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A Comparison of the Activity Reduction Occurring in Two Detergent-Assisted Protein (Cellulase and Lysozyme) Foam Fractionation Processes

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A Comparison of the Activity Reduction Occurring in Two Detergent-Assisted Protein (Cellulase and Lysozyme) Foam Fractionation Processes

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Abstract: Foam fractionation has the potential to be an inexpensive alternative to current protein drug concentration or separation methods; however, it has a few drawbacks. One is the fact that not all proteins form a foam layer when aerated at low concentrations. The other is the possible protein denaturation caused during the foaming process. Adding a detergent to the nonfoaming protein solution causes it to foam when aerated. Here, cellulase and lysozyme are studied as model proteins in this process. By themselves, both cellulase and lysozyme solutions hardly form a foam layer when aerated at concentrations below 1000 mg/L (1000 ppm). The addition of 100 mg/L of cetyltrimethylammonium bromide (CTAB) to a 200 mg/L cellulase solution increases the foam volume and makes it possible to almost quadruple (relative to the initial bulk concentration) the concentration of the resulting cellulase foam solution. The foaming, however, reduces the cellulase activity. Diluting the foam with β-cyclodextrin regains some of the lost activity because β-cyclodextrin strips CTAB away from the cellulase, which allows the cellulase to refold to its native state. CTAB detergent does not work well with lysozyme, but the addition of SDS detergent leads to a tripling of the concentration of lysozyme solution without any reduction in enzymatic activity.

Keywords: Activity, enzyme activity, protein, cellulase, lysozyme, foam fractionation, enzyme activity restoration

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INTRODUCTION

Protein separation and concentration are crucial to the recovery of protein drugs by the pharmaceutical industry. While many protein separation techniques may be suitable for laboratory work they are not always economical when applied to large-scale production (1). Foam fractionation is a simple and low-cost technique which can be used to separate or concentrate proteins (2–8). In general, protein enrichment by a foam fractionation process generally works best for dilute (as contrasted with concentrated) initial solutions. The protein solution cannot be too dilute, however, otherwise there will not be enough foam created to first fill and then to pass out of the foaming column. Higher concentrations often lead to high mass recoveries but, also, low protein enrichments. Therefore, an efficient foam fractionation process is achieved when there is neither too little nor too much protein in the prefoamed (starting) protein solution. In addition, foam fractionation is most likely to be effective with one or just a few proteins in solution in a downstream separation process because foam fractionation is usually not good at discriminating between the different proteins but, rather, is good at concentrating them. The foam height also plays an important role in a foam column, because, when the height is increased, the enrichment ratio also increases due to additional water drainage from the formed foam.

One concern with using foam fractionation to concentrate proteins is protein denaturation, mostly occurring at the gas-liquid interface (9). The hydrophobic parts of the protein prefer to be in the gas phase, so the protein reorients itself into a different conformation from its native state. A foam fractionation study of β-lactoglobulin suggests that the damage to the protein is reversible when the foam collapses back to a liquid (10). On the other hand, another study shows that several proteins do not refold to their native states following foam collapse to a liquid (11). In order for a foam fractionation process to be economically attractive, protein denaturation during processing needs to be reduced or even eliminated.

One relatively inexpensive renaturation method for recovering the activity of a damaged protein uses "artificial chaperones" (12, 13), a two-step process. The most commonly used detergent and cyclodextrin pair in this artificial chaperone process for protein renaturation are cetyltrimethylammonium bromide (CTAB) and β-cyclodextrin (β-CD). Artificial chaperones have been successfully applied to the renaturation of the following proteins (previously degraded by chemical denaturation): carbonic anhydrase B (14, 15), lysozyme (16–19), citrate synthase (15, 20), MM-creatine kinase (21), xylanase (22), insulin (17), and human growth hormone (23).

Artificial chaperones may be able to restore enzyme activity in physically denatured proteins from a foam fractionation process, since they can refold chemically and thermally denatured proteins. In order to apply a foam fractionation process to industrial downstream processing, significant process-derived protein denaturation resulting from foaming must be

corrected. The use of artificial chaperones in conjunction with a foam fractionation process may be a feasible and economical way to intrinsically reverse the activity loss from that process. To implement this recovery scheme, it is proposed that a detergent be added to the protein solution in the initial protein recovery step, such that the detergent is carried through the foam fractionation process, followed by a detergent-stripping step with the addition of cyclodextrin at the completion of the process.

The addition of detergents to an aerated protein solution can increase or even create foam, so that a nonfoaming protein can be concentrated by foam fractionation process when assisted by the addition of a detergent. Cellulase is used as the model nonfoaming protein/enzyme here because it is an enzyme of significant industrial importance and its enzymatic activity can be determined easily and rapidly. It is compared in this foam fractionation study to the better studied protein, lysozyme. Four performance criteria are looked at in order to evaluate the performance of the process: ER, MR, AE, and AR. The enrichment ratio (ER) is defined as the protein concentration (C) in the foamate divided by the initial protein concentration. The mass recovery (MR) is defined as the amount of protein in the foamate divided by the initial amount of protein. A is the activity per unit volume of solution. The activity enrichment (AE) is defined as the activity per unit volume of the foamate, A_f divided by the initial activity per unit volume of the solution before foaming, A_i. The activity recovery (AR) is defined as the total activity in the foamate divided by the total initial activity. The fraction of active enzyme (η) is defined as the ratio of the specific activity (activity per unit mass, S) of the enzyme in the foamate to the initial specific activity.

$$ER = \frac{C_{foam}}{C_{initial}} \tag{1}$$

$$MR = \frac{C_{foam} \times V_{foam}}{C_{initial} \times V_{initial}} \tag{2}$$

$$AE = \frac{A_{foam}}{A_{initial}} \tag{3}$$

$$AR = \frac{A_{foam} \times V_{foam}}{A_{initial} \times V_{initial}} = \eta \times MR \tag{4}$$

$$\eta = \frac{S_f}{S_i} \tag{5}$$

Both lysozyme and cellulase, as nonfoaming proteins, are compared, as both do not form a foam layer at low concentrations because they are "hydrophilic" proteins. Lysozyme is a single protein, unlike cellulase, which is a group of proteins acting in concert to catalyze the conversion of cellulose

substrates to sugars. Lysozyme (14.3 kDa) is smaller in size than cellulase; it has a net positive charge and a *pI* around 11. CBH I (65 kDa) is the most abundant protein in cellulase produced from *Trichoderma reesei*, and CBH I has a *pI* around 3.9 (24) and is generally nonfoaming. Comparing cellulase with lysozyme may help to better understand the cellulase foam fractionation.

MATERIALS AND METHODS

Cellulase from *Trichoderma reesei*, lysozyme from chicken egg white, lyophilized cells of *Micrococcus lysodeikticus*, sodium dodecylsulfate (SDS) and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma (St. Louis, MO). Cetyltrimethylammonium bromide (CTAB) was purchased from Fluka (Switzerland). Whitman filter paper No. 1, β-cyclodextrin, bicinchoninic acid (BCA) assay kit, and 96-well microplate were purchased from Fisher Scientific (Pittsburgh, PA). A measure of 10 mM pH 5.0 phosphate buffer was used as a solvent in cellulase experiment (7), and 10 mM pH 8.5 Tris-HCl buffer was used in the lysozyme experiment (25). The components used in preparing these buffer solutions were purchased from Sigma.

Foam Fractionation

Semibatch foam fractionation experiments are carried out in a glass column (inside diameter 2 cm, height 10 cm). The schematic drawing of the apparatus is shown in a previous publication (26). An amount of 12 mL of protein solution is introduced into the column and air from a compressed gas cylinder is introduced (at a selected rate) through a fritted disc sparger (pore size 40–60 μm) at the bottom of the column. Water loss in the effluent air stream is minimized by humidifying the air before it enters the column. Air continues to flow into the column until no more foam is generated. The produced foam is allowed to collapse into a liquid product: the foamate in the foam collector. Foamate volume is measured in a graduated cylinder. The column is aerated at air flow rates of 4 or 8 or 12 mL/min. Each experimental condition is repeated in triplicate.

Renaturation of Cellulase after Foam Fractionation

After foam fractionation of a cellulase and CTAB mixture, 350 μL of collected foamate is diluted with 150 μL of 13 mM β-cyclodextrin solution. The resulting solution is stored overnight before checking for its activity, both before and after addition of β-cyclodextrin. The filter paper activity test (27) is used to determine the cellulase activity. The DNS assay (28) is used to measure the amount of sugar produced in the filter paper test.

BCA Assay for Protein Concentration

The 20 μL of collected cellulase (in buffer solution) samples are put into a 96 well-microplate and the analysis is repeated in triplicate. Then, 180 μL of BCA reagent is added to each well-plate. The microplate is scanned after 30 min for the determination of absorbance at 562 nm (29). For lysozyme, 20 μL of the sample is added to a well in a 96 well-microplate. The 180 μL of BCA reagent is then also added to each well and again carried out in triplicate. The microplate is incubated at 37°C for 2 h before being read by a Bio-Tek μQuant plate reader at 562 nm. Longer incubation times and higher temperatures are used to increase the light absorbance signal from the BCA assay, when necessary.

Lysozyme Activity Assay

The protocol for this assay is taken from the Worthington Enzyme Manual (30). A total of 9 mg of lyophilized *Micrococcus lysodeikticus* cells is first diluted in 25 mL of 0.1 M potassium phosphate buffer at pH 7.0. Then, after dissolving the *Micrococcus lysodeikticus* cells, the buffer solution is added to bring the final volume to 30 mL. A total of 10 μL of the samples are added to a 96 well-microplate. The 290 μL of BCA reagent are then added to each well. The assay is carried out in triplicate. The change in absorbance at 450 nm is scanned every minute for 5 min using a Bio-Tek μQuant plate reader.

Circular Dichroism

A J-180 Jasco CD spectrometer (Jasco Inc., Easton, MD) is used to determine the cellulase secondary structure. The cellulase protein solution at three different stages in the process (before foaming, after foaming, and after renaturation) is scanned in the far-UV range (190–250 nm) across the 1 mm path length cell. Each of the three samples contains about 350 mg/L of cellulase. CD scans for samples before and after foaming are also conducted for lysozyme solutions.

RESULTS AND DISCUSSION

Cellulase Foam Fractionation

A solution containing cellulase alone hardly produces any foam when aerated at 4 to 12 mL/min, but the addition of CTAB to the system makes it possible to generate enough foam to rise up in the column and leave the fractionation

column. Besides increasing the foam volume, CTAB causes the foamate protein concentration to be higher than that of the initial solution. Without CTAB, the air flow rate needs to be around 60 mL/min to push the cellulase foam out of the column, but foaming at such a high air flow rate does not lead to meaningful enrichments. Figure 1 shows the enrichment ratio and mass recovery after foam fractionation for a solution containing cellulase and CTAB.

The enrichment ratio ranges between 2.6 and 3.8, with the highest value at the lowest air flow rate tested. It takes about 30, 15, and 10 min to complete foam fractionation at air flow rates of 4, 8, and 12 mL/min, respectively. The highest mass recovery, 0.55, is observed at the highest air flow rate tested, 12 mL/min. These enrichment ratio observations are similar to the foam fractionation of other proteins (3, 5, 6, 25, 31, 32). At low air flow rates, it takes a longer time for the foam to reach the top of the column, giving

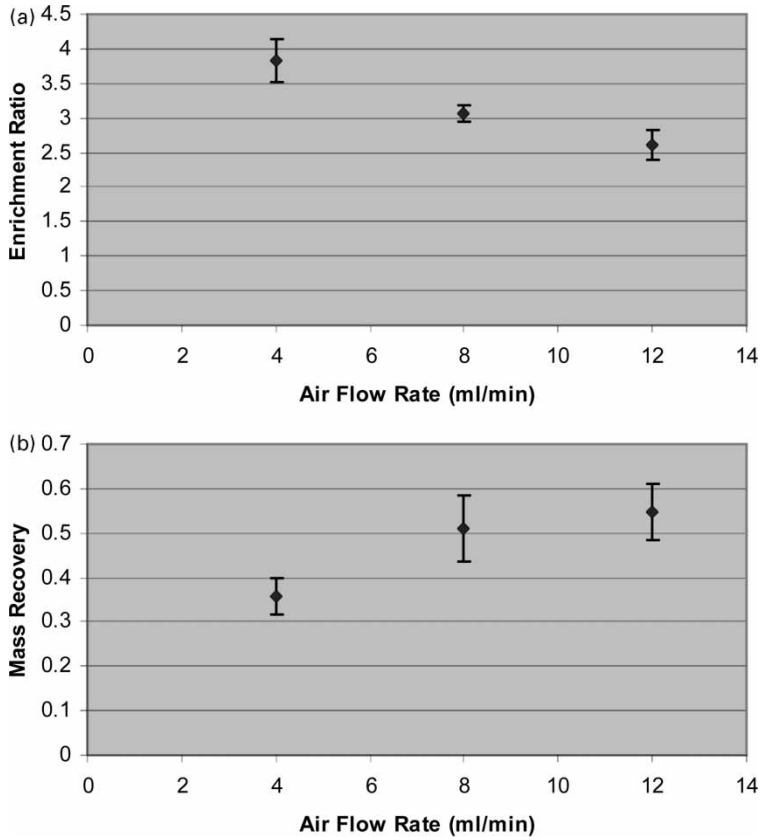

Figure 1. Cellulase (a) enrichment ratio and (b) mass recovery of the foam fractionation of a 200 mg/L cellulase and 100 mg/L CTAB solution at pH 5.0.

more time for drainage and resulting in less liquid hold-up. As a result, the foam becomes drier and more concentrated. At high air flow rates, the liquid hold-up is higher, thus the foamate is more dilute, leading to lower enrichment but higher mass recovery.

An enzyme's economical value depends on its activity (6), so the activity of cellulase in the foamate is checked both after foaming and after renaturation (Fig. 2). Although the foamate samples are more concentrated than the initial solution samples (Fig. 1A), the denaturation from the foam fractionation process causes the activity enrichment of the foamate to drop below 1, as seen in Fig. 2A. Protein denaturation leads to a reduction in specific activity, which can be more clearly seen in Fig. 3. For example, at 12 mL/min air flow rates, the initial specific activity (before foaming) is 0.5 unit/mg. After foaming, denaturation reduces the specific activity to 0.08 unit/mg.

To carry out the artificial chaperone renaturation process, foamate samples are diluted with 13 mM β-cyclodextrin following foam fractionation.

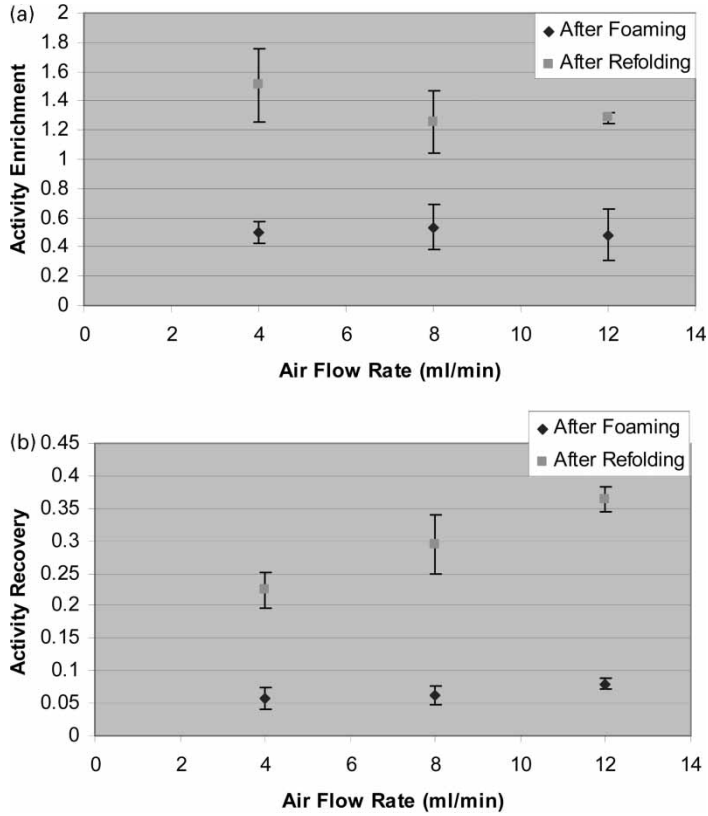

Figure 2. (A) Activity enrichment and (B) activity recovery of foam fractionation of 200 mg/L cellulase and 100 mg/L CTAB at pH 5.0.

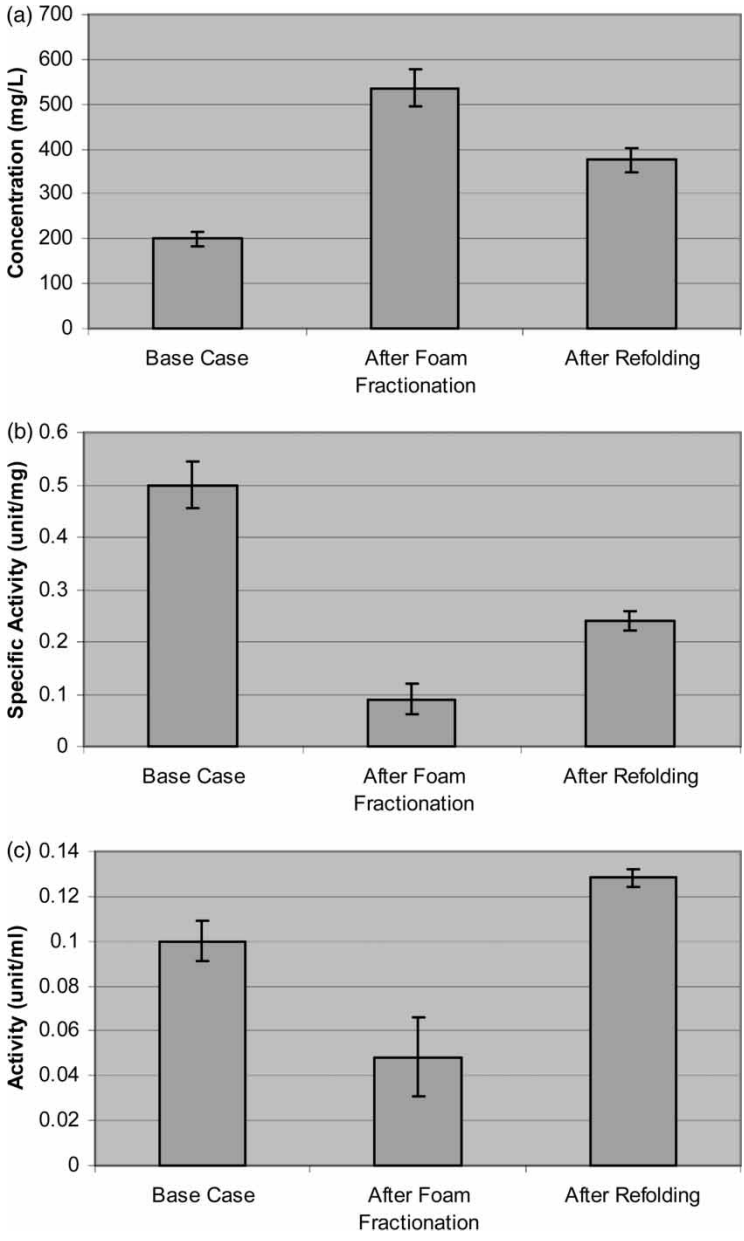

Figure 3. (a) Change in concentration of cellulase at three different stages of the process (before foam fractionation, after foam fractionation, and after diluting with β-cyclodextrin), (b) change in specific activity at three different stages of the process, and (c) change in activity at three different stages of the process. All data are from the foam fractionation of 200 mg/L cellulase and 100 mg/L CTAB with an air flow rate of 12 mL/min at pH 5.

The addition of cyclodextrin solution dilutes the mass enrichment ratio by 30% but does not change the mass recovery. Following dilution with β-cyclodextrin, the activity enrichment of the process increases two to three-fold. At air flow rates of 4, 8, and 12 mL/min, the activity enrichment of the foamate increases to 1.5, 1.25, and 1.28 from 0.5, 0.54, and 0.48, respectively. The highest activity recovery before refolding is 0.08 and the highest activity recovery after refolding is 0.36. The activity recovery increases significantly, since the activity per unit volume more than doubles. The increase in cellulase activity indicates that the artificial chaperones renature the cellulase. Since the activity enrichment is not as high as the mass enrichment ratio (e.g., at an air flow rate of 12 mL/min, the AE is equal to 1.3, but the ER is equal to 1.8), the artificial chaperones do not renature all the denatured cellulase (η is less than one). However, work by others has also shown that artificial chaperones do not completely renature denatured proteins (14). Figure 3 provides an overview for the case of foam fractionation of cellulase at an air flow rate of 12 mL/min.

The lowest concentration but the highest specific activity represents the cellulase base case, having the highest specific activity because it is not denatured. The cellulase solution is only concentrated in this study, not purified or separated from other proteins, so the specific activity cannot increase beyond the initial level. Since the cellulase purchased from Sigma is already purified, the specific activity (activity per protein mass) remains at or below the initial level with dilution and protein denaturation. The goal of the foaming/renaturation process here is to maintain the initial specific activity while concentrating the solution, so that the resulting concentrated product has a higher activity per unit volume (more mass in a given volume leads to higher activity per unit volume). Figure 3B shows that this processing achieves less than half of the maximum achievable recovery of activity per unit volume after renaturation. However, the concentration and the specific activity increase enough to make the activity (unit/mL) of the final solution more than what was present in the initial solution. That specific activity more than doubles after refolding clearly shows the benefit of incorporating an artificial chaperone system into a cellulase foam fractionation system.

Circular dichroism (CD) determination on cellulase samples both before and after foam fractionation provides information on the cellulase secondary structure. The shape of the spectrum after foaming is different than the spectrum of the sample taken before foam fractionation, with the profile change indicating possible denaturation of the secondary structure. This observation is consistent with previous findings that foaming can cause changes in the secondary structure of proteins/enzymes (10, 11). After refolding, the CD spectrum becomes closer to the spectrum of the initial state, showing that the addition of an artificial chaperone system (detergent and cyclodextrin) can help restore enzyme activity. We speculate that the cyclodextrin takes away CTAB from denatured cellulase based on earlier reports on artificial chaperones (14, 16, 33). By taking away CTAB, cellulase refolds to its native

Table 1. CD results at different steps of the process for foam fractionation of 200 mg/L cellulase and 100 mg/L CTAB at pH 5.0

	α-helix		β-strand			
	Regular	Distorted	Regular	Distorted	Turns	Unordered
Cellulase	0.02	0.05	0.2	0.12	0.2	0.41
Foam	0.12	0.08	0.2	0.13	0.2	0.27
Refold	0.02	0.04	0.22	0.14	0.18	0.4

state. The spectra changes are consistent with the measured changes in specific activity, which decrease after foaming but are partially restored to the initial state after refolding. The CD results using an online tool, DICHRO WEB (34) provided by the Birkbeck College Centre for Protein Membrane Structure and Dynamics are shown in Table 1.

There are at least two possible ways that cellulase can denature in this modified foam fractionation process. One way is that the addition of a surfactant chemically denatures the cellulase. Another way is that it can sustain damage from the foaming process itself. Figure 4 shows that cellulase can be in the presence of detergents at 50°C without much change in activity. The absorbance at 540 nm (shown in Figure 5) quantifies the amount of sugar released using the filter paper assay. There is no difference in activity between the cellulase samples with detergent and cellulase samples without detergent. It is suggested from the literature that the decrease in cellulase activity by foam fractionation is expected since foaming has previously denatured enzymes, mainly by molecular reorientation at a gas-liquid interface (9–11, 35).

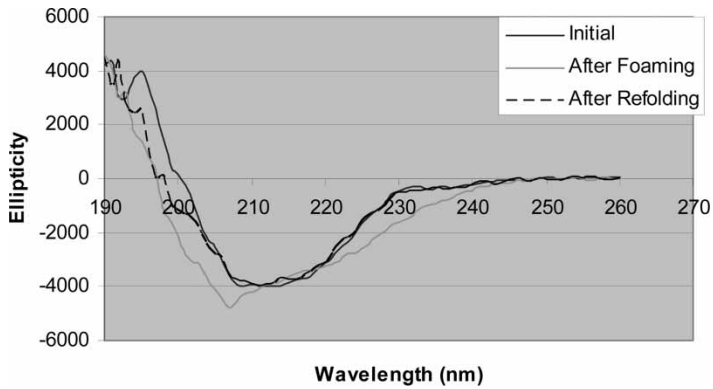

Figure 4. The CD spectrum at three different stages of the cellulase foam fractionation process.

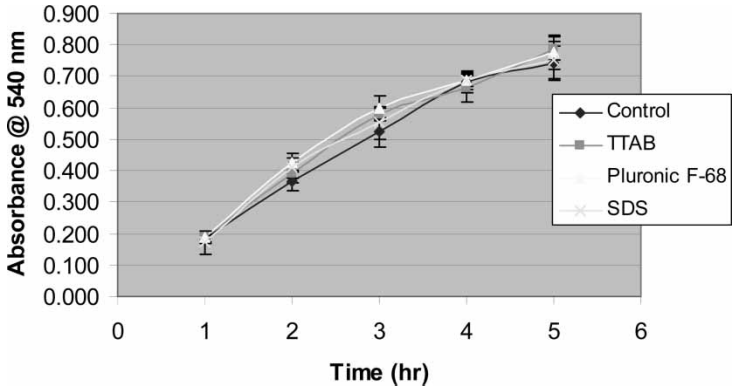

Figure 5. Effect of adding different surfactants to a 200 mg/L cellulase solution. Absorbance response to the filter paper assay is shown in this figure.

Lysozyme Foam Fractionation

The 10 mM Tris-HCl, pH 8.5 buffer, is used as the solvent for the lysozyme. The pH 8.5 is selected for this study because lysozyme can be renatured by artificial chaperones at this pH (16, 17). A 0.075 mg/mL (75 ppm) lysozyme solution is used in the foam column initially in order to check to see if aerated lysozyme at this concentration can produce any foam. No foam, in fact, is developed in the column when aerated at each of the three flow rates: 4, 8, and 12 mL/min. This observation confirms previously published observations (7, 36). Lysozyme is a hydrophilic protein when compared to bovine serum albumin (BSA) (37) and the lack of foaming is consistent with its known hydrophilic nature.

CTAB is added to the lysozyme solution to make the initial working detergent-assisted solution of 100 mg/L CTAB and 75 mg/L lysozyme. The CTAB concentration is the same one used in the previously discussed cellulase experiments and is foamed at three different air flow rates. The highest mass and activity enrichment ratios of 1.2 are achieved at 4 mL/min; the enrichment ratios decrease for both mass and activity as the air flow rate increases. Air flow rates above 12 mL/min are not studied since only enrichment ratios greater than one are desired. The foamate activity enrichments (1.2, 1.15, and 1.0, respectively) at each of the air flow rates do not differ significantly from the respective enrichment ratios. The foam fractionation of cellulase with CTAB provides higher enrichment ratios but also more protein denaturation. Since CTAB is cationic, it may not work well with lysozyme as it does with cellulase, because lysozyme has a known net positive charge at the processing pH. Thus, a similar charge may prevent CTAB and lysozyme from forming a stable complex. The resulting lysozyme mass recoveries are all very low for the three air flow rates studied. The highest mass recovery is 0.22, and the mass recovery increases linearly as the air flow rate

increases. The pattern of enrichment ratio and mass recovery ratio for the CTAB and lysozyme case is similar to the pattern shown by the foam fractionation of BSA (3). Since the lysozyme denaturation is insignificant, the mass recoveries are equal to the activity recoveries, and η from Eq. (4) equals 1.

Pluronic F-68 is added to the lysozyme solution as the detergent in another experiment to make a mixture of 75 mg/L lysozyme and 100 mg/L Pluronic F-68. Following foam fractionation of the Pluronic-assisted fractionation, the enrichment ratio is determined to be 1 at the tested air flow rates. The mass recovery from adding Pluronic F-68 is even lower than that from adding CTAB. The highest mass recovery using Pluronic F-68 is only 0.15. Since there is no apparent difference in the mass and activity recoveries for Pluronic F-68, it follows from Eq. (4) that $\eta = 1$. Another way of expressing this observation of $\eta = 1$ is that protein denaturation must be insignificant because a significant protein denaturation causes a change in specific activity, leading to a difference between the mass recovery and the activity recovery ($\eta < 1$).

SDS is used to prepare a third detergent solution comprising 75 mg/L lysozyme and 200 mg/L SDS. The resulting activity enrichment ratio is similar to the mass enrichment ratio (Fig. 6). This implies that there is very little or no protein denaturation. With SDS addition, the enrichment ratio clearly decreases as the air flow rates increase. The highest enrichment ratio achieved for both activity and mass using SDS is around 3.3 at an air flow rate of 4 mL/min. At the highest air flow rate of 12 mL/min, both enrichment ratios are equal to 1.4. The activity enrichment and mass enrichment ratios are slightly different at each of the air flow rates, but those differences are within the respective standard deviations. The mass recovery ratios and the activity recovery ratios are very close at all three measured air flow rates, since there is apparently very little or no protein denaturation (as shown in Fig. 7).

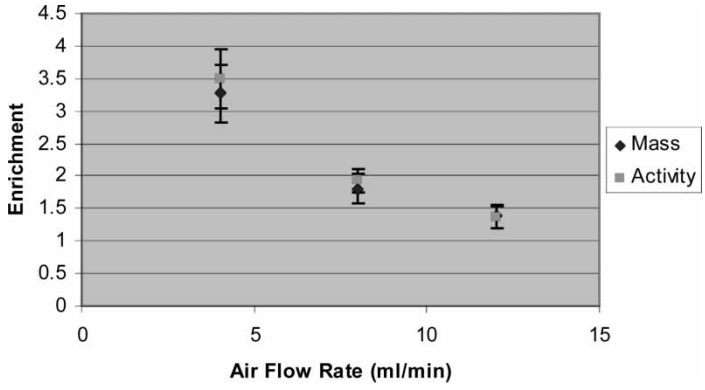

Figure 6. The lysozyme enrichment for the foam fractionation of a 75 mg/L lysozyme and 200 mg/L SDS solution as a function of the air flow rate.

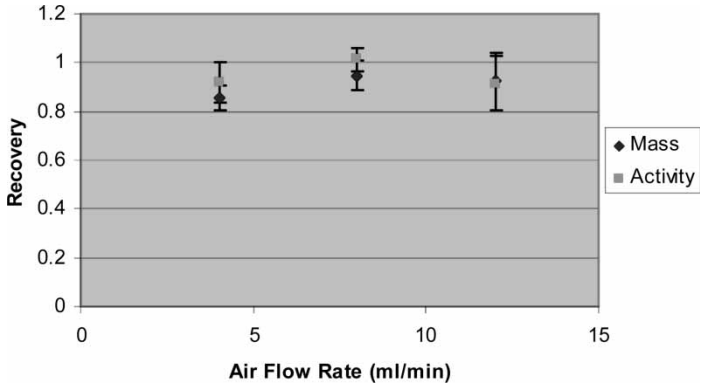

Figure 7. The lysozyme recovery for the foam fractionation of a 75 mg/L lysozyme and 200 mg/L SDS solution as a function of the air flow rate.

The mass recovery ratio is lowest at an air flow rate of 4 mL/min at the value of 0.85. The mass recoveries at air flow rates of 8 mL/min and 12 mL/min are very close together (0.94 and 0.92, respectively). The activity recovery ratio follows the same pattern as the mass recovery ratio.

SDS detergent appears to work the best of the three detergents studied in terms of both protein enrichment and recovery, as compared to CTAB and Pluronic F-68 detergents in assisting the foam fractionation of lysozyme. Since the isoelectric point, *pI*, of lysozyme is at pH 11, lysozyme has a net positive charge at pHs lower than its *pI*, and hence, at pH 8.5, which is used in this study. Thus, the recovery may be highest for the SDS-assisted system since SDS is an anionic surfactant and lysozyme is a positively charged protein. This possible electrostatic charge interaction can lead to the formation of a stable SDS-lysozyme complex, facilitating protein recovery.

Increases in protein concentration in the foamate indicate that there is protein adsorption on the bubble surface. Generally, when the activity enrichment equals the mass enrichment ratio, it implies that the protein denaturation is insignificant. SDS-lysozyme is a very different system than CTAB-cellulase, one where significant denaturation following foaming is noted.

Circular dichroism (CD) is used to examine the lysozyme denaturation in the foam fractionation process, as shown in Fig. 8. The CD spectrum indicates that there is very little change in the secondary structure of lysozyme during foam fractionation. Since activity measurements indicate that there is minimal activity loss for lysozyme, no β-cyclodextrin addition experiments to recover the activity are performed. The change in the lysozyme CD spectrum is not as dramatic as in the cellulase CD case (Fig. 4). The calculated results using DICHRO WEB (34) are shown in Table 2. The small change noted in the secondary structure is not enough to affect the measured enzymatic activity of the lysozyme. Some change in secondary structure is expected because changes in secondary structure of lysozyme at air-water

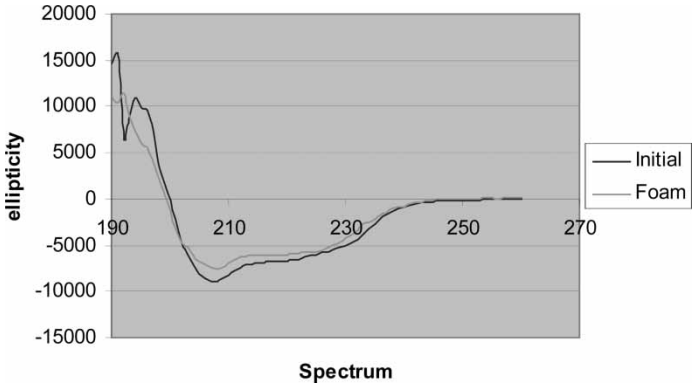

Figure 8. The CD spectrum at two stages of the lysozyme foam fractionation process.

interfaces due to adsorption were previously reported (38, 39). A neutron reflection method was used to determine that a small shift from an α-helix to a β sheet was not enough to influence the measured activity of lysozyme (39). Using an oppositely charged detergent makes it possible to specifically bind a particular protein and thus increase the process selectivity (40).

CONCLUSIONS

Artificial chaperones can be used to refold cellulase that has been physically denatured in a foam fractionation process. Addition of CTAB makes it possible to concentrate a nonfoaming enzyme like cellulase using foam fractionation. Circular dichroism (CD) measurements reveal that there is a significant change in the enzyme secondary structure of foam fractionated cellulase. This is in agreement with the measured cellulase activity enrichment values which are determined to be much less than 1, even with mass enrichments reaching 3.8. Dilution with β-cyclodextrin is the second part of the artificial chaperone system with the addition of a detergent being the first part. Together, they help increase the specific activity of cellulase following

Table 2. CD results before and after foam fractionation of 75 mg/L lysozyme and 200 mg/L SDS at pH 8.5

	α-helix		β-strand			
	Regular	Distorted	Regular	Distorted	Turns	Unordered
Lysozyme	0.18	0.06	0.17	0.11	0.16	0.32
Foam	0.09	0.09	0.18	0.1	0.21	0.33

foam fractionation. The CD measurements of the post-β-cyclodextrin-altered cellulase reveal that a shift in secondary structure back toward the initial native state occurred, confirming the activity restoration resulting from the use of the artificial chaperone system. This CD measurement of restored cellulase is consistent with the measured change in cellulase activity. The use of β-cyclodextrin makes it possible for the cellulase activity in the final state to be higher than the activity in the initial state. The lysozyme experiments show that an SDS-assisted foam fractionation process can be used to concentrate lysozyme without a reduction in its enzymatic activity.

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