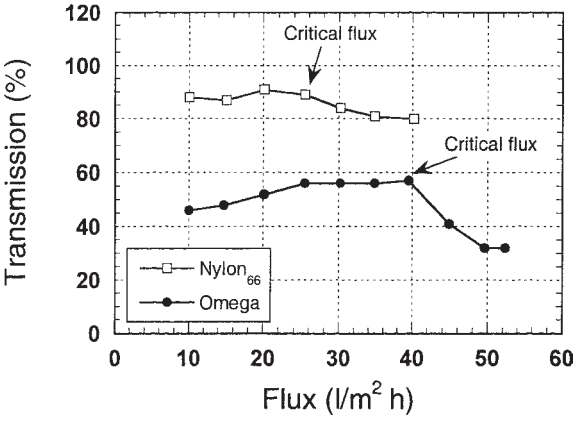

Fig. 3. Transmission of protein A through $Nylon_{66}$ membrane and Omega membrane as function of flux for whole broth. The pH was about 6.7 and the temperature was 24°C.

owing to a markedly lower flow velocity, and hence a lower mass transfer coefficient.

The experiment was repeated with an Omega membrane. The critical flux was found to be higher, about 40 L/(m²·h) (*see* Fig. 2). However, this flux is still significantly lower than the critical flux in the DMF module.

When the transmission was compared for the two membranes, it was found that it was much higher for the $Nylon_{66}$ membrane (90% at the critical flux) than for the Omega membrane (about 60%). The transmission decreased above the critical flux. The decline in the transmission above the critical flux was more pronounced for the Omega membrane than for the $Nylon_{66}$ membrane (*see* Fig. 3).

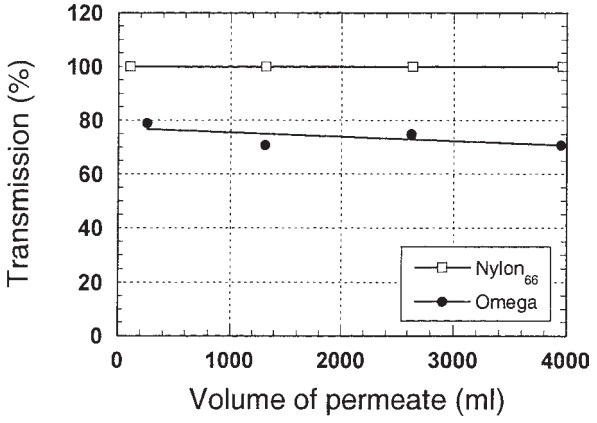

Fig. 4. Transmission of protein A through $Nylon_{66}$ membrane and Omega membrane as function of volume of permeate when using cell-free fermentation broth. The flux was constant (14.7 L/[m^2·h]), well below the critical flux; the pH was about 6.7; and the temperature was 24°C. The transmembrane pressure for the $Nylon_{66}$ membrane was 0.015–0.026 bar and for the Omega membrane was 0.01–0.02 bar.

Cell-Free Broth

The reason for a transmission below 100% may be the presence of the microorganisms in the solution. The protein can adhere to the microorganisms and remain within the retentate stream instead of passing through the membrane *(21)*. The microorganisms can also form small dynamic cakes on the membrane even though the critical flux has not been reached *(18)*. To establish whether this was the case, the transmission of protein A in cell-free fermentation broth was studied in the Centramate module. The transmission was higher than during filtration of whole broth for both membranes but was still surprisingly low for the Omega membrane. Figure 4 shows that the transmission for the $Nylon_{66}$ membrane was 100% and that for the Omega membrane was 70–80%. The transmission of protein A was not reduced for the $Nylon_{66}$ membrane but decreased slightly with time for the Omega membrane.

Influence of pH

Filtration of cell-free broth at different pH values was done to evaluate whether an alteration in protein and membrane surface charge could improve the transmission and flux through the membrane. The transmembrane pressure was kept constant, 0.05 bar, during the entire experiment. The flux was high initially (150–200 L/[m^2·h]) but declined with time. Each experiment lasted for 300 min. At the end of the experiments, the flux was 5–20 L/(m^2·h). The decline in flux was more pronounced for the Omega membrane than for the $Nylon_{66}$ membrane at pH 5.0 and 6.8, but almost the same at pH 3.0 (*see* Fig. 5).

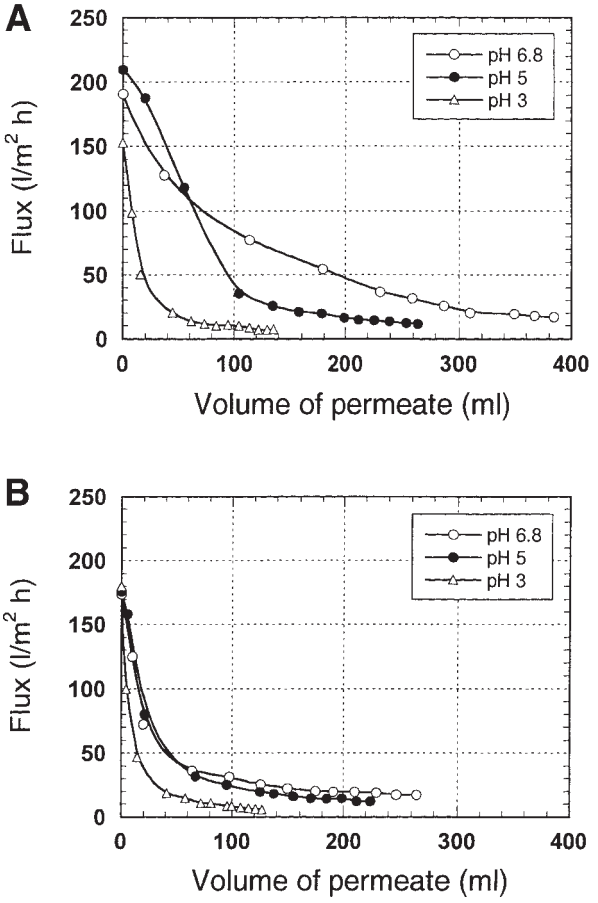

Fig. 5. Decline in flux of **(A)** $Nylon_{66}$ membrane and **(B)** Omega membrane as function of volume of permeate for cell-free broth with different pH values. The transmembrane pressure was 0.05 bar and the temperature was 24°C.

The highest transmission for both membranes was at pH 6.8 and the lowest at pH 5.0, the p*I* of protein A (*see* Fig. 6). The transmission through the $Nylon_{66}$ membrane was lower at pH 5.0 than at 6.8 and 3.0. A marked reduction in the transmission with time was found for the Omega membrane at all pH values.

Discussion

The Omega membrane is a high-porosity membrane with a smooth surface, and it is also low protein binding. This membrane seems thus to be well suited for cell separation of fermentation broth. This was also verified in a previous study in which the transmission of BSA was higher through the Omega membrane than through the $Nylon_{66}$ membrane *(8)*. However, in the present investigation, the transmission of protein A was found to be higher through the $Nylon_{66}$ membrane than through the Omega membrane.

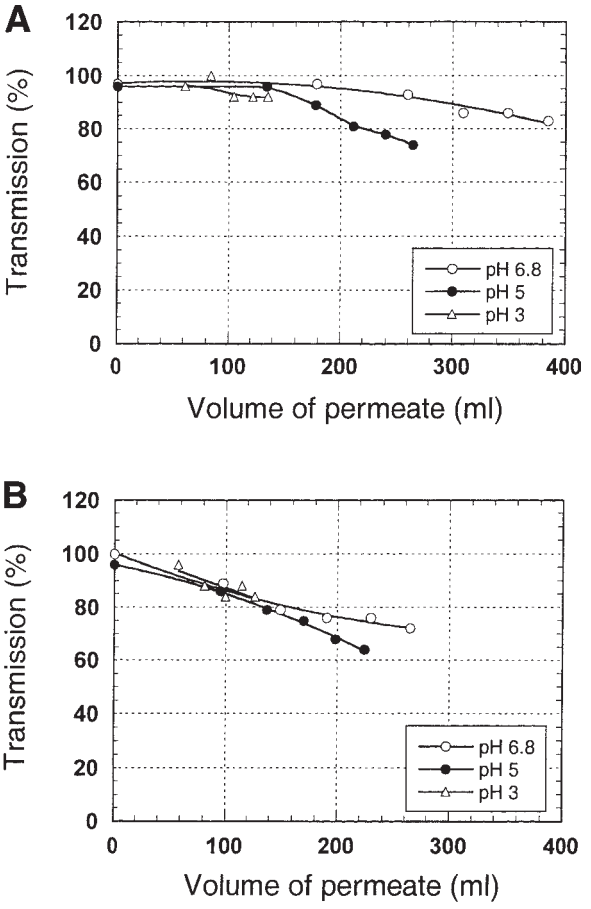

Fig. 6. Transmission of protein A through **(A)** $Nylon_{66}$ membrane and **(B)** Omega membrane as function of volume of permeate for cell-free broth with different pH values. The transmembrane pressure was 0.05 bar and the temperature was 24°C.

The molecular weight of BSA is greater than that of protein A (67,000 compared with 42,000 g/mol). On the other hand, the Stokes radius is larger for protein A (5.0 nm) than for BSA (3.48 nm) *(11,22)*. The larger Stokes radius of the smaller protein A may be owing to greater charges on the protein causing repulsion between the protein molecules and thus making the hydrodynamic volume of the protein larger. However, Palecek and Zydney *(23)* found no correlation between protein molecular weight and the transmission either for charged or for uncharged proteins.

Güell and Davis *(24)*, Franken et al. *(25)*, and Mueller and Davis *(26)* found that the membrane morphology (pore size, porosity, and surface roughness) affects the transmission far more than low-protein-binding properties of the membrane.

A membrane with a smaller pore size is more sensitive to the formation of protein agglomerates and/or electrostatic repulsive forces among

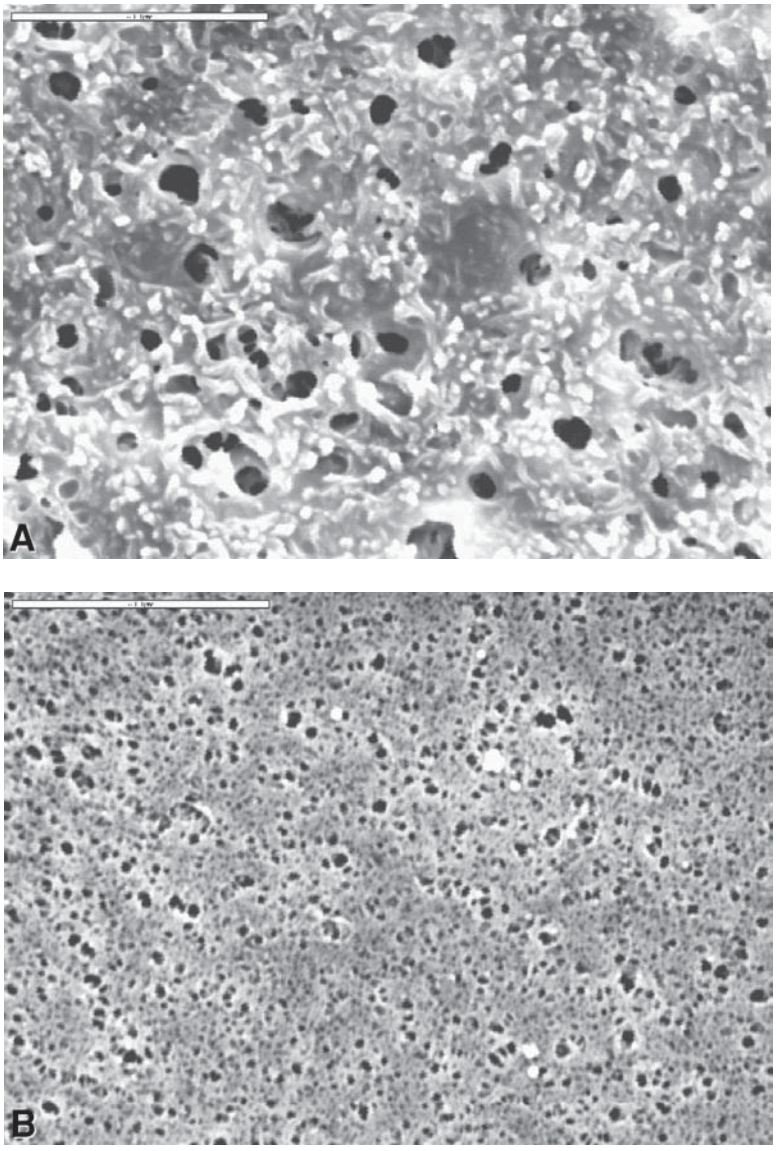

Fig. 7. Microscopic image of surface of **(A)** $Nylon_{66}$ membrane and **(B)** Omega membrane. The white bar in the upper left corner corresponds to 10 μm.

membrane, protein, and protein agglomerates. Since the pore size of the Omega membrane (0.16 μm) is smaller than that of the $Nylon_{66}$ membrane (0.2 μm) (*see* Fig. 7), the repulsive forces between the Omega membrane and the protein (and the growing protein agglomerates) are greater than for the $Nylon_{66}$ membrane. In addition, both membranes are negatively charged at pH 7.0 (the pH of the fermentation broth), but the Omega membrane has a higher charge density and this might increase the repulsive forces even further. The effect of the smaller pores of the Omega mem-

brane becomes even more pronounced when the protein also has a high charge density.

In the experiments in which the influence of pH was studied, the transmission was lower than 100% in all the experiments, even with the $Nylon_{66}$ membrane. This may be owing to the pretreatment of the microorganism-free fermentation broth. The fermentation broth had been frozen and then thawed before use. Kelly and Zydney *(4)* have shown that pretreatment of the protein and the protein solution may affect the result. Concentration polarization was higher in these experiments than in the experiments with the Centramate module because of the high initial flux. It is therefore likely that protein agglomeration was higher in these experiments than in the experiments with the Centramate module. The p*I* of protein A is at about pH 5.0, and because agglomeration of proteins occurs faster at the p*I*, an increase in the agglomeration rate is probably the reason for the lower transmission at this pH *(6)*.

At pH 3.0 the two membranes have opposite charges—the $Nylon_{66}$ membrane is positively charged and the Omega membrane is negatively charged—while the protein is positively charged. The similarity in decline in flux of the two membranes at pH 3.0 might be owing to an increase in ionic strength when the pH is adjusted by the addition of acid. These ions shield the protein charges from each other, which increases the agglomeration rate, and thereby the flux is lowered. The protein charges are also shielded from the charges on the membrane surface and in the membrane pores, and thus the membrane charge makes no difference in this case. The change in pH affects the charge of the protein and thereby also the charge density, and thus the Stokes radius might be changed. At pH 3.0 the Stokes radius for protein A might be smaller than at pH 6.8, and thus the protein passes through the membrane pores more easily, and the effect of difference in pore size is not so pronounced. This phenomenon might also be owing to the alteration in the broth when the pH is lowered. The adsorption and agglomeration properties of other substances present in the broth can be changed when pH is lowered, thereby altering the flux and transmission.

Conclusion

It has been previously shown that membranes with a smooth surface and a high membrane porosity are excellent for the cell separation step in the recovery process of protein because the transmission of the protein is high and thus the product recovery increases. If the membrane is low protein binding the transmission should be even higher. The Omega membrane is a high-porosity membrane with a smooth surface, which also is low protein binding. The good low-protein-binding properties of the Omega membrane have been demonstrated in a previous study *(8)*. However, in the present investigation the transmission of protein A through this membrane was low (60%) compared with a $Nylon_{66}$ membrane, which had

a transmission of about 90%. This could be owing to the smaller pores of the Omega membrane. The transmission through the Omega membrane thus becomes more sensitive to the electrostatic forces between the membrane and the protein.

The transmission of protein A was higher when no microorganisms were present in the solution. This was probably owing to the protein adhering to the microorganisms being retained together with the microorganisms, and/or the formation of a dynamic cake of microorganisms on the membrane that retains the protein.

It can thus be concluded that choosing the right membrane for a specific application is far more important than simply choosing a membrane showing low protein binding and good transmission of a well-known protein, such as BSA. It is very difficult to generalize about the best choice of membrane for a certain application. Experiments aiming at finding the membrane with the highest transmission and the optimal operating conditions with respect to critical flux and pH need to be performed in each case.

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