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Effect of β-Cyclodextrin in Artificial Chaperones Assisted Foam Fractionation of Cellulase

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Abstract

Foam fractionation has the potential to be a low-cost protein separation process; however, it may cause protein denaturation during the foaming process. In previous work with cellulase, artificial chaperones were integrated into the foam fractionation process in order to reduce the loss of enzymatic activity. In this study, other factors were introduced to further reduce the loss of cellulase activity: type of cyclodextrin, cyclodextrin concentration, dilution ratio cyclodextrin to the foamate and holding time. α-Cyclodextrin was almost as effective as β-cyclodextrin in refolding the foamed cellulase-Cetyltrimethylammonium bromide mixture. β-Cyclodextrin (6.5 m*M*) was almost as effective as 13 m*M* β-cyclodextrin in refolding. The dilution ratio, seven parts foamate and three parts β-cyclodextrin solution, was found to be most effective among the three ratios tested (7:3, 1:1, and 3:7). The activity after refolding at this dilution ratio is around 0.14 unit/mL The refolding time study showed that the refolding process was found to be most effective for the short refolding times (within 1 h).

Index Entries: β-cyclodextrin; artificial chaperones; foam fractionation; cellulase; protein denaturation; protein refolding.

Introduction

Foam fractionation is an adsorptive bubble separation process which can be used to concentrate or purify surface active chemicals from a dilute solution. This foam separation technique has been used in wastewater treatment and ore flotation *(1)*. Many studies also show that foam fractionation can be effective in recovering proteins from dilute solution *(2–5)*. Foam fractionation can also recover biosurfactants from a culture broth *(6)* or even be integrated into a fermentation process to recover the biosurfactant, surfactin, directly from *Bacillus subtilis* broth *(7)*. The low operating cost of foam fractionation makes it an attractive alternative for current protein concentration methods *(8)*. Because foam fractionation works well

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with dilute solutions, it is most useful when proteins are in low concentration as in the early stages of a downstream purification process or when coupled with a fermentation process. Foam fractionation usually does not, however, work with proteins that do not form a foam layer when aerated.

Protein denaturation at a gas–liquid interface is a concern when operating a protein foam fractionation process. The hydrophobic and hydrophilic parts of a protein reorient themselves during adsorption onto the surface of a bubble *(9,10)* in such a process, which causes the protein to shift away from its native state. This bubble surface denaturation can result in a loss of enzymatic activity for those proteins which are enzymes. In our previous work, an artificial chaperones system was integrated into the foam fractionation process. Because the system contained a surfactant, along with a detergent-stripping agent, it successfully reduced the amount of denatured cellulase at the end of the process, whereas simultaneously allowing foam to form using cellulase. Reducing the amount of denaturation makes it feasible to concentrate both foaming and nonfoaming enzymes such as cellulases by foam fractionation.

During foam fractionation, surface denaturation occurs because of protein adsorption at gas–liquid interfaces *(11–13)*. Adsorption causes the tertiary structure of proteins to change, and in some cases (e.g., pepsin) the secondary structure can change as well *(14–16)*. Previously, protein denaturation occurring during foam fractionation was minimized by setting the operating conditions to reduce protein adsorption at gas–liquid interfaces *(5,17)*. However, these conditions may not be the best settings strategy for concentrating enzymes since adequate bubble adsorption is a key to high enrichment and mass recovery of proteins. In this article, the effect of several operating variables such as cyclodextrin type, β-cyclodextrin concentration, dilution ratio of β-cyclodextrin to recovered protein and the effect of time on refolding will be evaluated in order to determine an effective combination to achieve the highest cellulase activity following the foam fractionation process.

Materials and Methods

Cellulase from *Trichoderma reesei* (cat. no. C-8546), 3,5-dinitrosalicylic acid (DNS), pluronic F-68, and sodium dodecyl sulfate (SDS) were purchased from Sigma Co. (St. Louis, MO). Bicinchoninic acid (BCA) protein assay reagent, Whatman No. 1 filter paper, Fisher brand 96 well-microplate and β-cyclodextrin were purchased from Fisher Scientific Co. (Pittsburgh, PA). Cetyltrimethylammonium bromide (CTAB) was purchased from Fluka Co. (Buchs, Switzerland).

Foam Fractionation

Batch foam fractionation experiments were carried out in a glass column a small column was used here to minimize the cost of cellulase used (which was $65/g in relatively pure form). The column inside diameter

was 2 cm, and the column height was 10 cm as previously described in Burapatana et al. *(18)*. A mixture of cellulase and detergent (pH 5.0; 10 m*M* phosphate buffer was used as a solvent) was placed in the column, and air from a compressed gas cylinder was introduced continuously through a fritted disk sparger (pore size 40–60 μm) at the bottom of the column. Water loss in the effluent air stream was minimized by humidifying the air before it entered the column. Air continued to flow into the column until no more foam was generated. The produced foam was allowed to collapse into a liquid product (foamate) in the foam collector.

Renaturation of Cellulase After Foam Fractionation

After foam fractionation of a cellulase and CTAB mixture, 350 μL of collected foamate was diluted with 150 μL of 13 m*M* β-cyclodextrin solution in water. The resulting solution was stored overnight before measuring the cellulase activity.

BCA Assay for Protein Concentration

Foamate samples of 20 μL were placed in a 96 well-microplate in triplicate. Then, 180 μL of BCA reagent *(19)* was added to each well-plate. The microplate was scanned after 30 min at 562 nm for absorbance determination.

Cellulase Activity

The filter paper assay *(20)* was used to determine the cellulase activity. The DNS reducing sugar assay *(21)* was used to measure the amount of sugar produced.

To determine the refolding effectiveness of α-cyclodextrin and γ-cyclodextrin as compared β-cyclodextrin, foamate of a cellulase-CTAB mixture collected from foaming at an air-flow rate of 8 mL/min was diluted with the three cyclodextrins. The concentration of β-cyclodextrin solution initially used was 13 m*M*. In additional experiments, refolding of the cellulase-CTAB mixture was conducted after dilution with 3.25 and 6.5 m*M* β-cyclodextrin solutions in order to see if lower concentrations of β-cyclodextrin could be used effectively. The dilution ratio of foamate to β-cyclodextrin was 7:3 initially, but 1:1 and 3:7 were also tested to see if higher refolding could be achieved. To investigate the process dynamics, the dilution ratio of 7:3 was divided into three equal size increments instead of a single increment, with the change in activity after each increment being tracked. Usually, the solution was left overnight after adding cyclodextrin. Because cellulase in solution can degrade over a period of time, the foamate of each cellulase-CTAB mixture (12 mL initial volume, 8 mL/min air-flow rate) was monitored over a period of 12 h following the addition of β-cyclodextrin. This time test was used to determine how long the postcyclodextrin waiting time should be.

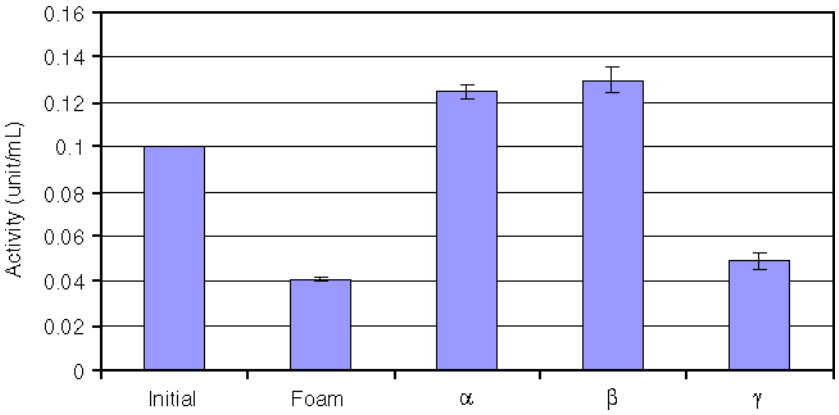

Fig. 1. Cellulase activity before and after foaming and after addition of three types of cyclodextrin to the foamate collected at an air-flow rate of 12 mL/min from a mixture of 200 mg/L cellulase and 100 mg/L CTAB.

Results and Discussion

Effect of Different Cyclodextrins

The artificial chaperones system consists of both a surfactant and a stripping agent, so the effect of different stripping agents on active protein recovery, specifically the type of cyclodextrin, was also investigated in this foam fractionation study. Twelve mililiters of a 200 mg/L cellulase and 100 mg/L CTAB mixture was aerated at an air-flow rate of 12 mL/min. Seven hundred microliters of the resulting foamate was then placed in a 1.5 mL microcentrifuge tube and diluted with 300 μL of a different type of cyclodextrin (α-cyclodextrin, β-cyclodextrin, or γ-cyclodextrin all at 13 m*M* concentration in water). The solutions were left overnight after adding β-cyclodextrin and the cellulase activities were measured the following day. The activity response results are shown in Fig. 1.

γ-Cyclodextrin was inefficient at renaturing cellulase, as the activity did not improve significantly as compared with the sample after foaming. However, α-cyclodextrin and β-cyclodextrin exhibited similar abilities to renature cellulase and improved the activity by a factor of three as compared with the foamate and even resulting in a higher activity than the initial mixture. This is consistent with another study, which has shown that α-cyclodextrin and β-cyclodextrin renatured chemically denatured pepsin similarly *(22)*.

Effect of Cyclodextrin Dilution Ratio

The dilution ratio of β-cyclodextrin recommended by Rozema *(23)* for artificial chaperones ratio was seven to three (denatured protein-detergent complex to β-cyclodextrin), and this ratio was tested to determine whether this is the optimal dilution ratio for inclusion in a cellulase foam fractionation/refolding system. Twelve mililiters of a 200 mg/L cellulase and 100 mg/L

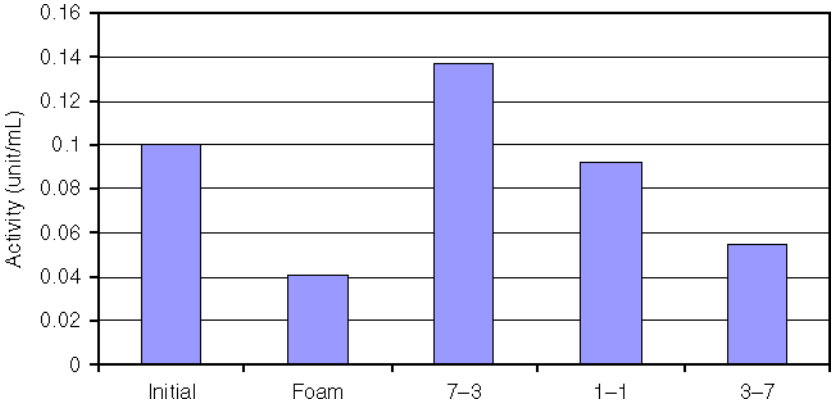

Fig. 2. Effect of the dilution ratio of β-cyclodextrin on cellulase activity before and after foaming and at different dilution ratios after foaming. The foamate was collected at an air-flow rate of 8 mL/min from a mixture of 200 mg/L cellulase and 100 mg/L CTAB.

CTAB mixture was aerated at an air-flow rate of 8 mL/min. For the 7:3 ratio, 700 µL of the foamate was placed in a 1.5 mL microcentrifuge tube and diluted with 300 µL of 13 m*M* β-cyclodextrin solution. For the 1:1 ratio, 500 µL of the foamate was placed in a 1.5 mL microcentrifuge tube and diluted with 500 µL of β-cyclodextrin solution. To achieve 3:7 ratio, 300 µL of the foamate was placed in a 1.5 mL microcentrifuge tube and diluted with 700 µL of β-cyclodextrin solution. The solutions were left overnight after adding β-cyclodextrin, and the cellulase activity for each was measured the following day. The 7:3 dilution ratio recommended by Rozema *(23)* for renaturing chemically denatured proteins was the best ratio of the three tested here for renaturing the foamate. Regardless of the dilution ratio, the specific activity remained around 0.27 unit/mg (the initial specific activity was 0.5 unit/mg). The higher the dilution with β-cyclodextrin, the more dilute the final solution and the lower the activity. Results from these trials are shown in Fig. 2.

Effect of Dilution Ratios

Because the original dilution was seven parts foamate to three parts β-cyclodextrin, 700 µL of foamate solution from 200 mg/L cellulase and 100 mg/L CTAB (aerated at an air-flow rate of 12 mL/min) was diluted with three separate 100 µL β-cyclodextrin volumes. The results are displayed in Fig. 3.

Cellulase activity per unit volume of solution increased as soon as the first dilution was made. However, the activity dropped slightly after the second dilution with β-cyclodextrin. When the final dilution was made, the activity increased to 0.11, which was slightly above the initial activity. However, it was still lower than the activity resulting from a single large dilution (the 7:3 ratio in Fig. 2). This could be owing to enzyme degradation, because the solution was left on the lab counter during the 3 h of the experiment.

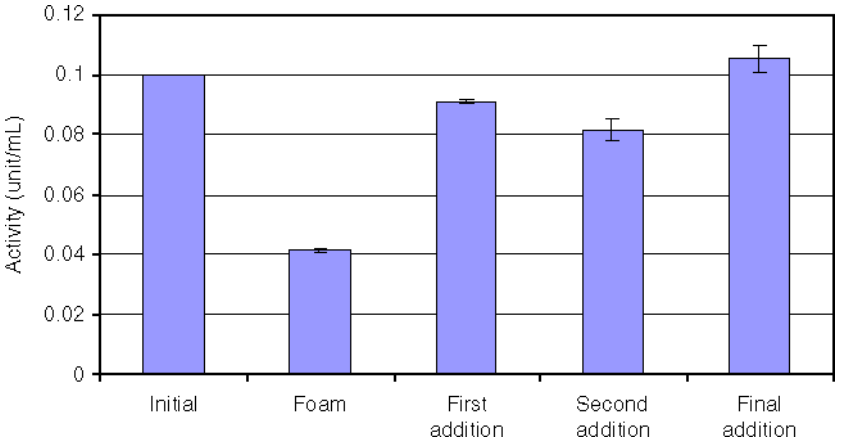

Fig. 3. Incremental addition to refold denatured cellulase, with activity measured before and after foaming and at each step of the dilution process. The foamate was collected at an air-flow rate of 12 mL/min from a mixture of 200 mg/L cellulase and 100 mg/L CTAB. β-cyclodextrin was added in three separate 100 µL volumes.

Effect of Standing Time on Refolding

The reference protocol *(23)* for investigation of refolding proteins over time suggested leaving the solution overnight to restore the protein activity. As (the physical) protein denaturation in foam fractionation is different than the chemically denatured protein studied in the reference, the time for this process to come to completion might be shorter or longer than specified. Foam fractionation was conducted at an air-flow rate of 8 mL/min for a 200 mg/L cellulase and 100 mg/L CTAB solution. Then, the foamate was diluted with 13 m*M* β-cyclodextrin (seven parts foamate to three parts β-cyclodextrin). The activity was measured as soon as the dilution was made, then after 4, 8, 12, and 24 h (the filter paper assay took 1 h to complete; therefore, there was effectively a 1-h delay in time measurement past the sampling time). These temperature variation experiments were also run at three different temperatures –4, 25, and 50°C. The results are shown in Fig. 4.

The recovered cellulase activity in the foamate was around 0.04 unit/mL before dilution with β-cyclodextrin, as seen in Fig. 3. After dilution with β-cyclodextrin, a dramatic increase in activity was observed. The recovered activity was highest at around (0.16) for a quadrupling of activity immediately following dilution. Because it takes about 1 h to complete the filter paper test for cellulase activity, refolding may have taken place before the filter paper test was completed (within 1 h) or even "instantaneously." The decrease in cellulase activity in time following the initial data point was probably owing to an unsuitable liquid environment which caused cellulase degradation. Degradation was fastest at 50°C. Keeping the solution in the refrigerator or at room temperature had a similar affect on the change in activity, so it

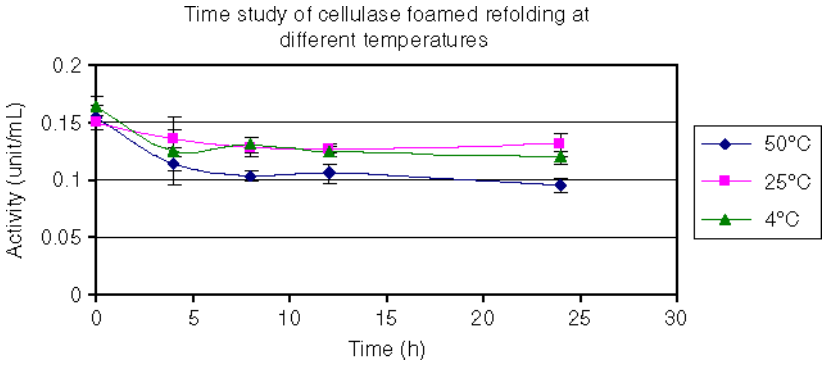

Fig. 4. Refolding of cellulase in the foamate as a function of standing time at three different temperatures. The foamate was collected from 12 mL of a 200 mg/L cellulase and 100 mg/L CTAB mixture aerated at 8 mL/min.

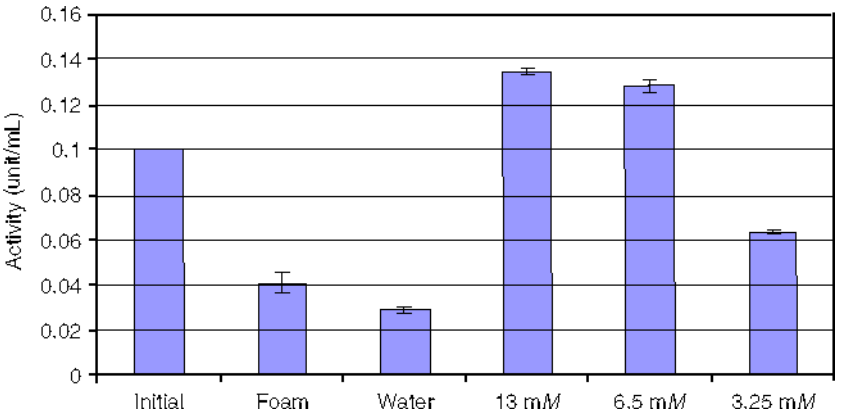

Fig. 5. Effect of β-cyclodextrin concentration on refolding of cellulase in the foamate. The foamate was collected from 12 mL of a 200 mg/L cellulase and 100 mg/L CTAB mixture aerated at 8 mL/min, 300 µL of β-cyclodextrin was added.

would be easier to run this process at room temperature on an industrial scale because it would save money on cooling cost.

Effect of β-Cyclodextrin Concentration

If the refolding β-cyclodextrin concentration used in the refolding process could be reduced, it would lower the cost of this process of concentrating cellulase. The β-cyclodextrin concentration normally used was 13 m*M*. The foamate solution from a 200 mg/L cellulase and 100 mg/L CTAB mixture that was aerated at 8 mL/min was individually diluted by 300 µL of β-cyclodextrin with different concentrations (13, 6.5, and 3.25 m*M*) or by deionized water. The dilution ratio was seven parts foamate and three parts β-cyclodextrin, with the results shown in Fig. 5.

Diluting the foamate with deionized water did not refold any of the cellulase, as expected in this control experiment. The activity decreased by the same ratio that the foamate was diluted with deionized water. As for β-cyclodextrin, a concentration of 13 m*M* worked best, but a 6.5 m*M* solution worked almost as well, 3.25 m*M* β-cyclodextrin renatured cellulase but not nearly as well as the other two concentrations. To save on chemical costs, it seems to be feasible to operate at 6.5 m*M* β-cyclodextrin instead of 13 m*M*. For industrial purposes, even corn dextrin might be used since it is inexpensive and as linear dextrin is known to successfully refold chemically denatured proteins *(24)*.

Conclusions

Three types of cyclodextrin were studied, and α-cyclodextrin was found to be as effective as β-cyclodextrin. The dilution ratio of cellulase-detergent to β-cyclodextrin was altered, but the specific activity of cellulase did not change for all three dilution ratios investigated. The concentration of β-cyclodextrin could be reduced by half without affecting the efficiency of renaturing the denatured cellulase. Pulse renaturation was applied in the dilution stage of cellulase foam fractionation. A single dilution volume was more effective than three separate dilution volumes (each 1/3 of the single one) for this process. Cellulase refolding was found to exhibit the highest activity immediately after β-cyclodextrin addition and only decreased with time. The refolding process was determined to occur optimally at room temperature.

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