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Review

Purification of Proteins Using Foam Fractionation

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Purification is an important step in the production of pharmaceuticals from recombinant proteins. The characteristics of industrial-scale purification schemes, such as conventional chromatography, have a significant impact on the cost of production. Foam fractionation, a novel separation technique based upon the differences in affinities of components for the gas/aqueous interface of a foam, has the potential to be a cost-effective component in a purification scheme. This review covers some of the more recent studies in understanding the process and applications of foam fractionation in protein-containing systems with special attention to the requirements of pharmaceutical products.

KEY WORDS: separation; foam fractionation; protein purification.

INTRODUCTION

In the past 10 years, recombinant protein technology has resulted in a great number of new therapeutic agents with even greater numbers of agents expected in the future. Successful production of these novel products is markedly dependent on the development of a suitable recovery scheme often composed of several separation techniques. The overall recovery process should ideally produce a product of very high purity at the lowest possible cost.

Recombinant protein pharmaceuticals present especially challenging problems from the standpoint of recovery and purification. These agents are typically produced in cell culture, the extracts of which are extremely complex mixtures containing a wide variety of contaminants such as lipids, nucleotides and other proteins very similar to the desired product. Therapeutic proteins may be prone to chemical or physical degradation under even relatively mild conditions, thus limiting the purification options. At present, isolation and purification of a protein pharmaceutical can require a number of sequential steps, often including multiple column chromatography operations. The direct scale-up of successful laboratory bench column chromatography methods to the industrial scale, however, can be difficult and expensive (1). Dwyer has reported separation costs in bioprocesses to account for between 40 and 90 percent of total production costs, with newer separation processes having higher expenses (2). Major contributors to the costs of separation and purification unit operations include high initial capital investment in specialized equipment, the expense of chromatography column matrices, regeneration of matrices and process validation, and the labor intensive nature of industrial scale chromatography. Replacement of even one column chromatographic operation by a simple, yet effective, separation step in an overall purification scheme could significantly reduce the expense of producing protein pharmaceuticals.

Foam fractionation is one member of a group of processes known as adsorptive bubble separation techniques. The basis of separation by foam fractionation is the difference in surface activity of molecules in a mixture coupled with the very high surface-to-volume ratio of a foam. Foaming has long been employed in the purification and concentration of conventional surfactants (3). The surface activity of proteins is well recognized and foam-formation of protein solutions has been studied in the food industry (4). The characteristics of foam fractionation, such as low equipment, operation, and labor costs suggest great promise in the cost-effective purification and recovery of pharmaceutical proteins from complex mixtures such as incubation broths.

As of yet, the application of foam fractionation to purification of recombinant protein pharmaceuticals has not been as well studied. The present work is intended to illustrate the potential of foam fractionation as one step in a separation or enrichment scheme for recombinant protein products. The advantages and disadvantages of foam fractionation will be highlighted in the hopes of stimulating further research into application to pharmaceutical processing.

THE FOAM FRACTIONATION PROCESS

Shown in Figure 1 is a schematic diagram of a batch-type foam fractionation device. Figure 2 illustrates the important phenomena occurring in a simple foam fractionation unit. At the bottom of the glass column, individual bubbles are produced by introducing an inert gas, pre-saturated with water, into the feed solution through a sintered glass frit or other porous device. As the bubbles rise through the feed solution, surface active species adsorb to the gas-liquid interface, the surface being dominated by those molecules with a greater rate and extent of adsorption. Bubbles leave the surface of the feed solution,

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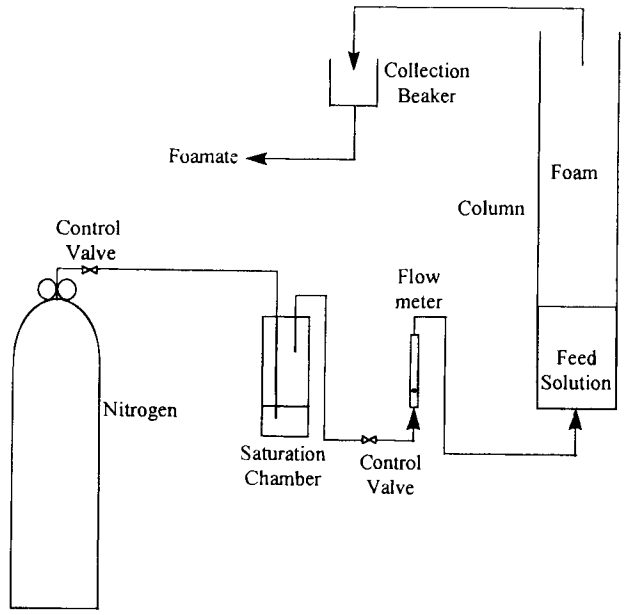

Fig. 1. Schematic diagram of a semi-batch foam fractionation system.

entraining both adsorbed solute and bulk liquid (in the interstitium between bubbles) into the rising column of foam. The interstitial liquid drains slowly through the lamella between the individual bubbles returning unadsorbed solute to the feed solution. Drainage results in thinning of the lamella, promoting interbubble gas diffusion and finally coalescence. At the exit point, foam is collected and collapsed, forming the foamate liquid enriched in the surface active component.

There are two modes of operation by which foam fractionation may purify a protein, the difference lying in the relative surface activities between the contaminants and the protein of interest. If the contaminants are more surface active, they will be removed via the foam, leaving the product in the residual solution. On the other hand, if the product of interest is more surface active, it will be enriched in the foam. Upon creation of a surface, the initial population of molecules at the interface is governed by a complex interaction of factors such as concentration, diffusivitity, molecular flexibility and hydrophobicity. Due to high molecular weight, proteins are slow to adsorb, typically exhibiting diffusion control (5). As the surface ages, molecules initially at the interface may be displaced by those with a higher affinity. The affinity of a protein for the surface tend to be high as the result of the summed interaction of many hydrophobic force-driven points of attachment to the interface. Such time-dependent competition for the air/water interface has been observed in protein-protein (6) and protein-detergent (7) mixtures.

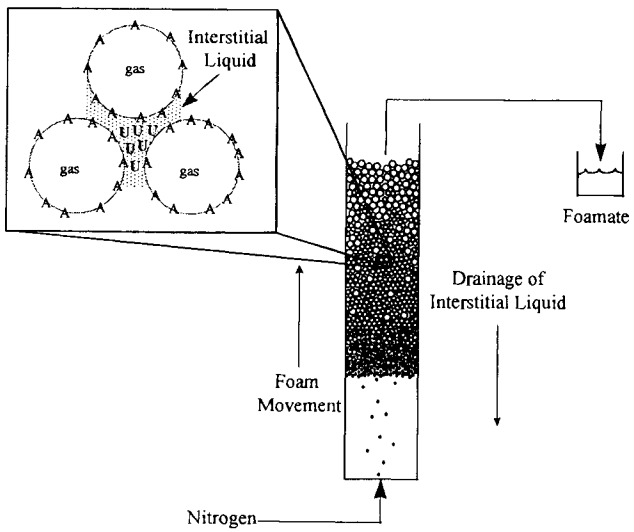

Fig. 2. Phenomenon occurring during foam fractionation. Rising bubbles produce an upward-moving foam containing both adsorbed solute and solute within the interstitial liquid subject to drainage back into the residual solution. Collapsed foam results in foamate solution. Inset: (A) Adsorbed solute; (U) Unadsorbed solute contained in the interstitial liquid.

RECENT USES AND POTENTIAL CONCERNS OF FOAM FRACTIONATION

Foam fractionation techniques have been shown to be successful in separating components of enzyme systems including pepsin from renin (8), amylase from catalase (9), streptokinase from culture media (10), and proteolytic enzymes from human placental extracts (11). Of particular importance to these studies has been the retention of enzymatic activity after foam fractionation. The biological activity of any protein is critically dependent upon the unique three-dimensional structure of the active site of the molecule. Surface chemical studies have shown that an extended period of residence at a quiescent gas/aqueous interface often results in unfolding of the tertiary structure of the macromolecule (4). Disruption of the structure of an enzyme by the anisotropic nature of the interface could result in the loss of activity when the protein is returned to the bulk state. In a limited number of studies reported, enzymatic activity after exposure to foam remains unchanged (8,12), slightly diminished (13,14) or greatly inhibited (12). Clearly, those instances where the structure of protein is damaged by the foaming process limits the use of the technique. The possibility of foam-associated damage to the structure of the protein appears to be greatest when it is the protein of interest that has the higher surface activity and is in more intimate contact with the interface. As of yet, means of predicting which proteins will be adversely influenced by the combined effects of shear and air/water interface in a foam have not been developed. In our own laboratory, we have observed no loss of enzymatic activity in lysozyme remaining in the feed solution when separated from bovine serum albumin by foaming at pH 7.4 (15). Changing of foaming conditions, such as minimizing the time of exposure of the protein to the interface, appears to minimize activity losses. It has been pointed out that conditions for optimizing recovery of enzymatic activity may conflict with those which optimize yield (14). For example, a rapid foaming brought about by the rapid introduction of gas to minimize exposure of the protein to the damaging effects of the interface may not allow sufficient time for adequate drainage of interstitial liquid, compromising enrichment.

One of the most important contaminants to be removed from protein preparations produced by recombinant methods is deoxyribonucleic acid (DNA). The separation of calf thymus DNA from both anionic (sodium dodecyl sulfate) and cationic

(cetylpyridium chloride) surfactants by foaming has been reported (16). Lalchev et al. (17) reasoned that the surface activity difference between a protein and the highly anionic DNA would result in successful separation by foaming methods. These authors went on to demonstrate removal of bovine serum albumin (BSA) or lysozyme from DNA by foam fractionation. For BSA-DNA mixtures, the separation was highly successful. Interestingly, the separation showed only a weak dependence upon pH, probably due to the strong foaming potential of BSA coupled with the extremely weak surface activity of DNA. For lysozyme-DNA mixtures, separation of the two macromolecules was successful only at values of pH > 10. In general, lysozyme exhibits a significant ability to form a stable foam only at high pH values where the tertiary structure of lysozyme is known to unfold (4). Caution should be exercised when employing extremes in pH to induce foam fractionation. Traboulsi and Bummer have shown that foaming of myoglobin at a pH of 4 results in a loss of heme from the hydrophobic pocket of the protein (18). Degradation reactions, such as deamidation, may also be enhanced by extremes in pH (19). Further studies are necessary to fully explore the potential of foam fractionation to separate contaminating DNA from recombinant protein preparations in a cost-effective manner.

SOLUTION CONDITIONS AND OPERATIONAL PARAMETERS

The experimental studies of foam fractionation of protein are essentially of two types: those that modify the conditions of the protein feed solution (pH, concentration, ionic strength) and those that modify the operational parameters of the column (gas flow rate, foam column height, bubble size). The goal of all of these investigations has been to determine those solution and column operating conditions that maximize separation of contaminants from the protein of interest.

A number of investigators have directly demonstrated the enrichment of BSA by foam fractionation is maximal at a pH around 4.7, the isoelectric point (pI) of the protein (20,21). Enrichment is defined here as the increase in the concentration of a surface active component in the foamate solution relative to the residual solution. The addition of inorganic salts such as aluminum sulfate, calcium chloride and sodium chloride enhance enrichment at pH values away from the pI, possibly as a result of electrostatic shielding at high ionic strengths. The observation that sodium sulfate enhances foam fractionation of BSA while sodium perchlorate inhibits the fractionation indicates that the effects of added electrolytes are complex (22). Other effects of added electrolyte on the relevant properties of protein foams have been noted in the food literature including alteration in the foam generation characteristics, protein solubility, and the rate and extent of drainage of interstitial liquid (4). In addition, Brown et al. have suggested that solution pH and ionic strength can significantly modulate the influence of foam column operational parameters upon the results of foam fractionation (23). The apparent interaction between the column operation parameters and solution conditions suggested by Brown et al. illustrates the point that direct comparisons of the results of different laboratories can be made only when identical procedures are employed by each.

The effects of pH and ionic strength on foam fractionation performance are not surprising. It has been well established that the surface activity of a protein is maximal at a pH equal to the pI (24). The stability of food protein foams also shows a similar pH dependence (4). Both of these phenomena are thought to be related to the maximal packing of the protein at the interface as a result of minimized electrostatic repulsion. Considering the central role of surface activity in foam formation, greater packing of protein at the gas/aqueous interface would be expected to enhance the performance of foam fractionation.

The concentration of protein in the feed solution has also been shown to significantly influence the performance of foam fractionation. A number of authors have reported that enrichment of a variety of proteins by foam fractionation decreases with increasing concentration of protein in the feed solution. The reason for this unusual behavior is unclear, but thought to be related to the capacity of the gas/aqueous interface for adsorbed protein. As expected, only a specific amount of protein may adsorb to the interface under specific conditions. Increasing the concentration of protein in solution up to about 0.1% w/v will tend to increase the amount adsorbed up to the point of saturation of the interface (4). Once the surface is saturated, further increases in solution concentration will significantly slow the rate of drainage of the foam (4,25), possibly due to enhanced surface dilatational and bulk viscosities (26). Under these conditions, large amounts of water are retained in the interstitial region and carried over into the foamate. Ultimately, the enhanced carry-over of water decreases the concentration of protein in the foamate. Keeping in mind that gel permeation chromatography often dilutes the molecule of interest, the characteristic of enhanced enrichment from a rather dilute protein solution illustrates an advantage of foam fractionation.

The effects of the operational parameters of the foaming column on the performance of foam fractionation have also been examined. Parameters such as gas velocity, bubble size, and foam column height have all been varied and been shown to influence foam fractionation results. Even the gas employed to form the foam may have a significant influence on the results. In all these cases, the effect of the operational parameters on foam drainage and competition for the interface are believed to be the primary mechanism by which enrichment of protein in the foamate is enhanced. For example, in those instances where foaming is carried out by a sufficiently slow rate of inert gas flow, molecules adsorbing initially to the interface may well be replaced by other components by the time the foam exits the column. On the other hand, for fast rates of gas flow, where the foam exhibits a short residence time in the column, insufficient time may not have elapsed for significant competition for the interface to develop. A further discussion of the effects of operation parameters on foam drainage will be found in the following section.

MODELING FOAM FRACTIONATION

Several groups have attempted to mathematically model the foam fractionation process with the intended goal of formulating a generalized approach to the choice of optimal column operation parameters and protein solution conditions. Ideally, the choice of conditions would be based strictly upon the physical dimensions and characteristics of the foaming column and the physicochemical characteristics of the molecular compo-

nents of the feed solution. In practice, models of foam fractionation have considerable empirical character.

A fully-empirical approach was chosen by Grieves and Bhattacharyya (27,28) who argued that the most important practical results of foam fractionation are the separation of species of interest and the amount of foam produced. In foaming studies, Grieves and Bhattacharyya fit their experimental data of separation and foam volume to an empirical function of the initial concentration of surfactant and contaminant. The authors show that, while it is possible to express separation in terms of foam column operating conditions, the model has no predictive power beyond the bounds of the variables studied.

Success in constructing truly predictive models of the foaming process devoid of a number of empirical adjustable parameters has been very limited. Figures 1 and 2 show that the number and complexity of the mass transport processes involved in foam fractionation makes rational model construction very difficult. Often, a great number of simplifying assumptions about the various phenomena occurring during foaming are required, leading to questions as to the physical relevancy of the resultant model. The two physical processes that are the most controversial with respect to the theoretical treatment are the coalescence of small bubbles into larger bubbles and the drainage of the interstitial liquid as the foam is propelled up the column.

When two bubbles coalesce into a single larger bubble, the total gas/aqueous interface is reduced. In the case of two 0.5 mm diameter spherical bubbles coalescing into one, a 20% reduction in total area results. Referring again to Figure 2, it would be expected that a sudden loss of gas/aqueous interface would influence the fraction of the surface active component(s) residing at the interface. With a loss of surface area, greater amounts of the surface active molecule would be forced into the interstitial liquid. Since solute in the interstitial spaces may influence drainage back to the feed solution, bubble coalescence has the potential to diminish the efficiency of foam fractionation.

Bubble coalescence is dependent upon a number of physical processes. Coalescence is promoted by the presence of only weakly surface active components in solution, by low viscosity solutions that drain in the interstitial spaces very rapidly, by disparity in bubble diameters which allows Laplace pressure differences to enhance interbubble gas diffusion resulting in the growth of larger diameter bubbles at the expense of smaller bubbles, and by thinner, easily ruptured lamella characteristic of larger bubbles. Direct visual measurement of bubble diameters at the foam-glass column interface (23) indicates that average bubble size grows as the foam is forced up the column. These studies have not been successful in determining which of the above processes is primarily responsible for coalescence. From a theoretical standpoint, these processes make coalescence extremely difficult to model. In earlier models of foam fractionation, the difficulties associated with bubble coalescence were considered so great that it was often assumed that coalescence did not occur, or was insignificant. In more recent models of foam fractionation, bubble coalescence is assumed to occur with some empirical relationship dependent upon the height of the foam column (23,29).

In addition to bubble coalescence, drainage of interstitial liquid has also been a difficult characteristic to incorporate into theoretical models. Rapid draining of this liquid back to the feed solution is advantageous in that it does promote the separation of solutes that are less surface active from those of greater activity. A number of characteristics, such as viscosities of bulk and surface films as well as the tortuous nature of the interstitial space appear to control the rate of foam drainage. The effect of the geometry of the interstitial space is particularly difficult to quantitate as it is sensitive to bubble size, shape and packing in the foam. For example, flow rates in the interstitial spaces have been modeled as that occurring through vertical capillaries (30), through parallel plates of varying thickness (31) and through plateau borders (23,31–33) including those of various orientations (34). The relative importance of drainage through films between bubbles compared to that through the plateau borders at the "corners" of dodecahedral bubbles remains an issue of active debate (23,30–34). Direct measurements of the rate of drainage of interstitial liquid in foams have been few. The x-ray attenuation method of Desai and Kumar (34) is noteworthy in that interstitial drainage of a small molecular weight surfactant system was found to be extremely rapid only in the first part of the foam, within one centimeter of the feed liquid-foam interface. Beyond one centimeter of foam, the rate of drainage of the interstitial fluid was found to be extremely slow. If this characteristic is common to protein-containing foams as well, these results suggest that attempts to increase the extent of drainage of the foam by lengthening the height of the foaming column may not be very productive.

In addition to bubble coalescence and drainage, the characteristics of protein adsorption are also thought to be key to understanding the foaming process. Many of the foaming models heretofore have been constructed assuming a surface active agent that rapidly attains true equilibrium between the bulk and surface phases. For typical surfactants such as sodium docecyl sulfate, this assumption is usually valid. Such an assumption on the behavior of proteins is not supported by experimental data where much slower kinetics of adsorption are the general rule. In addition, the structural flexibility of proteins often results in a slow rearrangement of conformation at the interface, a process that is thought to hinder the attainment of true equilibrium. By including a term for the kinetics of protein adsorption, Uraizee and Narsimham (29) have demonstrated improved predictive power under limited conditions. Additional improvements in this model may well provide the predictive power desired.

CONCLUSIONS

The purification of recombinant proteins is an important step in the production of pharmaceutical agents. Foam fractionation has the potential to be a cost-effective component of the purification scheme, but has yet not been applied to pharmaceutical proteins. Successful implementation of foam fractionation will depend upon optimization of the complex interactions between solution conditions, column operational parameters and protein structure. A concise mathematical model of foam fractionation would be helpful in choosing optimal parameters. Future research into the application of foam fractionation to recombinant protein products will likely require the combined efforts of pharmaceutical scientists and engineers specializing in dispersed systems, microbiologists and structural biologists.

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