Purification of Fermentation Products

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Purification of Fermentation Products

Applications to Large-Scale Processes

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FOREWORD

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that in order to save time the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable since symposia may embrace both types of presentation.

PREFACE

THE ADVENT OF GENETIC ENGINEERING through the use of recombinant DNA techniques has stimulated wide interest in creating useful biologically derived products. There exists today a significant family of products that has been traditionally synthesized through the action of microorganisms. Typically, the manufacture of a biologically derived product begins with mass cultivation of the desired microorganism, a process known as fermentation.

Fermentation, however, represents only one step in the overall scheme of product formation. It is equally important to recover and purify the desired product from the fermentation after the organism has been cultivated. Often, that product is present in relatively low concentrations compared to the other components of the fermentation, and the challenge lies in separating what is wanted from what is not in an efficient, economical, and timely fashion.

Although great strides have been made in the genetic manipulation of microorganisms, a concomitant increase in new product-recovery methods has lagged behind. Recently, though, many groups of researchers in both academia and industry have initiated active development programs in the area of fermentation product recovery and purification.

This book presents some of these newer approaches to product recovery. The emphasis is on large-scale processes where several approaches to product isolation are required to obtain pure material. Each author discusses state-of-the-art approaches to product purification that uniquely fit the material to be isolated.

Two separation technologies are highlighted here: filtration and chromatography. Both technologies have existed for some time, but only recently have they found wider use in fermentation processes. The chapters in this book describe some of the newest applications of these technologies to product purification. The authors provide a cross section of viewpoints from academia and industry and consider both the practical and theoretical aspects of biological processing.

We wish to thank all of the authors for their contributions to this book. We also thank the Division of Microbial and Biochemical Technology, and specifically, Dr. Larry Robertson, for sponsorship of this work. Finally, we

wish to express our appreciation to the editorial staff of the ACS Books Department with special thanks to Robin Giroux.

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Processing Cell Lysate with Tangential Flow Filtration

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Operating conditions and the corresponding performance of a membrane filter system have been examined for recovering proteins from a bacterial lysate. Specifically, the dependence of membrane flux on average trans membrane pressure and recirculation rate has been investigated for both whole cells and lysate. The recovery of an intracellular protein was simulated by adding a marker protein, IgG, to the lysate after cell lysis. Protein concentrations were measured at each step of the processing scheme to determine the distribution of IgG and how much could be recovered. Operating parameters that influence the flow of IgG through a microporous membrane have also been studied.

Since the introduction of genetic engineering on a practical scale in the 1970's, there has been increased interest in the production of biological products using large scale fermentation. Both new products that had not been commercially available previously in sufficient quantities, and old products whose production costs are now potentially cheaper have been in the spotlight. One of the difficulties that is encountered with some types of recombinantly derived proteins, however, is the purification steps. Because E. coli does not secrete recombinant proteins into the growth media, the cells must be lysed which increases the difficulty of separating the desired protein from all other biological constituents. Purification of a protein from a lysate requires a number of steps to first eliminate cell fragments and debris from soluble material and to then separate the desired protein from other soluble components. Techniques that have been classically used in lysate processing and protein isolation include: centrifugation, open column chromatography and precipitation. Tangential flow filtration can now be added to the list.(1)

Tangential flow filtration has been most extensively used for concentrating cells(2,3), concentrating and washing proteins(4) and removing pyrogens from solutions(5,6). By passing the process solution tangential or parallel to the filter, and recirculating this fluid back to the original container, it is possible to filter solu-

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tions that would normally clog a dead ended filtration system. Also, with filtration, it is possible to perform gross fractionations based on size. These two factors make it feasible to process cell lysates with membrane filters in order to separate cell debris from proteins, and to also concentrate the crude fraction prior to the next purification step. Filtration offers advantages over other techniques in that relatively little energy is needed compared to centrifugation, and the filtration system is closed and will not produce aerosols. Also, the equipment can be scaled up to handle large quantities.

The purpose of this work is several fold. First, we wanted to determine the magnitude of filtration rates that are possible with lysates compared to whole cells, and to identify those operational parameters that influence membrane flux. Second, an effort was made to investigate how several different methods of generating a lysate suspension would affect membrane processing, if at all. Third, we wanted to investigate the quantitatively recovery of a specific protein from a lysate with filtration, and last, an effort was made to determine what operational parameters are most important in maximizing the flow of protein through the filter? In short, this work was designed as the first step in documenting the performance of membrane systems for processing lysates.

To answer the above issues, a model system was devised in which a specific protein was added to an E. coli lysate. The lysate was then processed through the filtration steps and the protein recovered. The protein chosen was human IgG which has a relatively large molecular weight (160,000 Daltons). It was reasoned that if a large protein could be separated from cell debris and passed through a membrane and recovered, then a smaller protein that is typical of a recombinant process, should prove to be much easier.

Materials and Methods

Fermentations. E. coli was grown in a defined media with the following composition:

PO_4^{-2}	0.05M
$MgSO_4$	10^{-3}M
$CaCl_2$	10^{-4}M
$FeSO_4$	10^{-5}M
$(NH_4)_2\ SO_4$	0.2%
Glucose	0.5%

The antifoam used was 2% octadecanol in mineral oil (0.5ml/liter of fermentation broth). The fermentors were batched in 11 liter quantities, and growth lasted about 24 hours at 37°C. Rotor speed was 400 RPM and the aeration rate was 5 liters/minute. A Microgen (New Brunswick Scientific) or a Chemap 14 liter fermentor was used. Final viable counts were typically greater than 10^9/ml.

Membrane Separations. The membrane filter separation system

(Millipore Corporation, XX8140000) consisted of a membrane holder (Pellicon), a 4 gallon per minute rotary vane pump (Procon) and the membranes themselves. The filters were either 0.45 micron microporous (Durapore) or 100,000 NMWL ultrafiltration membranes. All tubing and connections were 1/2 inch. A manifold flow bypass was attached to the pump so fluid could be introduced into the filtration system without a sudden surge of pressure buildup (Bulletin AB822, Millipore Corporation). In all cases, 5 square feet of membrane were used.

Figure 1 shows a schematic of the equipment used and how it was plumbed together. All together there were three modes of operation used for various applications reported here. For concentrating cells or lysate, the system was operated exactly as shown in Figure 1. Filtrate was collected separately while the retentate was circulated back to the original holding tank. In the total recycle mode of operation, the filtrate line was placed in the cell or lysate holding reservoir so the cells or lysate concentration remained constant as a function of time. The reason for performing total recycle is to isolate process variables in determining the independent influence of trans membrane pressure or recirculation rate on flow through the membrane. A constant volume wash mode of operation is accomplished by continuously adding wash buffer to the cell or lysate suspension at the same rate that filtrate is collected. For all modes of operation, there was no filtrate back pressure.

The inlet pressure for the filter holder is measured on the upstream side of the membrane as the fluid enters. The outlet pressure is also monitored on the upstream side of the membrane as the retentate exits the filter holder device. The difference between the inlet and outlet pressure is proportional to the circulation rate while the sum of the inlet and outlet pressures is proportional to the average trans membrane pressure.

Cell Lysis. Cell lysis was accomplished either enzymatically with lysozyme or with sonication.

Sonication. Fermentor broths were reduced in volume via filtration to several liters or less. This reduced volume was then continuously circulated through a sonifer (Branson Cell Disrupter 185). Water from an ice bath was passed through the jacket to dissipate heat generated from the sonifer. Lysis was checked visually by monitoring the number of whole cells seen in a wet mount under an optical microscope at 1000X magnification.

Lysozyme. After fermentation, the cell broth was reduced in volume via filtration to one liter or less. The concentrated cells were then pelleted and washed with 0.85% saline. The washed cells were resuspended in 0.1M EDTA, pH 8.0 and allowed to sit at room temperature for about 45 minutes with gentle stirring. Lysozyme was then added to enough 0.5M NaCl, pH 8.0, to bring the total volume to 1/20th of the original culture volume. The lysozyme concentration was 2 mg/ml in this suspension. The mixture was suspended in 0.1% sodium desoxycholate to finalize the lysis. This procedure[7] produced a very viscous suspension. The viscosity was reduced by adding either streptomycin sulfate or DNAse. In either case, the resulting suspension was allowed to sit overnight with gentle stirring.

IgG, DNAse. Human, freeze dried IgG was purchased from Sigma Chemical (cat. no. HG-11) as Cohn fraction II. The IgG was resuspended in physiological saline or cell concentration filtrate and the IgG was added to the lysed cells to a final concentration of around 0.1%-0.2%. DNAse was also purchased from Sigma Chemical.

IgG Assay. Samples and controls to be assayed for IgG were collected in 5 ml quantities. From these samples, 750 microliters were centrifuged for 10 minutes at 1250 RPM. The supernant fluid was collected, and 250 microliters were injected into a Beckman ICS Analyzer II. The analyzer would mix appropriate amounts of anti IgG antibody with the sample automatically and measure the change in light scattering intensity as the antigen-antibody reaction progressed. The formation of the immunoprecipitin complex proceeds gradually at first, then rapidly and finally progresses through a peak value. If the sample IgG is within the correct concentration range, the final peak rate for the change in light scattering intensity is proportional to the sample IgG concentration. Normal human sera of known IgG concentration was used for calibration and controls. With lysate samples, this assay technique was reproducible to about 10%.

Results

A schematic illustrating how membrane filters are used to process lysates is shown in Figure 2. The overall purpose of the processing is to separate an intracellular product, typically a protein, from the bulk of large cell fragments or debris, and to concentrate the protein to a small workable volume. Basically, there are four filtration steps in this process. After the cells are grown, the cells are concentrated to a relatively small volume. This is accomplished in the first filtration step. After lysis, the lysate is concentrated and washed in the second and third steps respectively. Washing is performed in order to enhance the passage of protein through the membrane if the protein remains in the retentate during the concentration of the lysate. Both concentration and washing can use the exact same microporous membrane. In the last step, an ultrafiltration membrane is used to concentrate the crudely fractionated protein solution in preparation for the next purification step which is usually a chromatographic process of one sort or another.

The information presented in the following figures represents two types of results. First, there is the evidence for IgG recovery from membrane filtration as a function of different biochemical processing schemes. Second, there is the data which describes the flow rates of fluid or the flux through the membrane as a function of different operating conditions.

Figure 3 is a schematic representing a filtration process of cells, lysate and protein solution in which the lysate was formed by sonication. Initially, 22.3 liters or cells were concentrated down to 4.8 liters with 0.45 micron microporous membrane. The cells were lysed and the IgG added in the amount of 11.1 grams (6.8 liters total lysate). The lysate was then concentrated to 1.3 liters. During the lysate concentration, 7.6 grams of IgG passed through the 0.45 micron pore size membrane and 2.5 grams remained in the retentate. Respective fluid volumes were 5.5 liters and 1.3 liters. A constant volume wash was performed on the 1.3 liters of retentate in

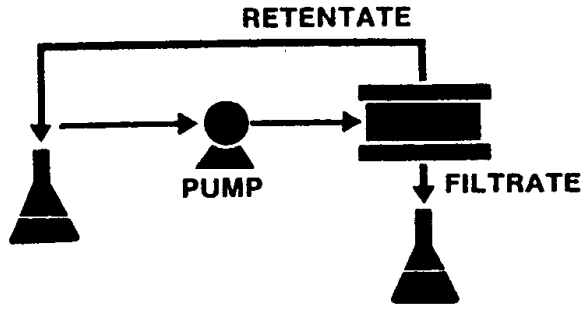

Figure 1. Schematic of equipment for processing lysates. The tangential flow filter is in the stacked sheet configuration which allows high inlet pressure.

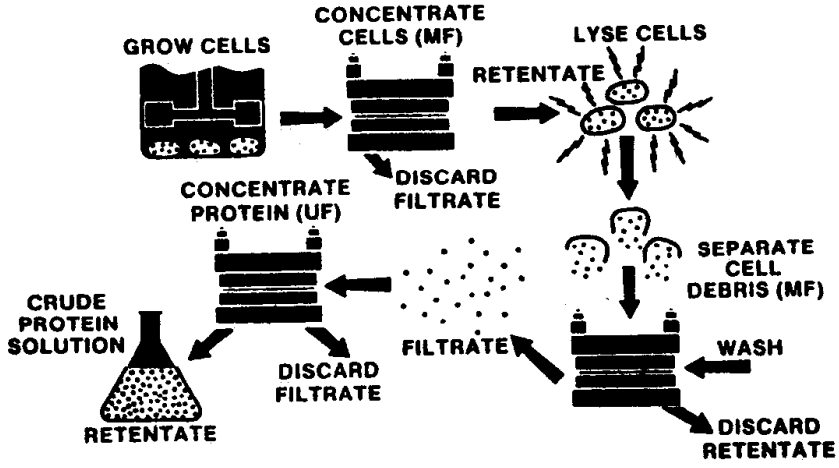

Figure 2. Filtration steps for processing lysate. All steps, except the protein concentration, use a microporous membrane. Protein concentration is accomplished by a UF membrane.

an effort to separate the remaining 2.5 grams of IgG from the cell debris. After 5 liters of wash was collected in the filtrate, 2.4 grams of IgG had also passed the membrane showing that the IgG can be recovered and effectively separated from the lysate. The 5.5 liters from the original lysate concentrate was then concentrated with a 100,000 MWCO UF membrane. In the concentration, only 5.2 grams of IgG was recovered, however, none of the IgG passed the UF membrane.

In an effort to determine if the loss of IgG in the UF concentration of the crude protein fraction shown in Figure 3 represented a real issue or just a matter of collecting all the retentate from the dead volume, the concentration step was repeated. This time, particular attention was paid to flushing out the retentate hoses and dead spots in the system. Figure 4 shows the results of this work. The lysate filtrate (10.5 liters, 7.2 grams of IgG) was split into two fractions of 5.9 liters and 4,8 liters respectively and concentrated under two separate operating conditions. Operating at an inlet pressure of 80 psi and concentrating the 5,9 liters to 1.4 liters, 3.7 grams of IgG was recovered immediately which represents 95% of that expected. Subsequently, 1.5 liters of physiological saline was recirculated through the filtration system for 5 minutes and then assayed for the remaining IgG. Recovery of another 0.2 grams of IgG (remaining 5%) was found, so together with the initial 3.7 grams of IgG, the total amount expected was recovered. For operating conditions of 40 psi inlet pressure and 0 outlet pressure, 88% of the IgG was recovered. Results from Figure 4 indicate that under both high and moderate inlet pressures, the IgG could be recovered from the concentration step.

Figure 5 shows the flow decay for the cell suspension concentration step of Figure 3. The flow rate decays gradually with time with an average final rate around 700 ml/min. Inlet pressure was 90 psi, and there was a 4.6X reduction in volume in about 15 minutes. The flow decay curve in Figure 5 is typical for an E. coli concentration with a new 0.45 micron pore size microporous membrane.

Figure 6 is the corresponding flow decay curve for the sonified lysate suspension. Again, this is a typical flow decay with time. The flux for the lysate is less than that seen for the whole cell concentration. The flow rate, however, is still significant and the equilibrium rate corresponds to 23 gallons per square foot per day (GFD). In other experiments, flow rates for lysate concentration are also seen to be relatively constant showing little decay.

Figure 7 is a plot of the flow rate over time for the constant volume wash step used to pass the IgG remaining in the retentate after the initial concentration of the lysate. As expected, with the lysate volume remaining constant at about 1.3 liters, there is little flow decay with time. The arrows in Figure 7 indicate the commencement of the addition of one liter aliquots of saline solution at a rate matching that of the filtrate. Five square feet of an 0.45 micron pore size filter was used for this procedure.

Figure 8 shows the respective flow decays for the crude protein fraction concentration with the 100,000 MWCO UF membrane. Inlet pressures were 80 psi and 40 psi respectively. The higher inlet pressure gave a higher flux compared to the 40 psi inlet pressure as would be expected. When the inlet pressure is increased, both the average TMP and also the recirculation rate increase. Based on the data in Figure 8 alone, it is not possible to determine if the higher

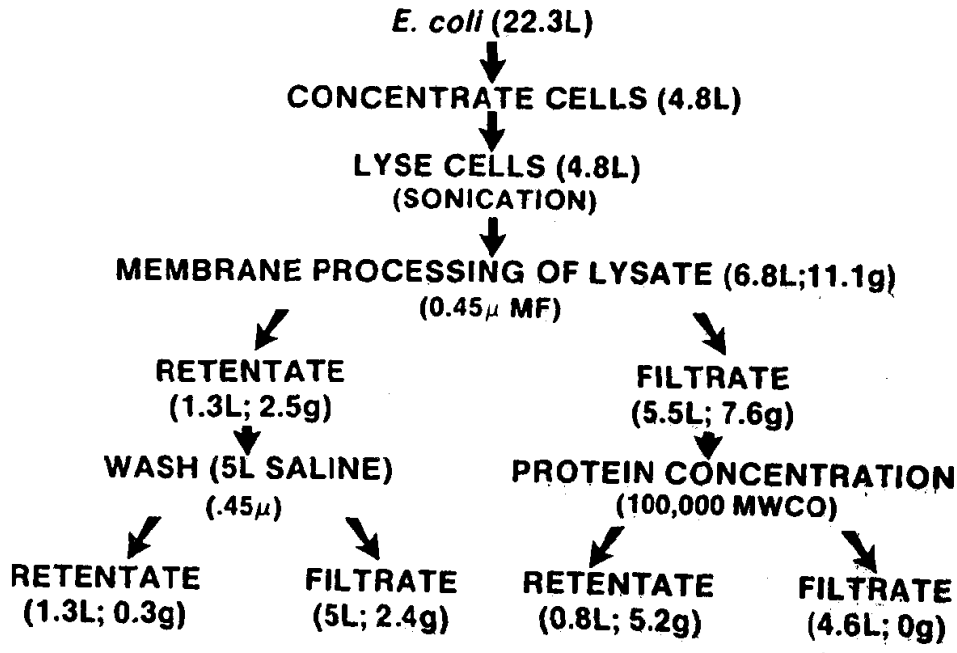

Figure 3. Distribution of IgC for the different process steps. Both the fluid volumes and the total IgC content are given for all retentates and filtrates throughout the whole sequence.

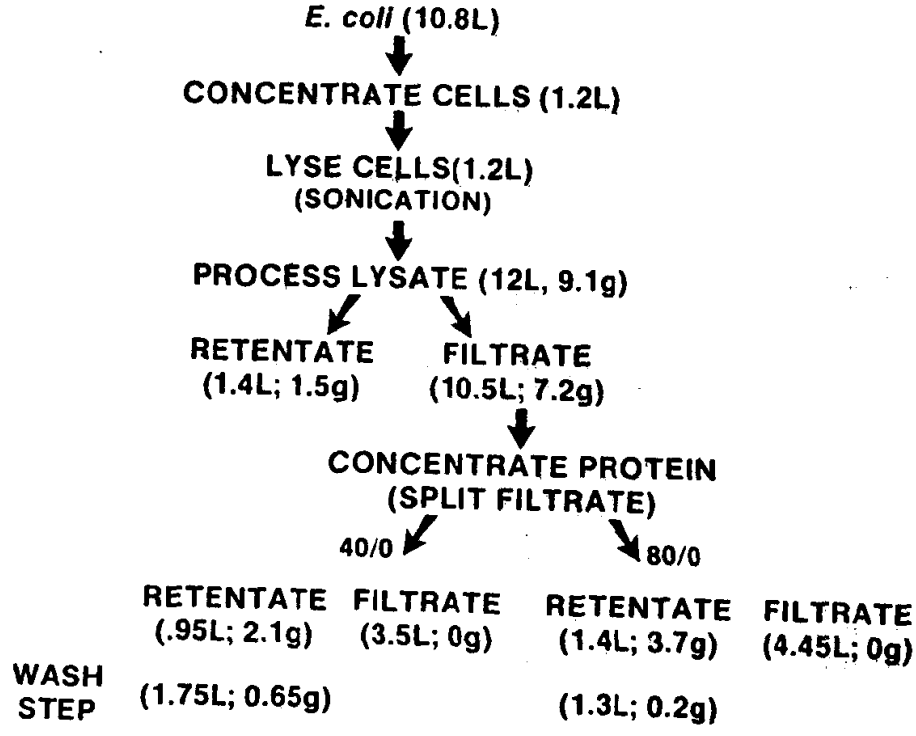

Figure 4. Concentration of protein under different operating conditions. IgC can be recovered quantitatively under two sets of operating pressures by a UF membrane.

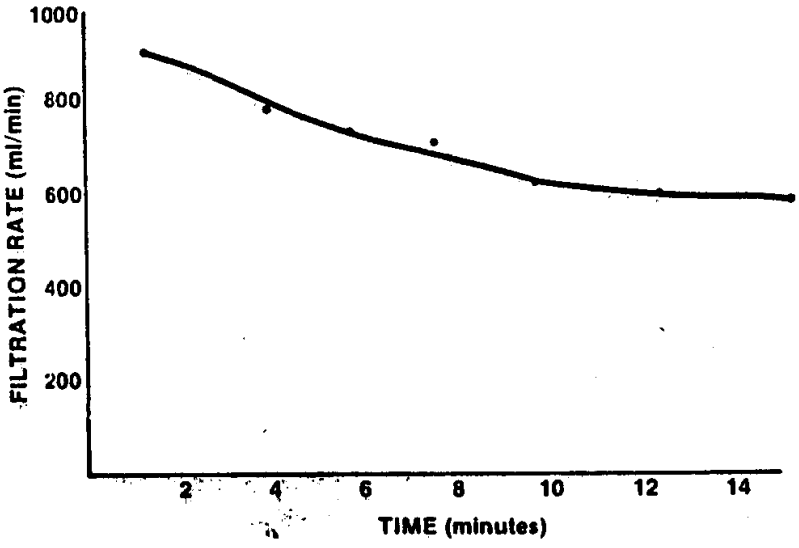

Figure 5, Flow decay curve for concentrating E. coli whole cells with a 0.45 μm microporous (Durapore) membrane. Inlet pressure was 90 psi. The initial volume was 22.3 liters; the final volume, 4.8 liters.

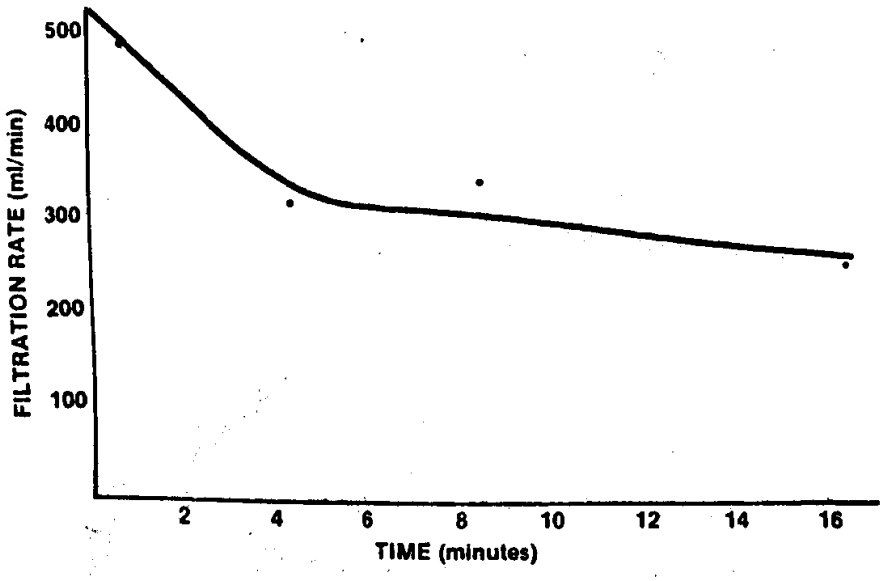

Figure 6. Flow decay curve for concentrating E. coli lysate with a 0.45 μm microporous (Durapore) membrane. Inlet pressure was 90 psi; outlet pressure, 30 psi. The initial volume was 6.8 liters; the final volume, 1.3 liters.

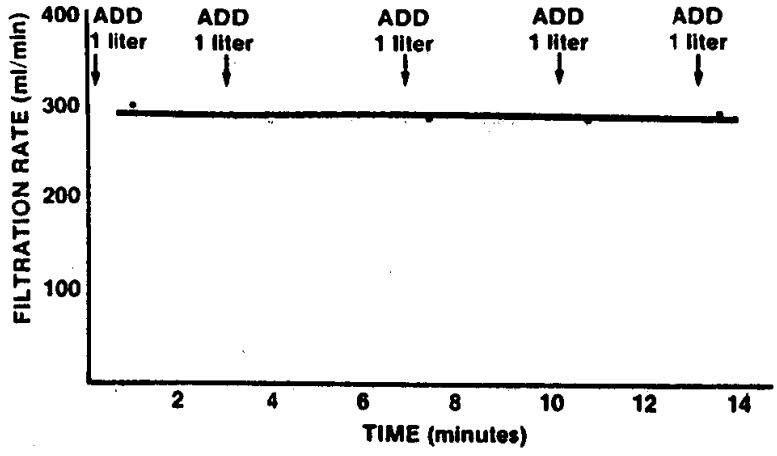

Figure 7. Flow versus time for the lysate wash with 5 square feet of a 0.45 μm microporous (Durapore) membrane. Lysate volume (1.3 liters) remains constant during the wash. Inlet pressure was 90 psi; outlet pressure, 30 psi.

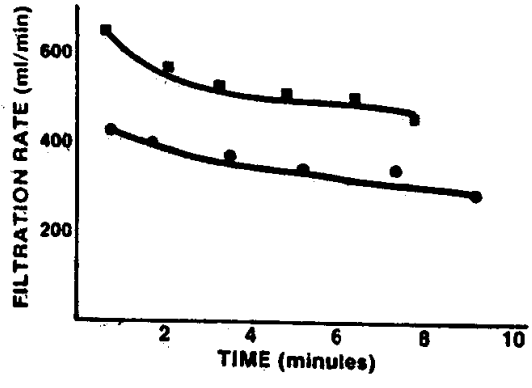

Figure 8. Protein concentration flow decay curves for two different operating conditions with a 100,000 MWCO UF membrane. Key: ■, P_{in} = 80 psi; ●, P_{in} = 40 psi.