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MECHANISMS OF PROTEIN FOULING IN MICROFILTRATION. I. DETERMINATION OF PROTEINS FOULED ON MICROFILTRATION MEMBRANES

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ABSTRACT

Protein fouling is one of the critical factors governing the effectiveness of many microfiltration processes. Although mechanisms have been proposed, direct correlation between protein amount fouled on membranes and filtration behavior is required. We developed a simple method to measure proteins on microfiltration membranes. The protein-fouled membranes were stained by amido black 10B first, then destained to remove excess dye not bound to protein. Finally, the dye associated with the protein on the membranes was eluted with 0.1 *N* NaOH. Three membranes (mixed esters of cellulose nitrate and acetate membrane, Durapore GVWP membrane, and nuclear-pore membrane) were examined by blotting, adsorbing, and depositing bovine serum albumin on them. The absorbency of the eluted solutions was measured at 620 nm. The absorbency of the eluted

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solutions was independent of staining time and eluting time, but decreased with prolonged destaining time. The results showed a good linear relationship between the absorbency and amount of protein on membranes in all conditions examined. Three other proteins were also examined and the results showed that different proteins have different slopes. These results indicate that the method could be used to quantify proteins fouled on membrane.

Key Words: Quantification; Protein fouling; Microfiltration

INTRODUCTION

Microfiltration of protein containing solutions is a widely used processing step in biotechnological industries. Typical uses include primary cell recovery from fermentation broth[1–3] and sterile filtration as the final step in the production of a protein product. However, protein fouling is a critical factor in many of these processes, resulting in a substantial reduction in membrane performance and a significant loss of valuable product.

The mechanisms of membrane fouling have been studied extensively. It is generally referred to the adsorption of proteins onto inner surface of membrane pores and deposition and accumulation of submicron particles on the membrane surface and within the pores of the membrane itself. In ultrafiltration, the protein molecules were partially or completely retained by ultrafiltration membranes, and protein accumulates to a high concentration at the membrane surface. This phenomenon leads to protein precipitation, gelation, or polymerization-type reaction, and results in protein deposition.[4,5] In microfiltration, however, the pore size is more than 10 times the size of protein molecules. The adsorption does not influence the filtration flux significantly,[6] and concentration, polarization, and gelation are negligible. However, dramatic decline in flux was also found in microfiltration. Bowen and Gan[7] found a sharp decline in flux during filtration of bovine serum albumin (BSA) through 0.22 μm capillary pore aluminum oxide membrane. They described the flux with standard filtration law and calculated the thickness of adsorbed BSA layer on pore wall. The thickness of the adsorbed BSA layer was about 55 nm, which resulted in a decrease in pore radius from 200 to 90 nm. The layer was thicker than monolayer. Bowen proposed that it was due to shear induced adsorption.

Most researchers consider deposition of protein aggregates as the main factor of membrane fouling. Chandavarkar and Coony[8] found that flux decline in their system strongly depended on the characteristics of the pumps used to

drive the protein solution through the device during microfiltration of BSA. Using quasi-elastic light scattering, they also found that prolonged pumping resulted in the formation of relatively large protein aggregates in the bulk solution. The fouling behavior was attributed to the deposition of these large aggregates on the membrane surface.

Kelly et al.[9] studied different commercial preparations of BSA through asymmetric polyethersulfone microfiltration membranes. They found that BSA fouling of microfiltration membranes is associated with the deposition of trace quantities of aggregated and/or denatured BSA, with these fouling species serving as initiation sites for the continued deposition of bulk protein. Kelly proposed a dual-mode fouling procedure.[10] They proposed that the initial flux decline was due to the deposition of large BSA aggregates on the membrane surface, with these aggregates then serving as attachment (or nucleation) sites for the continued deposition of bulk (native) protein.

Isotopic method has been used to determine the protein amount on membrane.[11–13] Matthiasson[11] measured the protein adsorbed on ultrafiltration membranes by ^{14}C-labeled BSA, and related both solution properties and membrane surfaces characteristics to adsorption kinetics, amount adsorbed, and hydraulic resistance of the adsorbed layer. Aimar et al.[12] studied adsorption of BSA on IRIS 3038 membranes using ^{125}I-labeled proteins, for pH values of 2.0, 4.7, and 7.2, and a concentration range from 0.1 to 50 g/L, without applied pressure. They found that adsorption isotherms followed Freundlich Law. In microfiltration, in which large volume of protein solution was needed, it is inconvenient to use radical methods. Some researchers[7,14,15] assayed concentration of solutions before and after adsorption to calculate the amount of protein on membranes. The method was only used in studying adsorption. Recently, an enzyme-linked immunoassay method[16] was published. Apparently, the method could only detect monolayer adsorption. In addition, because of space obstacle, it is difficult to determine proteins in membrane pores. Therefore, the method was not suitable for studying fouling of membrane. Most fouling mechanisms of microfiltration were concluded by analysis involving mathematical models describing the rate of the flux decline. Until now no direct measurements of proteins fouled on microfiltration membranes were reported. To get more details on fouling mechanism, a simple method to measure directly the protein on membranes is necessary.

The dye amido black 10B has been used to detect proteins in paper electrophoresis and cellulose acetate electrophoresis.[17] It was known that the sulfonic groups of amido black 10B can react with the basic groups of protein to form ionic bonds. And the correlation was good between bound amido black and the proteins' content of basic amino acids.[18] It was found that 1 mg of protein will bind 0.15–0.35 mg of amido black according to different types of proteins.[19] We introduced the method to determine proteins on microfiltration membranes. First, the

membranes fouled by proteins were soaked in staining solution that contained 7% acetate acid. In this procedure, the basic groups of proteins, which were positively charged in acid conditions, reacted with sulfonic groups in amido black 10B to form protein–dye complex. Then the stained membranes were rinsed in destaining solution to remove excess dye, and only dye–protein complexes were left on the membranes. In the dye–protein complex, the amount of amido black 10B was proportional to the amount of proteins. Finally, the dye that bound to proteins was eluted with 0.1 mol/L NaOH solution. In addition, the dye was determined with a colorimeter. The amount of proteins on membrane was calculated by the correlation between protein and dye. Three membranes were examined at three conditions, and four proteins were used in the experiments.

MATERIALS AND METHODS

Membranes: Millipore Durapore GVWP membranes (USA) with pore size of 0.22 μm was made of polyvinylidene fluoride. Mixed esters of cellulose nitrate and acetate membrane (which we abbreviate as "cellulose membrane") was purchased from Shanghai Xingya Company (Shanghai, China). Nuclear-pore membrane was purchased from Nuclepore Corporation (Pleasanton, CA). Bovine serum albumin was purchased from Boehringer Mannheim (Germany). Amido black 10B was purchased from Beijing Chemical Reagent Corporation (Beijing, China).

Protein solutions were prepared by carefully dissolving the lyophilized protein powder in a Tris–HCl buffer solution (pH 7.2) at room temperature. The buffer contains 10 mmol/L Tris that had been adjusted to pH 7.2 by 0.1 *N* HCl solution. Staining solution was composed of 0.5% (W/V) amido black 10B, 10% (V/V) acetic acid, and 50% (V/V) methanol. Destaining solution was composed of 5% (V/V) acetic acid, and 45% (V/V) alcohol.

Three procedures were used to simulate membrane fouling. (1) Blotting: the membranes were placed on Parafilm, and appropriate volumes of solution that contained desired amount of protein were pipetted, and then the solutions were added on membranes, respectively. If it could not reach the desired amount of protein by one pipette, another pipette of solution could be added after the membranes were dry. In the experiments, the concentration of protein was 1 mg/mL (when the total amount of protein blotted on one membrane was less than 100 μg) or 5 mg/mL (when the total amount of protein blotted on one membrane was more than 100 μg). (2) Adsorbing: 5 mL BSA solution was filtered through a membrane, and then the membrane was washed by filtrating of 4 mL water. The two filtrates were combined and the volume was made up to 10 mL with water. The protein concentration before and after filtration was determined by Bradford method. (3) Depositing: the BSA solution was incubated at 90°C for 7 min. The protein solution became turbid with BSA aggregates, but no precipitation appeared. Specific

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volumes of the solution were filtered through membrane, and the aggregates in these solutions were retained and deposited on the membrane.

The membranes fouled by protein were immersed into staining solution for 4 hr (or specified in the text), then were moved into destaining solution for another 4 hr (or specified in the text) to destain. During destaining, fresh destaining solution was changed three times. After destaining, the membranes were placed on filter paper to dry, and the membranes were stored until measurement. Before the measurement, the destained membranes were immersed in 4 mL 0.1 *N* NaOH to elute the dye for 4 hr. The absorbencies of eluted solutions were measured by Beckman DU 7500 (USA) at 620 nm. Clean membranes were used as control. The protein concentration was determined by Bradford method.[20]

RESULTS AND DISCUSSION

Influence of Staining Time

Cellulose membranes were blotted with 100 μg BSA, and immersed into staining solution for 0.5, 1, 2, 4, 6, 8, and 12 hr. After staining, the membranes were moved into destaining solution to destain for 4 hr with change of destaining solution for three times. The destained membranes were eluted with 4 mL 0.1 *N* NaOH for 4 hr, and the absorbency of eluted solution was measured. The results, as shown in Fig. 1, indicate that the absorbencies of eluted solutions were constant with staining time varying from 0.5 to 12 hr. This means that staining time does not influence the absorbency significantly.

Influence of Destaining Time

Cellulose membranes were blotted with 100 μg BSA, and immerged into staining solution to stain for 4 hr. Then the membranes were moved into destaining solution to destain for 0.5, 1, 2, 4, 6, 8, and 12 hr. The destained membranes were eluted with 4 mL 0.1 *N* NaOH for 4 hr, and the absorbency of eluted solution was measured (Fig. 2). It is shown that the absorbency decreased with increase in the destaining time. This may be caused by dissolving of protein-bound dye by destaining solution.

Influence of Eluting Time

Cellulose membranes were blotted with 100 μg BSA. The membranes were stained for 4 hr and then destained for another 4 hr. The destained membranes

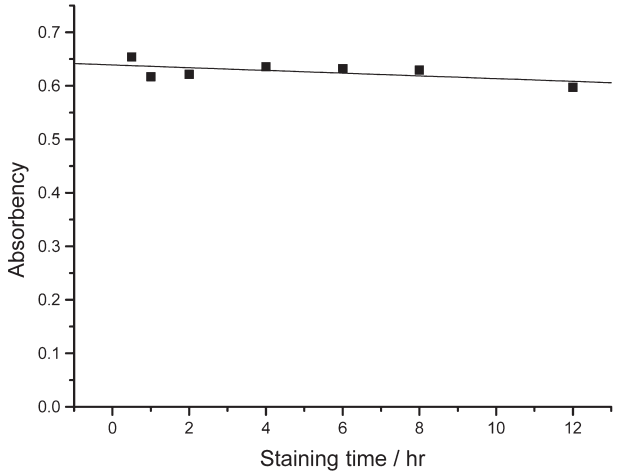

Figure 1. Influence of staining time. The dye was eluted with 4 mL 0.1 *N* NaOH.

were eluted with 4 mL 0.1 *N* NaOH for 10 min, 0.5, 1, 2, 4, 8, and 12 hr, and the absorbency of elute solution was measured. The absorbency of the eluted solution was also constant when eluting time changed from 10 min to 12 hr as shown in Fig. 3.

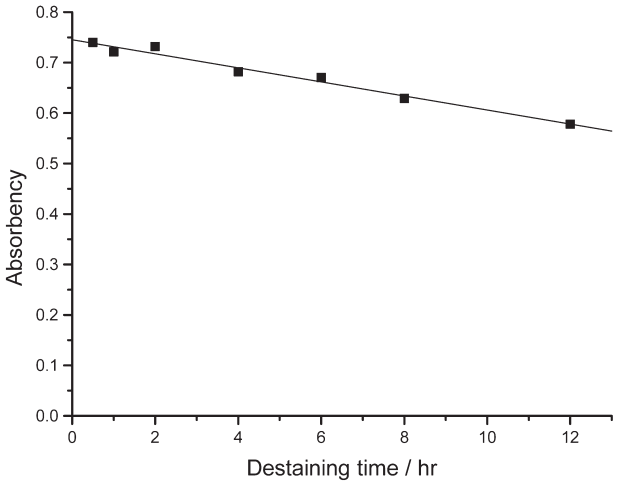

Figure 2. Influence of destaining time. The dye was eluted with 4 mL 0.1 *N* NaOH.

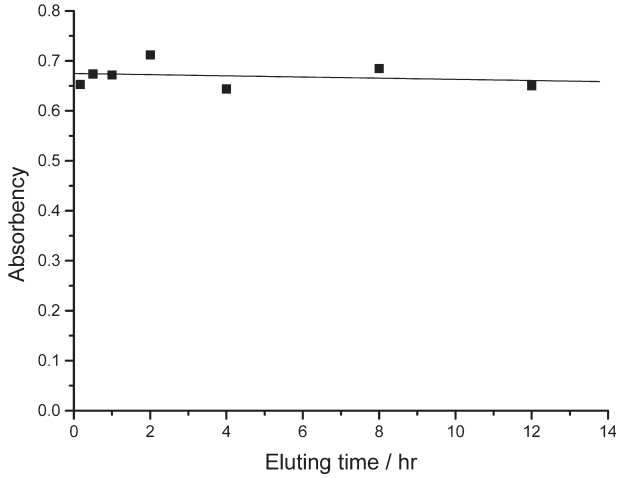

Figure 3. Influence of eluting time. The dye was eluted with 4 mL 0.1 *N* NaOH.

From Figs. 1–3 it was concluded that the staining time and eluting time did not influence the absorbencies of eluted solution significantly, but the destaining time influenced the result. The absorbency of eluted solution decreased about 20% when destaining time prolonged from 0.5 to 12 hr. If the destaining time were specific and varied within 0.5 hr, the error introduced would be less than 1%. Hence, this is a stable method by which constant absorbencies could be obtained when protein amount on membrane was constant. This result is important for correlating absorbencies of eluted solutions to protein amount on membranes. In practice, the staining time and eluting time are more flexible, but destaining time should be kept the same in a series of experiments so that the data could be comparable. We choose 1 hr for staining, 2 hr for destaining, and 1 hr for eluting as quantification conditions in our experiments later.

Quantification of Blotted Protein

From 20 to 500 μg protein was blotted on cellulose membrane and GVWP membranes (the area of membranes was about 2 cm^2). The membranes were stained for 1 hr and destained for 2 hr. Each membrane was eluted with 4 mL 0.1 *N* NaOH for 1 hr. The eluted solution that eluted the membrane blotted with 100–500 μg protein was diluted four fold with 0.1 *N* NaOH so that the absorbency was not too high. Figure 4(a) shows that the membranes were blotted with 20–100 μg protein and the membranes were eluted with 4 mL 0.1 *N* NaOH.

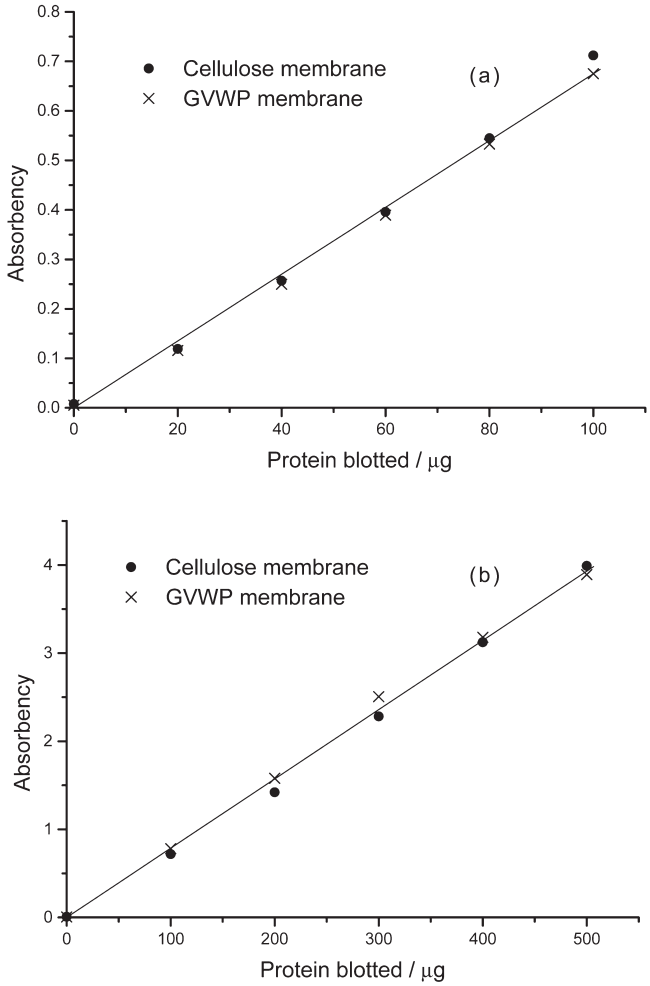

Figure 4. Absorbency of eluted solution vs. quantity of blotted protein. (a) The membranes were blotted with 20–100 μg protein. (b) The membranes were blotted with 100–500 μg protein.

The eluted solution was measured directly. Figure 4(b) shows the membranes, which blotted with 100–500 μg protein, were eluted with 4 mL 0.1 *N* NaOH. The eluted solution was diluted four fold by 0.1 *N* NaOH before measurement. The *Y*-axis is the value that was equivalent to 4 mL eluted solution. The absorbencies of eluted solutions were linearly related to protein amount, which ranges from 20 to 500 μg per membrane.

Quantification of Adsorbed Protein

Because Durapore GVWP membrane and nuclear-pore membrane adsorb little protein, we only used cellulose membrane in this experiment. A 5 mL BSA solution with concentration varying from 50 to 400 μg/mL was filtered through membranes, and then the membranes were washed by filtrating 4 mL water. The two filtrates were combined and the total volume was adjusted to 10 mL by adding water. The protein concentration was determined before and after filtration by Bradford method. The amount of proteins adsorbed on membranes was calculated by the difference of protein concentration. The membranes were stained for 1 hr and destained for 2 hr, and the dye was eluted with 4 mL 0.1 *N* NaOH. Figure 5 shows a linear relationship between absorbency and protein amount.

Quantification of Deposited Protein

A 400 μg/mL BSA solution was incubated at 90°C for 7 min. The protein solution turned turbid without any precipitation. This phenomenon showed that the particle size in solution reached the scale of a colloid. There were no molecules other than the BSA molecules that could aggregate to form colloidal particles, so the particles were BSA aggregates. Then the solution was diluted 10 folds with distilled water. Filtrated 2.5, 5.0, 7.5, 10, and 15 mL of solution

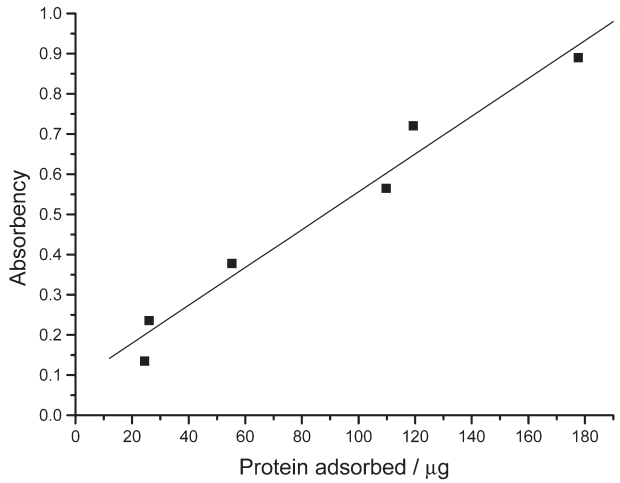

Figure 5. Absorbency of eluted solution as a function of adsorbed protein.

through cellulose membranes, GVWP membranes, and nuclear-pore membranes. The protein quantity deposited on the membranes was calculated by analysis of the protein concentration before and after filtration. The membranes were stained for 1 hr and destained for 2 hr, and the dye was eluted with 4 mL 0.1 *N* NaOH. Figures 6–8 show the absorbency of eluted solution as a function of the calculated protein quantity. For all three membranes, the relationship between absorbency and protein amount was linear.

Quantification of Different Proteins

From 20 to 100 μg of BSA, bovine hemoglobin, chicken egg albumin, and lysozyme were blotted on cellulose membrane (the area of membranes was about 2 cm^2). The membranes were stained for 1 hr and destained for 2 hr. Each membrane was eluted with 4 mL 0.1 *N* NaOH for 1 hr. All results from four proteins showed linear relationship between absorbency and protein amount, but the slope for different proteins was different, as shown in Fig. 9.

Adsorption and deposition are well-known procedures, while blotting is different from adsorption and deposition. In blotting, protein solution was loaded onto the membrane. A portion of the protein molecules was adsorbed onto the membrane surface and inner surface, while the protein molecules, which are in excess of the saturation amount of adsorption were simply left in membrane pores when water in the solution was evaporated. This portion of protein was different

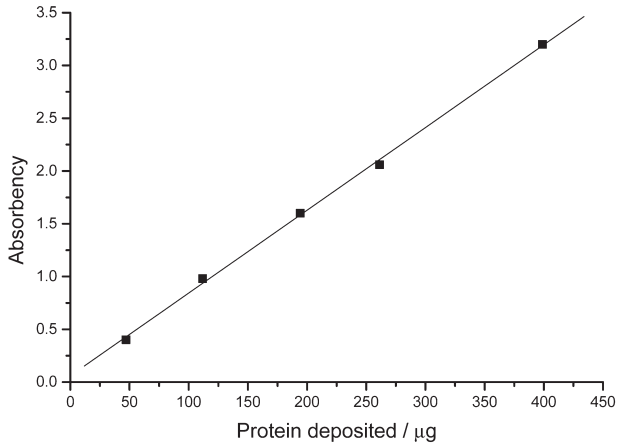

Figure 6. Absorbency of eluted solution as a function of deposited protein on cellulose membranes.

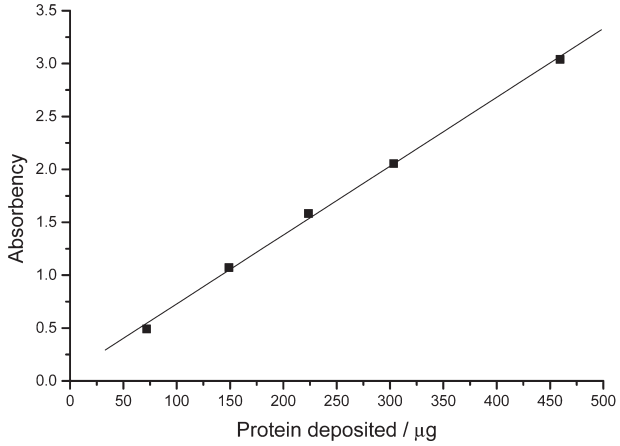

Figure 7. Absorbency of eluted solution as a function of deposited protein on GVWP membranes.

to the adsorbed or deposited aggregates of protein. From Figs. 4–9 it was concluded that, either for blotted, adsorbed, or deposited proteins on membranes, the absorbency of eluted solution was good linear with the amount of proteins on the membrane. The results that take from cellulose membrane and GVWP membrane were very consistent in quantification of blotted protein. However, the

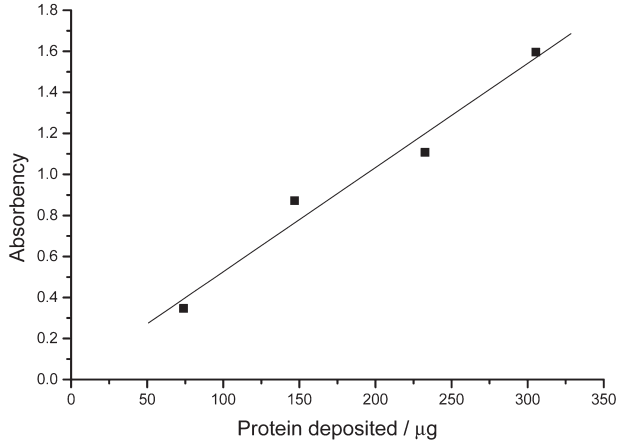

Figure 8. Absorbency of eluted solution as a function of deposited protein on nuclear-pore membrane.

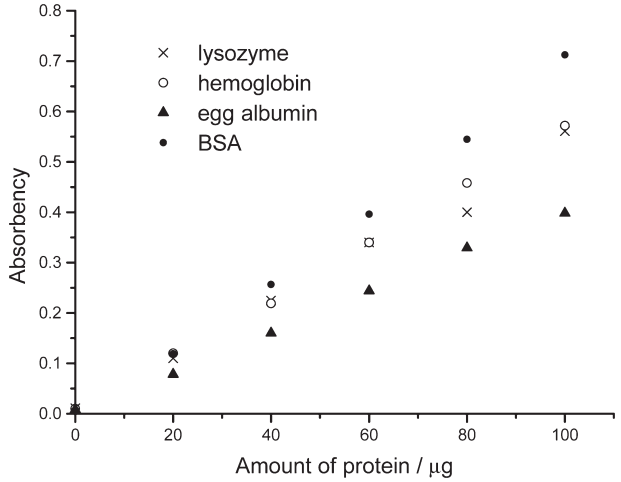

Figure 9. Quantification of different proteins.

result from deposited protein was dependent on type of membrane, and the slope of absorbency ranged from 0.0051 to 0.0075 with slope. Different proteins showed different slopes, and the slopes ranged from 0.004 to 0.007 with proteins examined.

CONCLUSION

The results clearly showed that staining fouled membranes with amido black 10B was a stable method for determining proteins on microfiltration membranes, and that the absorbency of eluted solution was directly proportional to the amount of protein on the membrane. These results indicate that it is possible to correlate absorbency of the eluted solution to protein amount on membrane. So, amount of protein fouled on membranes could be calculated by absorbency of eluted solution. The coefficient relating amount of protein on membrane to absorbency of eluted solution could be obtained by determining absorbencies of a series of membranes that had been blotted with a known quantity of proteins. The result of the adsorbed proteins determined by this method and that of the deposited protein was slightly different. This should be noticed in the application of the method. When deposition dominates the fouling procedure (for example, Durapore GVWP membrane and nuclear-pore membrane adsorb little proteins, or for long-term filtration in which adsorption was saturated and deposition dominates the fouling procedure), proteins on

membrane can be accurately quantified by the method, with blotted proteins as standard. In actual microfiltration, protein fouling of membranes is caused usually by adsorption and deposition of protein aggregates. In this case, there may be some difference between the calculated value and the absolute amount of proteins, and the difference was found to be less than 10%. This error is acceptable especially since we focus on the relative amount of proteins. Compared to isotopic methods and methods that calculated proteins adsorbed by determining concentration changes in solutions, the Amido Black 10B method was more convenient and it could determine protein amount, which is in deposited state. The newly developed ELISA methods have not been used in studying protein fouling of membranes. This apparently is because of the space obstacle for the entering of macromolecules into the pores and aggregates. The amido black 10B molecules are small molecules and it is easy for them to enter membrane pores and protein aggregates. Hence the method is suitable for quantification of proteins fouled on membrane.

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